

THE UNIVERSITY OF  
**SYDNEY**

# **Phytochemical and Biological Activity Study of Annona Atemoya**

A thesis submitted  
in fulfilment of the requirements for the degree of  
Doctor of Philosophy

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# STATEMENT OF AUTHENTICITY

This thesis is submitted to the University of Sydney in fulfilment of the requirements for the degree of Doctor of Philosophy. The work described was carried out in the School of Pharmacy, Faculty of Medicine and Health, University of Sydney, under the supervision of Professor Jane Hanrahan and Dr's Joanna Harnett and Dr Yanfei (Jacob) Qi.

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. The contribution of all co-authors in publications included in the body of the thesis have been declared and signed by each co-author. I hereby declare that I have not previously or concurrently submitted this material, either in full or in part, for a degree at this or any other institution.

Bassam Al Kazman

September 2023



## **Dedication**

***To Everyone Who Inspired and Encouraged Me to  
Pursue My Ambition***

***&***

***To the Love and Support from My Wife, Ashwaq,  
And My Children, Salem, Albandary and Eliana***

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# **Authorship attribution statement**

Declaration from the co-authors of publications included within this thesis as chapters are:

listed in the following pages.

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- Dr. Joanna Harnett

To whom it may concern,

I, **Prof. Jane Hanrahan**, declare that Bassam S. M. Al Kazman was the primary researcher within the papers submitted in the thesis. All experimental work and manuscript presentations were conducted by Bassam S. M. Al Kazman, except as specified in the following publications wherein I contributed as a supervisor to the experimental plan, data interpretation and editing.

1. **Bassam SM Al Kazman**, Joanna E. Harnett, and Jane R. Hanrahan. *"Traditional Uses, Phytochemistry and Pharmacological Activities of Annonaceae."* *Molecules* 27.11 (2022): 3462. ([Chapter 1](#))
2. **Bassam SM Al Kazman**, Joanna E. Harnett, and Jane R. Hanrahan. *"The Phytochemical Constituents and Pharmacological Activities of Annona atemoya: A Systematic Review."* *Pharmaceuticals* (14248247) 13.10 (2020). ([Chapter 2](#))
3. **Bassam SM Al Kazman**, Joanna Elizabeth Harnett, and Jane Rouse Hanrahan. *"Identification of Annonaceous Acetogenins and Alkaloids from the Leaves, Pulp, and Seeds of Annona atemoya."* *International Journal of Molecular Sciences* 24.3 (2023): 2294. ([Chapters 3 and 4](#))



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# List of publications

## Publications in support of this thesis

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## Conference proceedings

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2. **Bassam SM Al Kazman**, Joanna Harnett, and Jane R. Hanrahan. "Preliminary studies on phytochemical constituents of the leaves and fruits of *Annona atemoya*, in Proceedings of the 7th International Electronic Conference on Medicinal Chemistry, 1–30 November 2021, MDPI: Basel, Switzerland." (2021), online. Oral presentation.

## Abbreviations

|                            |                                                         |
|----------------------------|---------------------------------------------------------|
| <i>A. atemoya</i>          | <i>Annona atemoya</i>                                   |
| <i>A. cherimola</i>        | <i>Annona cherimola</i>                                 |
| <i>A. coriacea</i>         | <i>Annona coriacea</i>                                  |
| <i>A. cornifolia</i>       | <i>Annona cornifolia</i>                                |
| <i>A. crassiflora</i>      | <i>Annona crassiflora</i>                               |
| <i>A. glabra</i>           | <i>Annona glabra</i>                                    |
| <i>A. macroprophyllata</i> | <i>Annona macroprophyllata</i>                          |
| <i>A. muricata</i>         | <i>Annona muricata</i>                                  |
| <i>A. reticulata</i>       | <i>Annona reticulata</i>                                |
| <i>A. salzmännii</i>       | <i>Annona salzmännii</i>                                |
| <i>A. senegalensis</i>     | <i>Annona senegalensis</i>                              |
| <i>A. squamosa</i>         | <i>Annona squamosa</i>                                  |
| ABTS                       | 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) |
| ACT                        | Acetogenins                                             |
| AFL                        | Alcoholic fatty liver                                   |
| ALD                        | Alcoholic liver disease                                 |
| ALK                        | Alkaloids                                               |
| AP                         | African Pride                                           |
| ASH                        | Alcoholic steatohepatitis                               |
| A $\beta$                  | Amyloid- $\beta$                                        |
| BDNF                       | Brain derived neurotrophic factor                       |
| CC                         | Column Chromatography                                   |
| CCM2                       | Cerebral cavernous malformations 2                      |
| CEM                        | T lymphoblast cell line                                 |
| CHCl <sub>3</sub>          | Chloroform                                              |
| DCM                        | Dichloromethane                                         |
| DMEM                       | Dulbecco's Modified Eagle's Medium                      |
| DMSO                       | Dimethyl sulfoxide                                      |
| DPBS                       | Dulbecco's phosphate buffer saline                      |
| DPPH                       | 2,2-diphenyl-1-picrylhydrazyl                           |
| EC <sub>50</sub>           | Half maximal effective concentration                    |
| ECL                        | Enhanced chemiluminescence                              |
| EGFR                       | Epidermal growth factor receptor                        |
| ER                         | Endoplasmic reticulum                                   |
| ESO                        | Essential oils                                          |
| EtOAc                      | Ethyl acetate                                           |
| FBS                        | Fetal bovine serum                                      |
| FDA                        | Food and drug administration                            |
| FFA                        | Free fatty acids                                        |

|                        |                                                           |
|------------------------|-----------------------------------------------------------|
| FLA                    | Flavonoids                                                |
| FRAP                   | Ferric reducing antioxidant power                         |
| GCMS                   | Gas chromatography/mass spectrometry                      |
| GRK2                   | G Protein-Coupled Receptor Kinase 2                       |
| HCC                    | Hepatocellular carcinoma                                  |
| Hep 2,2,15             | Human hepatoblastoma cell line                            |
| Hep G2                 | Human liver cancer cell line                              |
| HUVECs                 | Human umbilical vein endothelial cells                    |
| KB                     | KERATIN-forming tumor cell line                           |
| <i>L. amazonensis</i>  | <i>Leishmania amazonensis</i>                             |
| <i>L. amazonensis</i>  | <i>Leishmania amazonensis</i>                             |
| <i>L. braziliensis</i> | <i>Leishmania braziliensis</i>                            |
| <i>L. chagasi</i>      | <i>Leishmania chagasi</i>                                 |
| <i>L. guyanensis</i>   | <i>Leishmania guyanensis</i>                              |
| LC-MS/MS               | Liquid chromatography mass spectrometry/mass spectrometry |
| LUHMES                 | Lund human mesencephalic cells                            |
| MBC                    | Minimum Bactericidal Concentrations                       |
| MeOH                   | Methanol                                                  |
| MIC                    | Minimum Inhibitory Concentrations                         |
| MRSA                   | Methicillin-resistant <i>Staphylococcus aureus</i>        |
| MS                     | Mass spectrometry                                         |
| MS/MS                  | Tandem mass spectrometry                                  |
| NAFLD                  | Non-alcoholic fatty liver disease                         |
| NASH                   | Non-alcoholic steatohepatitis                             |
| NMR                    | Nuclear magnetic resonance                                |
| ORAC                   | Oxygen Radical Absorbance Capacity                        |
| PA                     | Palmitic acid                                             |
| PBST                   | Phosphate-buffered saline with tween 20                   |
| PEI                    | Percutaneous ethanol injection                            |
| PHE                    | Phenolics                                                 |
| PVDF                   | Polyvinylidene fluoride                                   |
| RFA                    | Radiofrequency ablation                                   |
| SDS-PAGE               | Sulphate–polyacrylamide gel electrophoresis               |
| STE                    | Sterols                                                   |
| TACE                   | Transarterial chemoembolization                           |
| TLC                    | Thin Layer Chromatography                                 |
| UV                     | Ultraviolet                                               |
| VEGF                   | Vascular endothelial growth factor                        |
| WHO                    | World Health Organization                                 |

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# Objectives

The objective of this project was to study the chemical constituents of different parts of *Annona atemoya* such as leaves, fruits and seeds, and to investigate the biological activity of its constituents as anticancer agents *in vitro*.

## Specific aims

- i. To report an update literature review regards Annonaceae species in general and *Annona* genus in specific by:
  - Summarizing the literature of most well-known species of the *Annona* genus.
  - Providing a comprehensive summary of botanical features including biographical distribution, classification, diagnostic features, and traditional uses.
  - Reporting the various phytochemical constituents from different plant parts of *Annona* species.
  - Reviewing the pharmacological activities of crude extracts and isolated compounds from *Annona* species in both *in vitro* and *in vivo* assays.
- ii. To conduct a systematic review of all studies that reported the chemical constituents and the biological evaluation of *Annona atemoya*.
- iii. To characterise the phytochemical constituents of the leaves, fruit and seeds of the *Annona atemoya* cultivars collected from a farm located in southeast Queensland, using TLC NMR and MS.
- iv. To verify the chemical structure of isolated pure compounds.
- v. To evaluate the cytotoxic and protective activity of crude extracts of *Annona atemoya* using MTS assay *in vitro*.

## Thesis Abstract

Primary liver cancer ranks as the sixth most commonly diagnosed cancer worldwide and is the third leading cause of cancer-related deaths. Hepatocellular carcinoma (HCC) is the most prevalent type, accounting for 75 to 85% of liver cancer cases, and is the second leading cause of cancer-related deaths. The primary risk factors for developing HCC include chronic hepatitis B or C infections, obesity, excessive alcohol consumption, smoking, exposure to aflatoxin-contaminated foods, and type 2 diabetes. In Australia, the incidence of HCC has increased nearly fourfold, from 1.8 to 7.7 cases per 100,000 people between 1982 and 2016. Systemic therapies for advanced HCC are required and natural products, such as herbal extracts or pure compounds have garnered attention from scientists as potential alternative anti-cancer agents. In recent years the *Annona* species have been the focus of much interest for their anticancer activities.

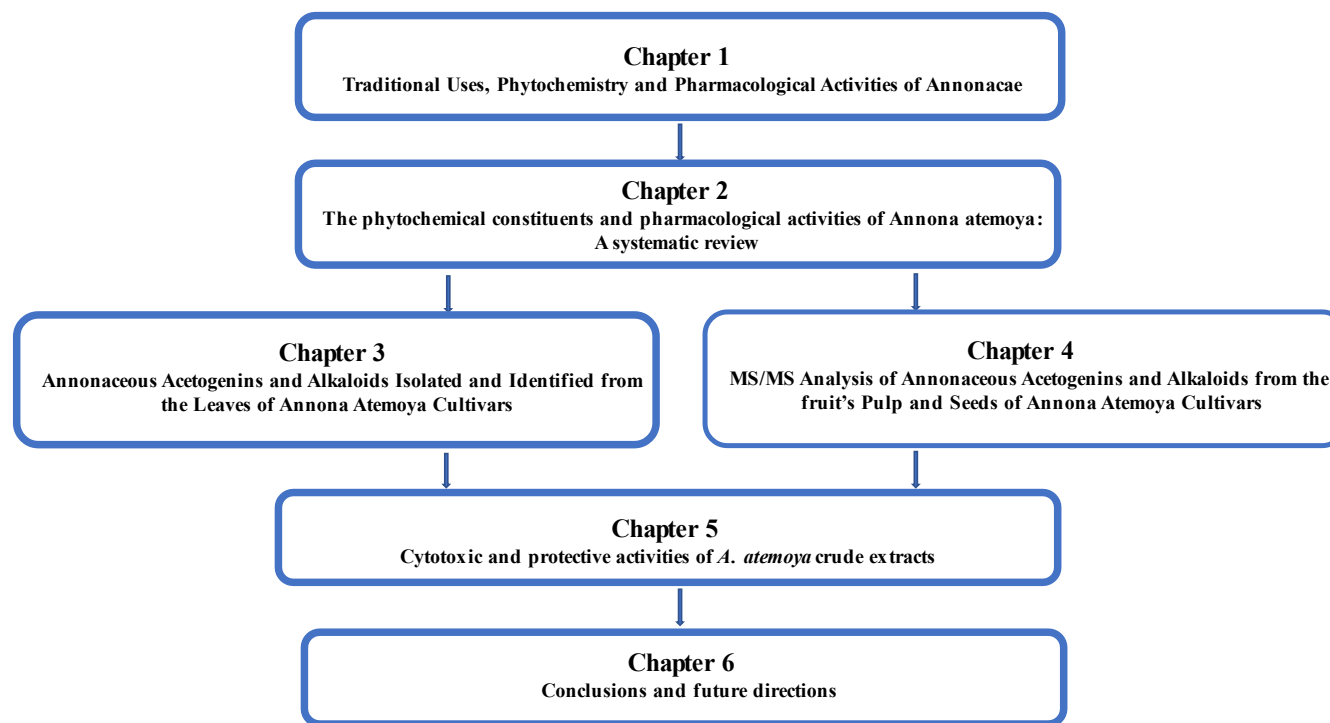
In 1789, the Annonaceae family was catalogued by de Jussieu. It encompasses tropical and subtropical plants which are widespread in distribution across various continents such as Asia, South and Central America, Australia and Africa. The genus of *Annona* is one of 120 genera of the Annonaceae family and contains more than 119 species of trees and shrubs. Most species are found in tropical America, where over 105 species have been identified. Due to its edible fruits and medicinal properties, *Annona* is the most studied genus of Annonaceae family. To date, only a limited number of these species have economic value, including *A. squamosa* L. (sugar apple), *A. cherimola* Mill. (Cherimoya), *A. muricata* L. (guanabana or soursop), *A. atemoya* Mabb. (atemoya), a hybrid between *A. cherimola* and *A. squamosa*, *A. reticulata* L. (custard apple), *A. glabra* L. (pond-apple) and *A. macrophyllata* Donn. Sm. (ilama). Phytochemically, several classes of secondary metabolites, including acetogenins, essential oils, alkaloids, terpenoids and flavonoids. The pharmacological activities of *Annona* species leaves and seeds include antibacterial, anticancer, antidiabetic and anti-inflammatory properties.

*Annona atemoya* also known as the custard apple is a hybrid between two Annonaceae species: Cherimoya (*Annona cherimola*) and the sugar apple (*Annona squamosa*). It is widely cultivated in tropical and subtropical continents including north and south America, Asia, Africa and Australia. Despite becoming an increasingly important commercial fruit plant due to its' creamy succulent flesh, compared to other Annonaceae species relatively few studies have investigated the phytochemistry and bioactivities of *A. atemoya*. Studies that evaluated *A. atemoya* extracts and its constituents were searched through the databases Scopus, PubMed and Embase from inception to June 2020. Constituents of *A. atemoya* include alkaloids, flavonoids, terpenes and acetogenins. The results of this systematic review indicate that the constituents of *A. atemoya* possess cytotoxic, anti-angiogenic, hypolipidemic, antioxidant, anti-inflammatory and neuroprotective activities. However, many of these studies are currently limited in quality and further phytochemical and pharmacological studies are required.

The study began with the screening of the phytochemical constituents of various parts including leaves, fruit's pulp, and seeds. Our initial findings indicated the presence of annonaceous acetogenins in the leaves of *A. atemoya* for the first time. Fifteen compounds were reported, eleven were acetogenins and four were alkaloids. Additionally, two compounds were isolated, and their structures and absolute configuration were determined by interpretation of the spectroscopic data and previous reported studies. The concentration of acetogenins in the hexane extract was very low compared to the ethyl acetate extract. Furthermore, the phytochemical investigations of *A.*

*atemoya* fruit and seeds have been screened in this project. The results of this study indicated the presence of annonaceous acetogenins in the fruit's pulp of *A. atemoya* for the first time. Eighteen compounds were identified in both cultivars, sixteen acetogenins and two alkaloids. The seeds of both cultivars were the richest in acetogenins, with sixteen compounds identified (1-11 and 16 to 20), compared with the pulp, which contained five acetogenins (1, 2, 3, 8 and 16). For the seed, both cultivars indicated some similarities (2, 3, 6, 7, 8, 10, 11 and 16) with more compounds reported in the AP cultivar. Moreover, the concentration of acetogenins in the pulp was very low compared to the seeds, and all three extracts of seeds illustrated almost the same acetogenins with more compounds reported in the ethyl acetate extract.

The biological activity of *A. atemoya* crude extracts were examined using Huh-7 cell line. Results from this study indicate that only two crude extracts, LH and SEY, displayed anti-cancer activity in Huh-7 cells, with IC<sub>50</sub> values of 67.21 and 22.55, respectively. Both extracts exhibited a dose-dependent inhibition of cell viability within 24 hours, as assessed by the MTS assay. For the remaining crude extracts that did not demonstrate cytotoxic effects, their protective effects were studied against PA. Most of these extracts were found to block PA by increasing the levels of anti-apoptotic proteins like Bax and Bim, while reducing the levels of pro-apoptotic proteins such as Bcl-XL and Mcl-1.



**Figure 1.** Flowchart of thesis components.

# CHAPTER 1

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## **Traditional Uses, Phytochemistry and Pharmacological Activities of Annonaceae**

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## Abstract

In 1789, the Annonaceae family was catalogued by de Jussieu. It encompasses tropical and subtropical plants which are widespread in distribution across various continents such as Asia, South and Central America, Australia and Africa. The genus of *Annona* is one of 120 genera of the Annonaceae family and contains more than 119 species of trees and shrubs. Most species are found in tropical America, where over 105 species have been identified. Due to its edible fruits and medicinal properties, *Annona* is the most studied genus of Annonaceae family. To date, only a limited number of these species have economic value, including *A. squamosa* L. (sugar apple), *A. cherimola* Mill. (Cherimoya), *A. muricata* L. (guanabana or soursop), *A. atemoya* Mabb. (atemoya), a hybrid between *A. cherimola* and *A. squamosa*, *A. reticulata* L. (custard apple), *A. glabra* L. (pond-apple) and *A. macrophyllata* Donn. Sm. (ilama). Phytochemically, several classes of secondary metabolites, including acetogenins, essential oils, alkaloids, terpenoids and flavonoids. Crude extracts of the leaves and seeds of *Annona* species exhibit pharmacological activities, encompassing antibacterial, anticancer, antidiabetic and anti-inflammatory properties.

**Keywords:** Annonaceae; *Annona*; custard apple; phytochemistry; bioactivity; ethnomedicinal pharmacological activity

## 1.1. Introduction

In 1789, the Annonaceae family was cataloged by de Jussieu [1, 2]. It encompasses tropical and subtropical plants, which are widespread in distribution across various continents such as Asia, South and Central America, Australia and Africa [3]. It is one of the largest Mangnoliidae families and the number of its genera and species is still debated [4-6]. Bailey and Popenoe believe that it has between 40 and 50 genera and from 500 to 600 species [6]; however, many studies have indicated that the Annonaceae family is comprised of more than 2400 species distributed in approximately 120 genera [4, 5]. The family of Annonaceae involves trees, lianas and bushes arranged in four large subfamilies: Malmeoideae, Annonoideae, Ambavioideae and Anaxagoreoideae [7, 8]. Economically, species of Annonaceae are important as a source of edible fruits, for instance, the pawpaw (Asimina), custard apple, sweetsop, soursop and cherimoya [1]. It has also been reported that some oils from the seeds might be used for the production of edible oils and as an ingredient in soaps, and the woods of some species have been reported for alcohol production [3]. Chemical studies of Annonaceae species have reported the isolation of a wide diversity of phytochemical components, including acetogenins, alkaloids and flavonoids from the bark, fruits, leaves, seeds and pulp of Annonaceae [9, 10]. This review aims to provide a comprehensive summary of the botanical features, phytochemistry, pharmacological properties, and the traditional and ethnomedicinal uses of the Annonaceae family and, specifically, Annona species.

## 1.2. Botanical Features of Annonaceae Species

### *1.2.1. Distribution and Classification*

Annonaceae has been listed as a diverse family of aromatic trees, bushes or shrubs, and climbers or lianas, which are predominantly found in the tropical and subtropical regions, with a limited number growing in temperate zones [1, 11]. In tropical America, the Annonaceae



species are usually shrubby and most grow in open grasslands [1]. In contrast, species that are climbers mostly grow in the tropical area of the old world [1]. In temperate zones like North America, the only genus reported is *Asimina* [1, 3]. In Brazil, more than 385 species have been reported, with the majority of them reported in the Amazonian region [2]. According to the Takhtajan system of flowering plant classification, the majority of Annonaceae plants can be found in both Asia and Australasia with approximately 51 genera and more than 950 species, while 40 genera with approximately 450 species are confined to Africa and Madagascar, and about 38 genera and 740 species are native to the American continent [3]. The first classification of the Annonaceae family was described by Dunal in 1817 and was limited to only fruit morphology [12]. Subsequently, a new classification of the Annonaceae family based on flower characteristics was introduced by Diels and Alder in 1932 [12]. However, a later classification by Fries in 1959 was found to be more comprehensive and authentic, using a combination of fruit morphology and flora characteristics [12]. The Annonaceae family are characterised by the presence of a variety of primitive and archaic features, leading to them being described by Darwin as “living fossil” due to their ability to survive the mass extinction [1, 12]. Under the Takhtajan system, the Annonaceae family is related to Magnoliaceae, which is one of the largest families of Magnoliales with other families such as Degeneriaceae, Canellaceae, Himantandraceae and Myristicaceae [1, 12].

### ***1.2.2. Diagnostic Features***

From one species to another, the botanical features of Annonaceae families vary greatly based on their origin, geography, and climate. Based on morphology and habitat, the Annonaceae family is known among the homogeneous plant families [1, 4]. The aromatic flowers are commonly open before other parts are entirely developed. The flowers are terminal, axillary, hermaphrodite, singular or grouped and regular [1, 12]. The stamens are typically abundant,

spirally arranged and hypogenous [1, 12]. The leaves are characterised by having a glaucous or metallic sheen, and they are alternate, exstipulate and regular [1, 12]. The fruits are typically made up of clusters of berries with an edible fleshy receptacle, particularly in the *Annona* genera and they are extensively consumed due to their high nutritional value [1, 12]. Finally, the seeds are enlarged and have a copious, irregular-surfaced endosperm with a minute embryo [1, 12].

### ***1.2.3. Traditional Uses***

Annonaceae species are famous in tropical regions and used traditionally across tropical regions due to their widespread distribution. Various parts of the species are used traditionally, including leaves, seeds, bark, fruit, stem, roots and twigs. A range of different methods for preparation is reported, such as infusions, pastes and decoctions [12]. For instance, the fresh fruit of *Annona dioica* is used for wound healing in Brazil [12]. The dried leaves of *Annona muricata* are used orally for analgesic effects in some parts of Indonesia [12]. In Burkina-Faso, the bark and roots of *Annona muricata* are used for dysentery and as an anthelmintic medicine, whereas the leaves are utilized for both fever and dysentery [13]. In the northwestern part of Brazil, both leaves and twigs of *Duguetia chrysocarpa* are ground and the extract of this mixture are utilized for treating gastrointestinal ulcers as well as a remedy for bowel disease [12]. A decoction of the stem bark of *Annickia chlorantha* is used orally as a remedy for the treatment of wounds and fever in Cameroon [14]. Further data on the traditional uses of the most widely used Annonaceae species are presented in (Table 1.1).

Table 1.1. Uses of most commonly used Annonaceae family in traditional medicines.

| Annonaceae Species                                   | Region    | Local Name                              | Medicinal Uses                                                                                                                   | Part Used                    | Mode of Usage                                                                                         | References |
|------------------------------------------------------|-----------|-----------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|------------------------------|-------------------------------------------------------------------------------------------------------|------------|
| <i>Alphonsea javanica</i><br>Scheff.                 | Indonesia | Aku Battu                               | Rheumatism and edema                                                                                                             | Leave                        | Ethanollic extract                                                                                    | [15]       |
| <i>Annickia chlorantha</i><br>(Oliv).                | Cameroon  | African yellow wood (c)<br>Moambe Jaune | Treatment of sores<br>Antipyretic<br>Antiemetic<br>Stimulant<br>Tuberculosis<br>Treatment of jaundice<br>Urinary tract infection | Bark                         | Powder<br>Crushed bark and drink extract<br>Decoction<br>Decoction in baths<br>Decoction<br>Decoction | [13, 14]   |
| <i>Annickia affinis</i><br>(Exell) Versteegh & Sosef | Cameroon  | African yellow wood                     | Wound healing<br>Antiemetic<br>Antipyretic                                                                                       | Stem bark                    | Decoction of stem bark                                                                                | [14]       |
| <i>Anonidium floribundum</i> Pellegr                 | Cameroon  | Eboum, Libanga<br>Ebom                  | Poison antidote<br>Dysentery<br>Antipyretic                                                                                      | Roots<br>Root/Bark<br>Leaves | Decoction taken orally                                                                                | [13, 16]   |
| <i>Anonidium mannii</i><br>(Oliv.)                   | Cameroon  | Ebome; Npole<br>Wapo'o, Ebome<br>Afan   | Antipyretic                                                                                                                      | Stem bark                    | Decoction of stem bark                                                                                | [14]       |
| <i>Boutiquea platypetala</i><br>(Engl.)              | Cameroon  | Not reported                            | To treat fresh wounds                                                                                                            | Leaves                       | Pounded fresh leaves                                                                                  | [13]       |

|                                                                 |                    |                                                      |                                                                              |                         |                                                                                            |      |
|-----------------------------------------------------------------|--------------------|------------------------------------------------------|------------------------------------------------------------------------------|-------------------------|--------------------------------------------------------------------------------------------|------|
| <i>Cananga odorata</i><br>(Lam.) Hook and Thomson               | Malaysia and India | Kenanga utan, Perfume tree, Cananga oil, Ylang ylang | Rheumatism<br>Ophthalmic inflammation and wound healing                      | Bark                    | Bark extract eye drops for inflammation and decoction are used to wash fresh wounds        | [12] |
| <i>Duquetia chrysocarpa</i> Maas                                | Brazil             | Pindaíba-da-mata                                     | Bowl and rheumatism inflammation                                             | Leave and twigs         | Leaves and twigs extract taken to relieve inflammation                                     | [17] |
| <i>Enicosanthellum pulchrum</i> (King) Heusden                  | Malaysia           | Disepalum                                            | Rheumatism fever, edema and asthma                                           | Leave                   | Decoction can be used for asthma and rheumatism                                            | [18] |
| <i>Enantia chlorantha</i> var. <i>soyauxii</i> Engler and Diels | Africa             | African yellow wood                                  | Arthritis and wound healing                                                  | Bark                    | Powdered bark with citrus lemon used as dressing                                           | [12] |
| <i>Friesodielsia enghiana</i> (Diels.) Verdc                    | Cameroon           | Lonkosso                                             | Analgesic                                                                    | Bark                    | Decoction of bark is taken orally                                                          | [16] |
| <i>Friesodielsia gracilipes</i> (Benth.) Steenis                | Cameroon           | Ntonda                                               | Treatment of sores, skin infection, ulcers, and jaundice                     | Bark and wood           | Decoction of bark and wood                                                                 | [13] |
| <i>Fissistigma oldhamii</i> (Hemsl.) Merr                       | Southern China     | Oldhamii                                             | Rheumatoid arthritis                                                         | Stems and roots         | Powdered of stems and roots and orally ingested                                            | [12] |
| <i>Greenwayodendron Suaveolens</i> (Engl and Diels) Verdc       | Not reported       | Otounga                                              | Aphrodisiac and vermifuge<br>Rheumatic pains, fevers, headache, stomach-ache | Root<br>Leaves and bark | Chew roots<br>Pulverized leaves or bark and mixed with seeds of <i>Aframomum melegueta</i> | [16] |

|                                                  |                                    |                                                 |                                                                     |                              |                                                                                                 |          |
|--------------------------------------------------|------------------------------------|-------------------------------------------------|---------------------------------------------------------------------|------------------------------|-------------------------------------------------------------------------------------------------|----------|
| <i>Isolona hexaloba</i><br>(Pierre) Engl & Diels | Democratic<br>Republic of<br>Congo | Bodzungu                                        | Malaria                                                             | Stem bark                    | Decoction of stem bark                                                                          | [19, 20] |
| <i>Monodora myristica</i><br>(Gaertn.) Dunal     | Ivory coast                        | M Kpo. Abidjan<br>district                      | Eye diseases and<br>hemorrhoids, febrile<br>pains and headache      | Fruits Seed                  | Fruits and seeds<br>consumed whole or<br>ground to be used in soup<br>and stews                 | [21]     |
| <i>Monodora tenuifolia</i><br>Benth              | Not reported                       | African nutmeg<br>Ebom osoé<br>Grandes feuilles | Toothache<br><br>Dysentery and fevers                               | Root<br><br>Bark and<br>root | Clean the roots, boil and<br>rinse the mouth<br>Prepared as a decoction<br>and used as an enema | [13]     |
| <i>Polyalthia suaveolens</i><br>Engl and Diels   | Cameroon                           | Diels; Otungui;<br>Ntounga                      | Analgesic,<br>Antiepileptic<br>Antipyretic<br>Treatment of jaundice | Stem bark                    | Decoction of stem bark                                                                          | [14]     |
| <i>Polyalthia longifolia</i><br>(Sonn.) Thwaites | India                              | Ashoka                                          | Fever                                                               | Bark                         | Decoction of bark                                                                               | [22]     |
| <i>Xylopi aethiopica</i><br>(Dunal) A.Rich       | Sudan                              | Ethiopia or Negro<br>pepper                     | Rheumatism, colic<br>pain, headache, and<br>neuralgia               | Fruits                       | Ethanollic fruit extract or<br>dried fruits are used as<br>whole                                | [23]     |
| <i>Xylopi aromatic</i><br>Lam. Mart              | Columbia                           | Monkey pepper                                   | Pulmonary<br>inflammation and<br>hemorrhoids                        | Roots<br>Leaves              | Insertion of root<br>pieces into rectum<br>and leaves burnt and<br>smoke inhaled                | [24]     |
| <i>Xylopi parvifolia</i><br>Hook.f. and Thomson  | East and Central<br>Africa, India  | Netawu/Athu<br>ketiya                           | Gastrointestinal ulcers<br>Analgesic                                | Roots                        | Decoction Finely<br>dried powder                                                                | [25]     |
| <i>Xylopi staudtii</i> Engl<br>& Diels           | Not reported                       | Ntom, Odjobi<br>Bush pepper (c)                 | Cold and headache<br>treatment                                      | Bark                         | Powder                                                                                          | [13]     |

|                                     |          |            |                                                                                  |           |                                  |      |
|-------------------------------------|----------|------------|----------------------------------------------------------------------------------|-----------|----------------------------------|------|
| <i>Monodora tenuifolia</i><br>Benth | Cameroon | Ebome osso | Joint and muscle pain,<br>promotion of breast<br>milk production and<br>headache | Stem bark | Decoction of stem bark<br>powder | [14] |
| <i>Uvaria acuminata</i><br>Oliv     | Cameroon | Nosonaback | Typhoid and Yellow<br>fever<br>Headache and epilepsy                             | Stem bark | Decoction of stem bark           | [14] |

### 1.3. Phytochemistry of Annonaceae Family

A wide array of chemical compounds from various parts of Annonaceae plants have been discovered, isolated and characterised. The results of both phytochemical investigations and biological studies on various plants from this family have led to the identification of a wide diversity of compounds such as annonaceous acetogenins, flavonoids, alkaloids and essential oils, as summarized in (Table 1.2). These phytochemical constituents have been found to exhibit a broad range of biological activities such as immunosuppressive, antineoplastic, cytotoxic, antimicrobial, anti-inflammatory effects (Table 1.3). However, it is the *Annona* genera that are the most widely used as a food source and in traditional medicines.

Table 1.2. Representative phytochemicals isolated from plants of Annonaceae.

| Species                                               | Part                | Compounds                                                  | Class | References |
|-------------------------------------------------------|---------------------|------------------------------------------------------------|-------|------------|
| <i>Anaxagoma dolichocarpa</i><br>Sprague and Sandwith | Fruits              | p-Cymene<br>Spathulenol<br>Caryophyllene oxide<br>Guaiene  | ESO   | [5]        |
| <i>Anomianthus dulcis</i> (Dunal) J.<br>Sinclair      | Stem                | (-)-Anolobine<br>(-)-Anonaine                              | ALK   | [26]       |
| <i>Artabotrys pierreanus</i> Engl. &<br>Diels         | Stem bark           | Cyperene<br>Caryophyllene oxide<br>Cyperermone<br>Cadalene | ESO   | [5]        |
| <i>Artabotrys hexapetalus</i> (L.f.)<br>Bhandari      | Aerial parts        | 9-Oxo-asimicinone<br>Artapetalin-A<br>Artapetalin-B        | ACT   | [27, 28]   |
| <i>Goniothalamus giganteus</i><br>Hook.f. & Thomson   | Bark                | Pyranicin<br>Pyragonicin<br>Goniotrionin                   | ACT   | [29]       |
| <i>Miliusa balansae</i> Finet &<br>Gagnep             | Leaves and branches | Ombuine<br>Chrysosplenol<br>Pachypodol<br>Chrysosplenol C  | FLA   | [30]       |

ALK (Alkaloids), ACT (Acetogenins), ESO (Essential oils) and FLA (Flavonoids).



Table 1.3. Pharmacological activities of some isolated compounds from Annonaceae species.

| Species                                                     | Part Used                | Isolated Compounds                          | Pharmacological Activity    | Mechanism of Action                                                                                                                                                                       | References |
|-------------------------------------------------------------|--------------------------|---------------------------------------------|-----------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>Alphonsea javanica</i><br>Scheff                         | Leaves                   | (+)-Altholactone<br>(+)-Goniothalmin        | Anti-inflammatory           | Inhibited lipopolysaccharide (LPS) induced NO production in RAW 264.7 macrophages with IC <sub>50</sub> = 0.8 $\mu$ M.                                                                    | [15]       |
| <i>Artabotrys hexapetalus</i><br>(L.f.) Bhandari            | Roots, stems, and leaves | Artabonatine B<br>Squamolone                | Anticancer                  | Exhibited activity against 2,2,15 and Hep G2 cell lines with IC <sub>50</sub> 11.0 and 9.1 $\mu$ g/mL. Displayed activity against Hep G2 cell lines with IC <sub>50</sub> 2.8 $\mu$ g/mL. | [31]       |
| <i>Cananga odorata</i> (Lam.)<br>Hook.f. & Thomson          | Fruits                   | Cleistopholine                              | Cytotoxic                   | Exhibited cytotoxicity against both Hep 2,2,15 and Hep G2 cell lines with IC <sub>50</sub> 0.54 and 0.22 $\mu$ g/mL, respectively.                                                        | [32]]      |
| <i>Goniothalamus tamirensis</i><br>Pierre ex Finet & Gagnep | Stem bark                | Dielsiquinone                               | Cytotoxic                   | Displayed cytotoxic activity against U251, RPMI, MCF7, HT029 and A549. with ED <sub>50</sub> 0.37, 0.11, 0.11 1.12 and 0.11, respectively.                                                | [33]       |
| <i>Guatteria blepharophylla</i><br>Mart                     | Bark                     | Isocoreximine                               | Anti-proliferative activity | Exhibited activity against UACC-62, NCI-H460, HT-29 and MCF-7 with TGI > 764.52 $\mu$ M.                                                                                                  | [34]]      |
| <i>Rollinia sylvatica</i> A.St.-<br>Hil                     | Leaves                   | Hinesol<br>z-Caryophyllene<br>beta-Maaliene | Anti-inflammatory           | Leukocytes migration was significantly reduced at concentrations of 36.04–45.37 $\mu$ g/mL.                                                                                               | [35]       |

## 1.4. *Annona* Genera

The genus of *Annona* is one of the 120 genera of the Annonaceae family and contains more than 119 species of trees and shrubs, most of them distributed in tropical areas of the Americas and Africa [6]. The majority of these species are found in tropical America, with more than 105 species (26 of them are endemic) and 10 species distributed in tropical Africa [11, 36]. It has been reported that this genus is the second or the third largest genus in the Annonaceae family [37]. Its generic name derives from the Latin Hispaniolan Taino “annual harvest” [6, 37]. Due to its edible fruits and medicinal properties, *Annona* is the most important genus of Annonaceae family [2]. Numerous *Annona* species furnish edible fruits like *Annona muricata* (“graviola”), *Annona crassiflora* (“araticum”) and *Annona squamosa* (“fruta do conde”) [2]. Most of the fruits are consumed either in fresh form or used in desserts, juices and ice cream preparations [36]. Despite *Annona* having many species, only limited species of this family are economically important such as *A. squamosa* L. (sugar apple), *A. cherimola* Mill. (Cherimoya), *A. muricata* L. (guanabana or soursop), *A. atemoya* Mabb. (atemoya), a hybrid between *A. cherimola* and *A. squamosa*, *A. reticulata* L. (custard apple), *A. glabra* L. (pond-apple) and *A. macrophyllata* Donn. Sm. (ilama) [6]. Phytochemically, several classes of secondary metabolites such as acetogenins, essential oils, alkaloids, terpenoids and flavonoids have been described in this genus [36, 38, 39]. A variety of pharmacological activities have been reported from various parts of *Annona* species specially leaves and seeds including applications against antibacterial [40], antinociceptive [41], anticancer [42, 43], anticonvulsant [44], antidiarrhea [45], antidiabetic [46], antimalarial [42], anti-inflammatory [47], antioxidant [48], antileishmanial [49], antiulcer [50] and antidepressant [51].

## 1.5. Botanical Features

Generally, *Annona* species are small trees or shrubs with a height from 5 to 11 m depending on various factors including soil, climate, species, and crop management [2]. In relation to the botanical characteristics of *Annona* species, the majority of them are moderately erect with brown bark that is frequently furrowed (Table 1.4) [11, 52]. The stems are rust-coloured (ferruginous) and covered with densely matted hairs (tomentose) when young, becoming smooth and hairless (glabrous) as they mature [6, 11]. It has thin lateral roots and a taproot that is not generally pronounced [2]. With regard to the flowers, they are hermaphrodites, solitary or fascicle containing from two to four flowers. The flowers are usually fragrant, with six petals and three green sepals, in a circular arrangement of two verticils [6]. Flowering of the plant usually starts at 3 to 4 years and flower opening usually occurs by separation of the apex of external petals [6, 11]. Finally, the leaves may be shiny or hairy and have an impressed vein on the upper side, and the fruits are syncarpous and comprised of seeds and many carpels [6, 11].

Table 1.4. Botanical information of some *Annona* species.

| Species                  | Synonyms                                                                                                                                       | Local Names                                                                                 | Geographic Distribution                                                                                                              | References |
|--------------------------|------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>A. cherimola</i>      |                                                                                                                                                | Chirimoya<br>Chirimolia<br>Cerimoya<br>Cherimoyer Momona                                    | South Africa, China<br>Egypt<br>Eritrea<br>Myanmar<br>Philippines<br>India<br>France<br>Italy<br>Mexico, Ecuador<br>Portugal<br>Peru | [6, 53]    |
|                          | <i>A. tripetala</i> Aiton<br><i>A. pubescens</i> Salisb                                                                                        |                                                                                             |                                                                                                                                      |            |
| <i>A. coriacea</i>       | <i>A. coriacea</i> var. <i>amplexicaulis</i> S. Moore,<br><i>A. coriacea</i> var. <i>cuneate</i> , <i>A. coriacea</i> var. <i>pygmaea</i> Warm | Marolo<br>Araticum<br>Marolino                                                              | Brazil (Cerrado, Caatinga)                                                                                                           | [11, 54]   |
| <i>A. cornifolia</i>     | <i>A. walkeri</i> S. Moore                                                                                                                     | Araticum-mirim                                                                              | Brazil                                                                                                                               | [55]       |
| <i>A. crassiflora</i>    | <i>A. macrocarpa</i> Barb<br><i>A. rodriguesii</i> Barb                                                                                        | Araticum<br>Pinha-docerrado<br>Cerrado pinecone<br>Marolo<br>Cabeça de negro                | Brazil                                                                                                                               | [56]       |
| <i>A. macrophyllata</i>  | <i>A. diversifolia</i> Saff                                                                                                                    | Ilama,<br>Papauce<br>Anona blanca                                                           | Mexico<br>China<br>India                                                                                                             | [57, 58]   |
| <i>A. montana</i> Macfad | <i>A. Montana</i> f. <i>marcgravii</i> (Mart.) Porto                                                                                           | Mountain soursop<br>False graviola<br>jacá do Pará<br>Araticum grande<br>Shan di fan li zhi | Southern Asia,<br>South America Amazon<br>Rainforest and<br>Atlantic Forest                                                          | [11]       |

|                             |                                                                                                                                                                                                                                                                                                                                                                 |                                                                                                                          |                                                                                               |         |
|-----------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------|---------|
| <i>A. muricata</i>          | <i>A. macrocarpa</i> Barb<br><i>A. muricata</i> Guanabanus<br><i>A. cearensis</i> Morales                                                                                                                                                                                                                                                                       | Brazilian pawpaw Soursop,<br>ci guo fan li zhi, Graviola<br>Araticum grande Mullu<br>Raama Phala, Corossol<br>Catuche    | Tropical regions of<br>Americas<br>Malaysia, Myanmar,<br>Pakistan, India, Indonesia,<br>China | [6, 11] |
| <i>A. reticulata</i>        | <i>A. excelsa</i> Kunt<br><i>A. laevis</i> Kunth<br><i>A. longifolia</i> Moc<br><i>A. riparia</i> Kunth                                                                                                                                                                                                                                                         | Custard apple<br>Bullock's heart                                                                                         | Indonesia, West Indies,<br>Bangladesh, China, India                                           | [59]    |
| <i>A. sclerophylla</i> Saff | <i>Sulcata</i> Urb<br><i>A. spinescens</i> Mart                                                                                                                                                                                                                                                                                                                 | Not reported                                                                                                             | Brazil                                                                                        | [11]    |
| <i>A. senegalensis</i>      | <i>A. senegalensis</i> var. <i>arenaria</i><br>Sillans<br><i>A. senegalensis</i> var. <i>Bail.</i> Wild<br><i>A. senegalensis</i> var. <i>glabrescens</i><br>Oliv<br><i>A. senegalensis</i> var. <i>cuneata</i><br>Oliv<br><i>A. arenaria</i> Thonn<br><i>A. chrysophylla</i><br><i>A. chrysophylla</i> var. <i>porpetac</i><br>Bail<br><i>A. porpetac</i> Bail | Wild soursop<br>Sour soup<br>Abo<br>Uburuochaand Gwandar                                                                 | Nigeria                                                                                       | [6]     |
| <i>A. squamosa</i>          | <i>A. asiatica</i> L.<br><i>A. squamosa</i> f. <i>Parvifolia</i><br>Kuntze<br><i>A. cinerea</i> Dunal<br><i>Guanabanus squamosus</i> (L.)<br>M.Gómez                                                                                                                                                                                                            | Custard apple<br>Sweetsop<br>Tiep baay<br>Amritaphala<br>Chirimoya<br>Fruta do conde<br>Squamosus<br>Gomez<br>Guanabanus | Egypt, Sudan, Pakistan,<br>Thailand, China, India,<br>Costa Rica,                             | [6, 11] |

### 1.5.1. *Annona Cherimola*

*Annona cherimola* Mill (Cherimoya) belongs to the genus *Annona* in the Annonaceae family in magnolias order, which means “cold seeds”, and is a small tree that produces heart-shaped and conical edible fruit [60]. It is a steep, semi-momentary and a low bunched tree that is widespread in Ecuador and Peru and distributed throughout Asia, South Europe, America and Africa [61]. In Mexican traditional medicine, this plant has been used to treat various diseases such as diabetes, cough, fever, headache, worms and inflammation either alone or in combination with other plant species [53, 62-65]. Recently, various parts of *A. cherimola* have been phytochemically profiled and contain various polyphenols and alkaloids. The leaves were found to be a source of bioactive compounds with potential for use as treatments for skin and eye diseases and gastric, cardiovascular and intestinal disorders [60].

### 1.5.2. *Annona Squamosa*

*Annon squamosa* L., commonly known as custard apple, is a tropical, endemic species of the West Indies, Ecuador, Peru, Brazil, South and Central America, Mexico, Bahamas, Bermuda, and Egypt [66]. This plant is extensively cultivated in various states of India, including Maharashtra, Gujarat, Madhya Pradesh, Chhattisgarh, Assam, Uttar Pradesh, Bihar, Rajasthan, Andhra Pradesh, and Tamil Nadu. The total area of cultivation has been reported by the Indian Council of Agricultural Research (ICAR) as 40,000 ha [67]. Its tree grows as a small sapling from 3 m to 8 m, with large branches having brownish or light brownish bark and it has thin leaves and is known for its edible fruit [66]. In the Aligarh district village in Uttar Pradesh, *A. squamosa* is well-known for its antidiabetic properties [68]. Its seeds, bark and leaves possess various pharmacological properties, mainly anti-tumour properties [69].

### 1.5.3. *Annona Muricata*

*Annona muricata* is commonly known as soursop and graviola and is native to Central and South America. It is a small tree 5–10 m tall and 15–83 cm in diameter; it has low branches and edible fruit that are used commercially for the production of candy, juice and sherbets [70]. Traditionally, the aerial parts of this plant have been used to treat various diseases like diabetes and malaria and nowadays, it is widely used by people diagnosed with cancer [71]. Moreover, this species possesses several pharmacological properties, including vasodilator, cardio-depressive, antispasmodic, antimutagen, anticonvulsant, antiviral, antidiabetic and antihypertensive effects [72]. Both the leaves and seeds of *A. muricata* have been evaluated for their constituents resulting in the identification and isolation of more than 50 mono-THF acetogenins, alkaloids, terpenoids, saponins, flavonoids, coumarins, cardiac glycosides, phenols, tannins and anthraquinones [72].

### 1.5.4. *Annona Reticulata*

*Annona reticulata* Linn is a traditionally important plant utilized in traditional medicines [73]. It is indigenous to the West Indies and widely distributed in tropical and subtropical regions of the world [59]. It is a small tree with a height between 6 and 7.5 m and contains numerous lateral branches [59]. It has a cylindrical stem that contains lenticels and very short coffee-colored hairs [59]. The leaves of *A. reticulata* are lanceolate, membranous, oblong, and rounded or curate at the base. Fruits are edible, rough, somewhat heart-shaped and yellow in color that shifts to yellowish-red on ripening, and the seed is smooth and blackish in color [74]. Traditionally, *A. reticulata* has been utilized for the treatment of epilepsy, dysentery, cardiac problem, constipation, haemorrhage, bacterial infection, parasite and worm infestations, fever, ulcers and as an insecticide [73, 74]. Its leaves are used for helminthiasis treatment while bark is a powerful astringent and used as a tonic [73, 74].

#### 1.5.5. *Annona Coriacea*

*Annona coriacea* Mart. is a species belonging to the *Annona* genera, commonly known as “marolo”, “araticum” and “araticum-liso” [75]. This plant is distributed across Paraguay and Brazil, with little available information about its ethnomedicinal uses [76]. It is a small tree (3–6 m) and its edible fruit consist of an ovoid-obtuse syncarp and weighing up to 1.5 kg [77]. The leaves are glabrous on the ventral surface, obovate, and the base is frequently cordate and margin undulate [78]. The flowers are terminal, thick, solitary and having fleshy petals with colors shifting between orange and pink [78]. The leaves are traditionally used as carminatives, anthelmintics, antirheumatics and in the treatment of stomatitis, headaches, abscesses, neuralgia, rheumatism, ulcers and dermatitis [79, 80]. Both seeds and fruits are toxic when crushed and exhibited effects against ectoparasites like lice [80].

#### 1.5.6. *Annona Senegalensis*

*Annona senegalensis* is a small tree 2–6 m tall that is commonly known as wild custard apple and wild soursop [81]. This plant is native to tropical east and northeast, west and west-central, and southern Africa and islands in the western Indian Ocean [81]. Its leaves are simple, alternate, oblong, green to bluish-green, ovate or elliptic, and mainly lack hairs on the upper surface and brownish hairs on the lower surface [82]. This plant has been used in traditional medicine as a pain reliever, antioxidant, antidiarrheal, antitrypanosomal, antimalarial, anti-inflammatory, antimicrobial, antiparasitic, anticonvulsant and as an anti-snake venom [83]. It has been reported that the leaves of *A. senegalensis* are used for the treatment of tuberculosis, yellow fever and smallpox, whereas stem bark is reported for the treatment of injury from venomous animals [84]. The root was also reported for treating erectile dysfunction, tuberculosis, gastritis, reproductive deficiency and in the management of malaria and diabetes [85].



### 1.5.7. *Annona Vepretorum* Mart

*Annona vepretorum* is commonly recognized as ‘bruteira’, is a small tree of 2.5–10 m high native to the Brazilian biome Caatinga [86]. The fruits of *A. vepretorum* can be consumed either raw or as juice for nutritional purposes [87]. Traditionally, a decoction of the leaves have been used to bathe in for the treatment of allergies, yeast, skin diseases and microbial infections, whereas the root is traditionally used to treat snake and bee bites, inflammatory conditions and heart pain [86].

### 1.5.8. *Annona Salzmannii*

*Annona salzmannii* is a tree of 6–20 m high that known as “araticum-da-mata” and “araticum“apé” [88]. It is commonly cultivated in Brazil especially in the States of Bahia, Pernambuco, and Paraíba [88]. Its root, seeds and leaves are used in folk medicine for treating several illnesses like ulcers, dysentery and inflammatory conditions [88]. The leaves and bark of *A. salzmannii* are utilized for the treatment of tumors, diabetes and inflammatory conditions [89].

### 1.5.9. *Annona Crassiflor*

*Annona crassiflor* is known as araticum of cerrado or cerradão [90]. It is a small tree that bears a typical fruit known as araticum of cerrado or cerradão [91]. The fruits are highly consumed “in natura” by native people and can be used to make juice, jelly and ice-cream [90, 91]. In folk medicine, the seeds are used to treat scalp infections, and infusions of the leaves and seeds are utilized for their antidiarrheal and antitumor properties [90]. For more details about the botanical characteristics and traditional uses of *Annona* species, see (Tables 1.4 and 1.5).

Table 1.5. Traditional uses of common *Annona* species.

| Species                     | Region                            | Local Name                                                  | Medicinal Uses                                 | Part Used       | Mode of Usage                              | References   |
|-----------------------------|-----------------------------------|-------------------------------------------------------------|------------------------------------------------|-----------------|--------------------------------------------|--------------|
| <i>A. ambotay</i> Aubl      | French Guiana                     | Not reported                                                | Treating fever                                 | Leaves and bark | Leaves and bark crushed and rubbed on body | [19]         |
| <i>A. cherimola</i> Mill.   | Tropical America                  | Cherimola                                                   | Abortion                                       | Aerial parts    | Not reported                               | [92, 93]     |
|                             |                                   | Cherimoya                                                   | Anti-anxiety                                   | Fruit           |                                            |              |
|                             | Asia                              | Chirimoya                                                   | Cough                                          | Leaf            |                                            |              |
|                             | Gabon                             | Custard apple                                               | Diarrhea                                       | Root            |                                            |              |
|                             | Cultivated in Spain and Australia | Mao ye fan li zhi                                           | Hypercholesterolemia                           | Seed            |                                            |              |
|                             |                                   |                                                             | Infections                                     | Stem            |                                            |              |
| <i>A. coriacea</i> Mart.    | Brazilian (Cerrado, Caatinga)     | Araticum<br>Marolino<br>Marolo                              | Painful inflammations                          |                 | Not reported                               | [94]         |
|                             |                                   |                                                             | Parasitic                                      |                 |                                            |              |
|                             |                                   |                                                             | Sedative                                       |                 |                                            |              |
|                             |                                   |                                                             | Anthelmintic                                   | Leaves          |                                            |              |
|                             |                                   |                                                             | Chronic diarrhea                               | Root            |                                            |              |
|                             |                                   |                                                             | Inflammation                                   | Seeds           |                                            |              |
| <i>A. cornifolia</i> St-Hil | Bolivian and Brazilian savannah   | Not reported                                                | Leishmaniasis                                  |                 | Not reported                               | [95]         |
|                             |                                   |                                                             | Malaria                                        |                 |                                            |              |
| <i>A. crassiflora</i> Mart. | Brazil (Cerrado)                  | Araticum of the Cerrado<br>Araticum-mirim<br>Marolo<br>Panã | Rheuma                                         |                 | Not reported                               | [47, 96, 97] |
|                             |                                   |                                                             | Antiulcerative (green fruit)                   | Seeds           |                                            |              |
|                             |                                   |                                                             | Analgesic                                      | Leaves          |                                            |              |
|                             |                                   |                                                             | Antimicrobial                                  | Root bark       |                                            |              |
|                             |                                   |                                                             | Antirheumatic                                  | Root wood       |                                            |              |
|                             |                                   |                                                             | Carminative                                    | Seeds           |                                            |              |
|                             |                                   |                                                             | Digestive                                      | Fruit           |                                            |              |
|                             |                                   |                                                             | Rheumatism, Anti-inflammatory<br>Wound healing |                 |                                            |              |

|                                          |                                                                                         |                                                                                                |                                                                                     |                                            |                                                    |            |
|------------------------------------------|-----------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------|--------------------------------------------|----------------------------------------------------|------------|
| <i>A. cuneata</i> (Oliv.)<br>R.E. Fr     | Congo                                                                                   | Not reported                                                                                   | Asthenia<br>Female sterility<br>Hernia<br>Parasitic infections<br>Venereal diseases | Root bark<br>Stem bark                     | Not reported                                       | [98]       |
| <i>A. dioica</i> A. St.-<br>Hil.         | Brazil<br>(Cerrado,<br>Pantanal)                                                        | Ceraticum and<br>ariticum                                                                      | Diarrhea<br>Rheumatism                                                              | Fruits<br>Leaves                           | Dried leave pastes<br>and<br>fresh fruit decoction | [12, 35]   |
| <i>A. diversifolia</i><br>Saff           | Tropical forest<br>of Central<br>America<br>China                                       | Ilama<br>Papausa<br>White anona,<br>Yi ye fan li zhi                                           | Arthritic pain<br>Anti-spasmodic                                                    | Leaves<br>Seeds                            | Not reported                                       | [44, 58]   |
| <i>A. foetida</i> Mart                   | Brazil                                                                                  | Araticum-da-<br>caatinga                                                                       | Malaria                                                                             | Bark and leaves                            | Decoction of bark<br>and leaf                      | [99]       |
| <i>A. glabra</i> L                       | Caribbean                                                                               | Mamain                                                                                         | Fever                                                                               | Leaves                                     | Not reported                                       | [100]      |
| <i>A. glauca</i><br>Schumach. &<br>Thonn | West Tropical<br>Africa<br>(Senegal,<br>Ghana,<br>Suriname)                             | Dangan<br>Mampihege,<br>Mandé sunsun<br>Tangasu                                                | Arachnicides<br>Blennorrhoea<br>Diuretic<br>Fish-poisons<br>Insecticides            | Roots<br>Seeds                             | Not reported                                       | [101]      |
| <i>A. haematantha</i><br>Miq             | French Guiana<br>South<br>American<br>tropical<br>rainforest                            | Not reported                                                                                   | Fever                                                                               | Leaves<br>Bark<br>Roots                    | Leaves and bark<br>crushed rubbed on<br>body       | [19],[102] |
| <i>A. montana</i><br>Macfad.             | South America<br>Southern Asia<br>The Amazon<br>Brazil (Mata<br>Atlántica,<br>Pantanal) | Mountain soursop<br>Shan<br>Di fan li zhi false<br>Graviola<br>Araticum grande<br>Jacá do Pará | Against snake bite<br>Against obesity                                               | Leaf<br>Pulp juice<br>Seed<br>Stem<br>Twig | Not reported                                       | [103, 104] |

|                                         |                                                                                                                                                              |                                                                                                         |                                                                                                                                                                                               |                                                                                                            |                                                                                                   |            |
|-----------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------|------------|
| <i>A. muricata</i> Linn                 | Brazil                                                                                                                                                       | Araticum<br>Condessa<br>Graviola                                                                        | Anthelmintic<br><br>Analgesic, neuralgia,<br>rheumatism, arthritis<br>pain                                                                                                                    | Fruit, juice, and<br>crushed seeds<br>Fruit and leaves                                                     | Juice of fruit<br><br>Water extraction of<br>the leaf                                             | [71]       |
| <i>A. pickelii</i> (Diels)<br>H. Rainer | Mexico<br>Caribbean<br>Central<br>America<br>Venezuela<br>Colombia<br>Belize<br>Central<br>America<br>South America<br>Southern Asia<br>Africa<br>Madagascar | Sincollo<br>Soncoyo<br>Bullock's-heart<br>Custard apple<br>Anona blanca<br>Anona<br>Niiu xin fan li zhi | Contraceptive<br>Blood dysentery<br>Cold<br>Stomachache<br>Fainting spinal<br>disorders<br>Fever<br>Hysteria<br>Influenza<br>Mental depression<br>Skin diseases<br>Unhealthy ulcers<br>Wounds | Leaf<br>Leaf<br>Root<br>Stem<br>Seed<br>Aerial parts<br>Bark<br>Fruit<br>Leaf<br>Root<br>Seed<br>Stem bark | Not reported                                                                                      | [105, 106] |
| <i>A. reticulata</i> Linn               | West Indies                                                                                                                                                  | Ramphal                                                                                                 | Bronchitis<br>Asthma<br><br>Bowel inflammation                                                                                                                                                | Fruit<br>Seeds<br><br>Leaves                                                                               | Decoction of fruit<br>Oral ingestion of<br>powdered seeds<br>Oral ingestion of the<br>leaf powder | [107]      |
| <i>A. senegalensis</i><br>Persoon       | Nigeria                                                                                                                                                      | Ukopko (Idoma)                                                                                          | Anti-inflammatory<br>Analgesic<br>Anthelmintic<br>Cancer<br>Diarrhea<br>Epilepsy<br>Infectious diseases<br>Inflammations<br>Sleeping sickness                                                 | Leaves<br>Seed<br>Stem<br>Bark<br>Root bark                                                                | Roots and bark are<br>ground together, and<br>their decoction is<br>used                          | [108]      |

|                              |          |                                                 |                                                                                                                |        |                                  |
|------------------------------|----------|-------------------------------------------------|----------------------------------------------------------------------------------------------------------------|--------|----------------------------------|
|                              |          |                                                 | Snakebite<br>Cardiovascular<br>diseases<br>Diabetes<br>Febrile seizures<br>Gout<br>Mental disorders<br>Painful |        |                                  |
| <i>A. squamosa</i> Linn      | Cameroon | Sugar apple<br>(English); Kedahan<br>(Yambetta) | Vomiting, abscesses,<br>muscle aches, fever,<br>and skin disease                                               | Leaves | Decoction of leaves [14]         |
| <i>A. vepretorum</i><br>Mart | Brazil   | Araticum<br>Bruteira                            | Analgesic and<br>anti-inflammatory                                                                             | Leaves | Methanolic leaf<br>extract [109] |

## 1.6. Traditional and Ethnomedicinal Uses of Annona Genus

Traditionally, the *Annona* species have been used widely. For instance, antidiarrheal effects have been reported for *A. reticulata*, *A. muricata* and *A. salzmannii*, whereas *A. cherimola*, *A. squamosa* and *A. reticulata* have been reported for their antiparasitic effects (Table 5) [11]. Moreover, both *A. vepretorum* and *A. salzmannii* have been also reported for anti-inflammatory effects [11]. *A. purpurea* and *A. reticulata* have been used to treat fever, while anticancer effects have been reported for *A. senegalensis* and *A. muricata* [110, 111]. Furthermore, *A. foetida*, *A. muricata* and *A. glabra* have been traditionally used to treat rheumatism [112], while *A. reticulata*, *A. salzmannii*, *A. foetida* and *A. squamosa* have been described for treating ulcers [4]. In Indonesia, the fruit juice of *A. muricata* has used as a diuretic and to treat liver ailments and leprosy [113], whereas leaves was used to treat spasms, boils and as an aphrodisiac [38]. The leaves of *A. diversifolia* have been used as anti-inflammatory, anticonvulsant and analgesic agents [57]. Ethnobotanically, despite reports of the toxicity of *A. muricata* seeds, the powder of toasted seeds has been reported to be used as an emetic and cathartic in the traditional Mexican pharmacopeia [38]. To Southeast Asian people, the immature fruit of *A. reticulata* was used to treat both dysentery and diarrhea, and a decoction of roots was used to cure toothache and as an antipyretic [114]. Additionally, a decoction of leaves has been used internally against worms and topically to treat abscesses and boils [114]. Finally, the leaves of *A. squamosa* have been used as tonic and cold remedy in tropical America and systemically to cure dysentery in India [113].

## 1.7. Phytochemistry of Annona Species

A wide range of secondary metabolites, including acetogenins, flavonoids, alkaloids and essential oils (Figure 1.1), from nearly every part of *Annona* plants, have been discovered, isolated and characterised (Table 1.6). The plants of the *Annona* genera are also found to be

rich in minerals and vitamins, for instance, calcium, potassium, magnesium, sodium, copper, zinc, selenium, phosphorus, iron, vitamin C, pantothenic acid B5, thiamine and riboflavin [6].

Table 1.6. Compounds isolated from plants of *Annona* genus.

| Species                           | Part   | Isolated Compounds                                                                                                                                                                                                                                                                                                                                                                                                                                        | References |
|-----------------------------------|--------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>A. amazonica</i><br>R.E. Fries | Stems  | Cassythicine<br>Liriodenine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                         | [115]      |
| <i>A. cherimola</i>               | Root   | Corytenchine, Isocoreximine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                         | [116-121]  |
|                                   | Fruit  | $\alpha$ -Pinene, $\alpha$ -Thujene, Terpinen-4-ol, Germacrene D (ESO)                                                                                                                                                                                                                                                                                                                                                                                    |            |
|                                   | Seed   | 2,4- <i>cis</i> -Annocherinones, Annocherin, 2,4- <i>trans</i> -Isoannonacins, Annocherimolin, Annomolin, Annomocherin, Annomontacin, Annonacin, Asimicin, Tucumanin, 2,4- <i>trans</i> -Annocherinones, 2,4- <i>cis</i> -Isoannonacins, <i>cis</i> -Annonacin, Annogalene, Annosenegalin, Annomolon A, Annomolon B, Cherimolacyclopeptide C (ACT)                                                                                                        |            |
|                                   | Stem   | Annocherine A and B, Artabonatine B, Romucosine H, Cherianoine (ALK)                                                                                                                                                                                                                                                                                                                                                                                      |            |
| <i>A. coriacea</i> Mart.          | Bulb   | Crolechinic acid, Crolechinic acid (methyl ester), Annonene, Annonalide (ESO)                                                                                                                                                                                                                                                                                                                                                                             | [122-128]  |
|                                   | Seed   | Gigantecin, Coriapentocin A and B, Bullacin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                         |            |
|                                   | Leaf   | Quercetin-3-O- $\beta$ -(6''-O- $\beta$ -glucosyl)-glucoside, Quercetin-3-O- $\beta$ -(6''-O- $\alpha$ -rhamnosyl)-galactoside, Trigonelline, Rutin, Hyperin, Hyperin, Isorhamnetin-3-O- $\beta$ -glucoside, Isorhamnetin-3-O- $\beta$ -galactoside, Isoquercitrin, Isoquercitrin, Nicotiflorin, Biorobin, Keioside, Cacticin, Isorhamnetin-3-O- $\beta$ -glucoside, Narcissin, Rutin (FLA)                                                               |            |
|                                   | Root   | Coriacin, Coriadienin, Coriaheptocin A and B, Coriacyclodienin, Coriacycloenin, 4-Deoxycoriacin, Annoheptocin A and B (ACT)                                                                                                                                                                                                                                                                                                                               |            |
| <i>A. crassiflora</i>             | Leaf   | Kaempferol-3-O- $\beta$ -diglucoside, Kaempferol-3-O- $\beta$ -glucoside, Quercetin-3-O- $\beta$ -D-galactopyranoside, Epicatechin, Quercetin-3-O- $\beta$ -L-arabinopiranoside (FLA)                                                                                                                                                                                                                                                                     | [129]      |
| <i>A. foetida</i>                 | Bark   | Annomontine, <i>N</i> -Hydroxyannomontine, Liriodenine, <i>O</i> -methylmoschatoline (ALK)                                                                                                                                                                                                                                                                                                                                                                | [130-132]  |
|                                   | Leaf   | ( <i>E</i> )-caryophyllene, Bicyclogermacrene, $\alpha$ -Copaene (ESO)                                                                                                                                                                                                                                                                                                                                                                                    |            |
|                                   | Branch | Atherospermidine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                    |            |
| <i>A. glabra</i>                  | Fruit  | 16 $\alpha$ -17-Dihydroxy-ent-kauran-19-oic acid, 16 $\alpha$ -Hydro-ent-kauran-17-oic acid, 16 $\beta$ -Hydroxy-17-acetoxy-ent-kauran-19-oic acid, 16 $\alpha$ -Hydro-19-al-ent-kauran-17-oic acid, 16 $\beta$ -Hydro-ent-kauran-17-oic acid, 19-nor-ent-Kauran-4 $\alpha$ -ol-17-oic acid, Annoglabasin A and B, ent-Kaur-15-ene-17,19-diol, ent-Kaur-16-en-19-ol, ent-Kaur-16-en-19-oic acid, Methyl-16 $\alpha$ -hydro-19-al-ent-kauran-17-oate (ALK) | [133-137]  |



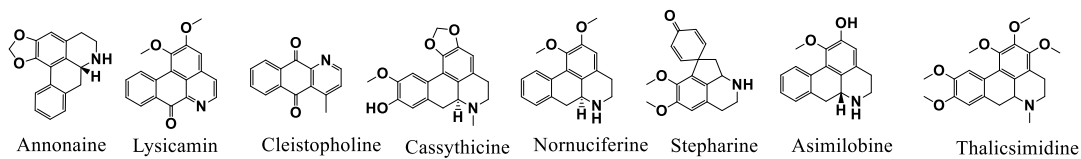
|                                               |                     |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |           |
|-----------------------------------------------|---------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
|                                               | Fruit & stem        | Annoglabin A, B, C, D, E and F, (-)-Anonaine, (-)-Asimilobine, (-)-Kikemanine, (-)-Nornuciferine, (+)-Stepharine, Blumenol A, Liriodenine, <i>N-p</i> -Coumaroyltyramine, (-)- <i>N</i> -Formylanonaine, (+)-Nordomesticine, Annobraine, Dehydrocorydalmine, Lysicamine, <i>N-trans</i> -Feruloyltyramine (ALK),<br>6- <i>O</i> -Palmitoyl- $\beta$ -sitosteryl-D-glucoside, $\beta$ -Sitosteryl-glucoside, Stigmasteryl-D-glucoside, $\beta$ -Sitosterol, Stigmasterol (STE)                                                                                            |           |
|                                               | Seed                | Isodesacetyluricin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |           |
|                                               | Leaf                | Bullatanocin, Glabracins A and B, Javoricin, Glacins A and B (ACT),<br>3- <i>O</i> - $\alpha$ -L-Arabinopyranoside, 3- <i>O</i> - $\beta$ -D-Glucopyranoside (GLU)<br>(-)-Actinodaphnine, (-)-Asimilobine, (-)-Anolobine, (-)- <i>N</i> -Methylactinodaphnine, (-)-Roemeroline, (+)-Boldine, (+)-Norisodomesticine, (+)-Stepharine, Liriodenine, (-)-Pallidine, (+)-1S,2S-Reticuline <i>N</i> -oxide, (+)-Magnoflorine, (+)-Reticuline (ALK)<br>Quercetin, Quercetin-3- <i>O</i> - $\beta$ -D-galactopyranoside (FLA)                                                    |           |
| <i>Annona leptopetala</i> (R.E.Fr.) H. Rainer | Leaves and branches | Laurotetanine, Nornuciferine, Corypalmine, NorannuradhapurineAnonaine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              | [138]     |
| <i>A. montana</i>                             | Leaf                | Annolatine, Annoretine, Liriodenine, Argentinine (ALK), $\beta$ -Sitosterol- $\beta$ -D-glucoside, $\beta$ -Sitosterol (STE), Montanacin-K, L, C, D, B and E, Annonacin-10-one, Annonacin-A, <i>cis</i> -Annonacin-10-one, Annonacin, <i>cis</i> -Annonacin (ACT)                                                                                                                                                                                                                                                                                                        | [139-142] |
|                                               | Seeds               | Montalicens G, Montalicens H Monlicins A & B, Murisolin, 4-Deoxyannomontacin, Muricatin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |           |
|                                               | Stem                | <i>N-trans</i> -Feruloyltyramine, <i>N-p</i> -Coumaroyltyramine, <i>N-trans</i> -Caffeoyltyramine (PHE)                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |           |
| <i>A. muricata</i>                            | Seed                | 2,4- <i>cis</i> -Gigantetrocinone, 2,4- <i>trans</i> -Isoaiinonacin, 2,4- <i>trans</i> -Gigantetrocinone, 2,4- <i>trans</i> -Isoannonacin-10-one, Gigantetrocin-A, Muricatenol, Annomontacin, Gigantetronenin, Annonacin A, Annoreticum-9-one, <i>cis</i> -Annomontacin, Murisolin, Muricin H, Xylomaticin, Muricin I, <i>cis</i> -Annonacin, <i>cis</i> -Goniothalamycin, <i>cis</i> -Annonacin-10-one, Arianacin, Javoricin, Donhexocin, Murihexol, Cohibins C, Cohibins D, Gigantetrocin B, Longifolicin, Muricin A, B, C, D, E, F and G, Annomuricatin B and C (ACT) |           |
|                                               | Stem bark           | Muricatin A, B and C (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |           |
|                                               | Fruit               | Epomuricenins-A and B, Epomuricins-A and B, Epomuricins-A and B, Muricin J, K and L (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | [143-151] |

| Asimilobine, Nomuciferine, Annonaine (ALK) |              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |               |
|--------------------------------------------|--------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------|
|                                            | Fruit & Root | Sabadelin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |               |
|                                            | Leaf         | Annonacin, Annomuricin C, Muricatocin C, (2,4- <i>cis</i> )-10 <i>R</i> -annonacin-A-one, (2,4- <i>trans</i> )-10 <i>R</i> -annonacin-A-one, Annohexocin, Annomutacin, Annopentocins A, B and C, Annomuricine, Muricapentocin, Annomuricins A and B, <i>cis</i> -annomuricin-D-ones, <i>trans</i> -annomuricin-D-ones, Muricatocins A and B, Murihexocin A and B, Muricoreacin, Murihexocin C (ACT)<br>( <i>R</i> )-4- <i>O</i> -methylcoclaurine, ( <i>R</i> )- <i>O</i> , <i>O</i> -dimethylcoclaurine, ( <i>R</i> )-Anonaine, Annonamine, ( <i>S</i> )-Norcorydine, Anonaine, Isolaureline, Xylopine (ALK)<br>Catechine, Epicatechine, Gallic acid, Chlorogenic acid, Kaempferol, Kaempferol-3- <i>O</i> -rutinoside, Quercetin-3- <i>O</i> -rutinoside, Quercetin-3- <i>O</i> -glucoside, Quercetin-3- <i>O</i> -neohispredoside, Quercetin-3- <i>O</i> -robinoside, Annoionols A and B, Annoionoside (FLA) |               |
|                                            | Leaf & seed  | Annonacin, Annocatacin A and B, Annonacinone, Annocatalin, <i>cis</i> -Corossolone, Goniiothalamycin, Isoannonacin, Corossolone (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |               |
|                                            | Pericarp     | Annonacin, Annonacin A, Annomuricin A (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |               |
|                                            | Root         | Annonacin, Muridienins-1, 2,3 and 4, Chatenaytrienins-1, 2 and 3, Muricadienin, Montecristin, <i>cis</i> -Panatellin, <i>cis</i> -Reticulatacin-10-one, <i>cis</i> -Uvariamicin IV, Coronin, <i>cis</i> -reticulatacin, <i>cis</i> -Solamin, Cohibins A and B (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |               |
| <i>A. purpurea</i>                         | Leaf         | Lirinidine, 7-Formyl-dehydrothalicsimidine, 7-Hydroxy-dehydrothalicsimidine, <i>N</i> -Methylasimilobine, <i>N</i> -Methylaurotetanine, Thalicsimidine, Norpurpureine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | [110, 152]    |
|                                            | Root         | Annomontine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |               |
| <i>A. reticulata</i>                       | Leaf         | Dopamine, Salsolinol, Spathenelol, Muurolene, Coclaurine, Copaene, Eudesmol (ESO), Squamone, Solamin, Rolliniastatin 2, Annoreticuin-9-one, Annomonicin, Annonaretin A (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    | [59, 153-157] |
|                                            | Stem bark    | Dopamine, Salsolinol (ESO), Reticullacinone, Rolliniastatin-2, (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |               |
|                                            | Root         | Liriodenine, Norushinsunine, Neoannonin, Reticuline (ALK)<br>Spathenelol, Copaene, Eudesmol, Muurolene (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |               |
|                                            | Bark         | Reticulatacin, Liriodenine, Copaene, Coclaurine (ALK)<br>Patchoulane (ESO)<br>Molvizarin, Bullatacin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |               |

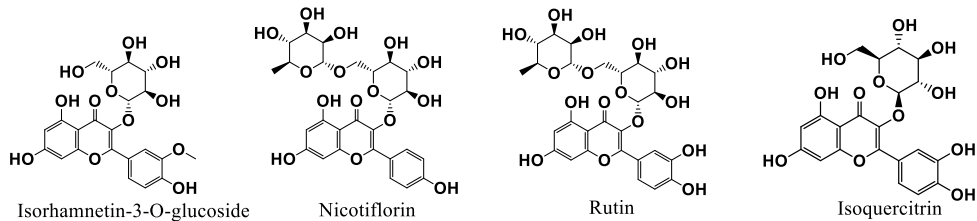
|                                |              |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |            |
|--------------------------------|--------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
|                                | Seed         | Squamocin, <i>cis</i> -/ <i>trans</i> -isomurisolenin, Bullatacin, <i>cis</i> -/ <i>trans</i> -Bullatacinone, Anoreticuin, Anoreticuin-9-one, Solamin, Annonomicin, Isoannonareticin, Rolliniastatin-1, 2 Squamone, Annonareticin, 2, 4- <i>cis</i> -Isoannonareticin, Solamin, Murisolin, Reticulacinone, Annonomicin, Sitosterol, Anoreticuin (ACT), Myrcene, Limonene, Germacrene D (ESO)                                                                                                                                                                                                                                                                        |            |
|                                | Fruit        | Terpinen-4-ol, Germacrene D, Limonene, Pinene, Myrcene (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |            |
|                                | Root bark    | Anonaine, Michelalbine, Reticuline, Oxoushinsunine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |            |
| <i>A. senegalensis</i>         | Leaf         | (-)-Roemerine, $\alpha$ -Humulene, $\gamma$ -Cadinene, Germacrene D, $\beta$ -Caryophyllene (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | [158, 159] |
|                                | Aerial parts | (-)-Anonaine, (-)-Asimilobine, (+)-Nornantenine (ALK)<br>(+)-Catechin (FLA)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |            |
|                                | Seed         | Annogalene, Annosenegalin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |            |
| <i>Annona sericea</i><br>Dunal | Leaf         | Nornantenine, Nornuciferine, Isoboldine, Lysicamine, Hydroxynornuciferine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | [160]      |
| <i>A. squamosa</i>             | Leaf         | (-) Anonaine, O-Methylarmepavine, $\beta$ -Caryophyllene, $\beta$ -Cedrene, (E)-Caryophyllene, Germacrene D, Bicyclogermacrene, Quercetin-3-O-glucoside (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       | [161, 162] |
|                                | Bark         | 2,4- <i>cis</i> -Mosinone A, 2,4- <i>trans</i> -Mosinone A, Anoreticum-9-one, Mosin B and C, Bullatacin, Bullatacinone, Squamone (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                              |            |
|                                | Pulp fruit   | $\alpha$ -Pinene, Limonene, Sabinene (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |            |
|                                | Stem         | 11 <i>ent</i> -Kauranes, 10-nor- <i>ent</i> -Kaurane-4 $\alpha$ ,16 $\beta$ ,17-triol, 16 $\alpha$ ,17-Dihydroxy- <i>ent</i> -kauran-19-oic acid, 16 $\beta$ ,17-Dihydroxy- <i>ent</i> -kauran-19-al, 16 $\beta$ -Hydro- <i>ent</i> -kauran-17,19-dioic acid, 17-Hydroxy-16 $\beta$ - <i>ent</i> -kauran-19 oic acid, <i>ent</i> -Kaur-16-en-19-oic acid, 16 $\alpha$ ,17-Dihydroxy- <i>ent</i> -kauran-19-al, 16 $\alpha$ -Hydro-19-al- <i>ent</i> -kauran-17-oic acid, 16 $\beta$ ,17-Dihydroxy- <i>ent</i> -kauran-19-oic acid, 16 $\beta$ -Hydroxy-17-acetoxy- <i>ent</i> -kauran-19-oic acid, 4 $\alpha$ -Hydroxy-19-nor- <i>ent</i> -kauran-17-oic acid (ALK) |            |
|                                | Seed         | Neoannonin-B, Annosquamins A, B and C, Annosquacin-I, Annosquamin A, B and C, Annosquatin A and B, Annotemoyin-1 and 2, Cherimolin-1 and 2, Diepomuricanin A and B, Dieporeticenin, Dieposabadelin, Squadiolin A, B and C, D, E, F, G, H, I, J, K, L, M and N, Squamostanin A, B, C, D, E and F, Cyclosquamosin A, B, C, D, E, F, G, H and I, Squamin A and B (ACT)                                                                                                                                                                                                                                                                                                 |            |
| <i>A. vepretorum</i>           | Leaf         | Spathulenol, Bicyclogermacrene, $\alpha$ -Phellandrene (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | [121, 163] |

ALK (Alkaloids), ACT (Acetogenins), ESO (Essential oils). STE (Sterols), FLA (Flavonoids), PHE (Phenolics).

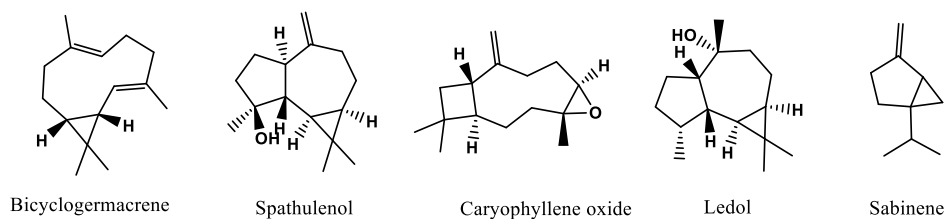
### Alkaloids



### Flavonoids



### Terpenoids



### Acetogenins

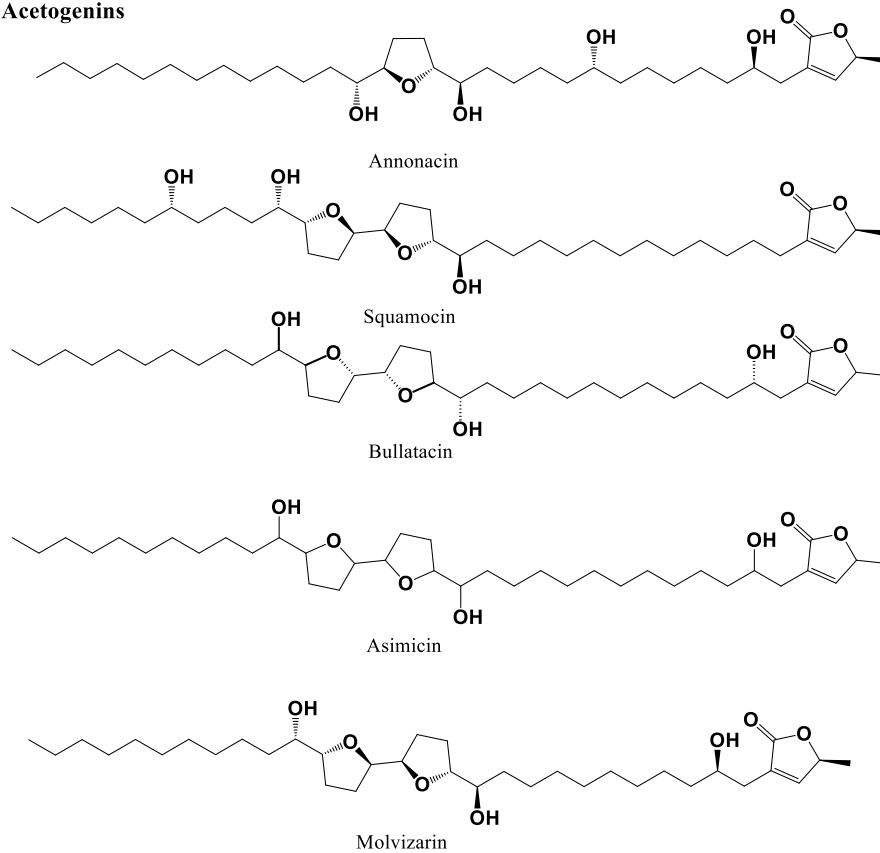


Figure 1.1 Structure of selected compounds identified in *Annona* species.

## 1.8. Pharmacological Properties of *Annona* Species

The species of the *Annona* genera have been reported to elicit a diversity of biological activities such as antitumor, anti-inflammatory, antioxidant, antinociceptive, antiprotozoal, antipyretic, antiulcer, antihyperglycemic, anthelmintic, antileishmanial, antimalarial, antidiarrheal, antifungal and antimicrobial promoted by whole extracts, fractions, or pure compounds (Table 1.7).

Table 1.7. Pharmacological activities of *Annona* species.

| Species             | Biogeographical Distribution                      | Used Part                                             | Traditional Use                                                                                                                       | Pharmacological Activities                                                                                                                             | Extract/Compound Evaluated                                                                                                                                                       | References |
|---------------------|---------------------------------------------------|-------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>A. ambotay</i>   | South American tropical rainforest                | Trunkwood                                             | Antipyretic                                                                                                                           | Antimicrobial                                                                                                                                          | Alkaloids                                                                                                                                                                        | [164]      |
| <i>A. bullata</i>   | Endemic of Cuba                                   | Bark                                                  | Not reported                                                                                                                          | Antitumoral                                                                                                                                            | 32-Hydroxybullatacinone                                                                                                                                                          | [165]      |
| <i>A. cherimola</i> | Tropical America, Asia, Spain<br>Australia, Gabon | Aerial parts<br>Fruit<br>Leaf<br>Root<br>Seed<br>Stem | Abortion<br>Anti-anxiety<br>Cough<br>Diarrhea<br>Hypercholesterolemia<br>Infections<br>Painful inflammations<br>Parasitic<br>Sedative | Antidepressant<br>Antifungal<br>Antiprotozoal<br>Antitumoral<br>Antihypercholesterolemic<br>Antiulcerative<br>Antiviral<br>Insecticidal<br>Vasodilator | Acetogenins: Molvizarin, Squamocin, Cherimolin-1, Motrilin, Aherradurin, Tucumanin<br>Annomocherin, Annonacin, Annomontacin,<br>Alkaloids: Roemerine, Anonaine, Dehydroroemerine | [40, 166]  |
| <i>A. coriacea</i>  | Brazilia (Cerrado and Caatinga)                   | Leaf<br>Root<br>Seed                                  | Leishmaniasis<br>Malaria<br>Rheuma<br>Anthelmintic<br>Chronic diarrhea<br>Inflammation                                                | Antifungal<br>Anti-inflammatory<br>Antitumoral<br>Insecticidal<br>Leishmanicidal<br>Trypanocidal                                                       | Acetogenins: Annoheptocins A-B, coriacin, 4-Deoxycoriacin, Coriaheptocins A-B, Coriadienin, Gigantecin                                                                           | [11]       |

|                        |                                                           |                                                                 |                                                                                                                                                                |                                                                                                                                                                                         |                                                                                                                                                                                       |            |
|------------------------|-----------------------------------------------------------|-----------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>A. muricata</i>     | America, Asia,<br>Africa                                  | Bark<br>Leaf<br>Fruit<br>Root<br>Root bark<br>Seed<br>Stem bark | Anthelmintic<br>Antiscorbutic<br>Asthma<br>Cancer<br>Cough<br>Cystitis<br>Diabetes<br>Diuretic                                                                 | Anti-arthritic<br>Antidepressant,<br>Antidiabetic<br>Anti-inflammatory<br>Antimicrobial<br>Antimalarial<br>Antiviral<br>Hepatoprotective                                                | <u>Acetogenins:</u><br>Solamin, Muricin H,<br>Muriacin I, cis-<br>Annonacin, Muriacins A-<br>G, Muriacoreacin,<br>Muriacapentocin,<br>Gigantetrocin A,<br>Annonopentocins A-C         | [167, 168] |
| <i>A. salzmannii</i>   | Brazil                                                    | Bark<br>Leaf                                                    | Not reported                                                                                                                                                   | Antioxidant<br>Antimicrobial                                                                                                                                                            | <u>Alkaloids:</u><br>Reticuline, Anonaine,<br>Laurelliptine,<br>Isoboldine                                                                                                            | [106]      |
| <i>A. senegalensis</i> | Madagascar,<br>Comoros, Cape<br>Verde, Tropical<br>Africa | Leaves<br>Seed<br>Stem<br>Bark<br>Root bark                     | Anti-inflammatory and<br>Analgesic<br>Anthelmintic<br>Cancer<br>Diarrhea<br>Epilepsy<br>Infectious diseases<br>Inflammations<br>Sleeping sickness<br>Snakebite | Anticonvulsant<br>Antidiabetic<br>Antidiarrheal<br>anthelmintic<br>Anti-inflammatory<br>Antimalarial<br>Antimicrobial<br>Antioxidant<br>Insecticidal<br>Hepatoprotective<br>Antitumoral | Aqueous extract.<br>Ethanol extract<br>Terpenoids, coumarins,<br>flavonoids, tannins,<br>alkaloids, quinones.<br>Methanolic extract<br>containing<br>Annonosnegalin,<br>Annonogalene. | [11, 169]  |

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|                    |                                  |                                   |                                                                                                                                    |                                                                                                                      |                                                                                                                                                                                                                                     |
|--------------------|----------------------------------|-----------------------------------|------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>A. squamosa</i> | Tropical America, Asia Australia | Seed<br>Stem<br>Bark<br>Root bark | Analgesic<br>Anthelmintic<br>Antirheumatic<br>Cancer<br>Digestive<br>Headache<br>Anti-inflammatory<br>Antimicrobial<br>Carminative | Antibacterial<br>Antidiabetic<br>Antilipidemic<br>Antioxidant<br>Antimalarial<br>Antigenotoxicity<br>Antileishmanial | Acetogenins: [11]<br>Squadiolins A and B,<br>Squafosacin B,<br>Bullatacinona,<br>Squamona,<br>Tetrahydrosquamone<br>Monoterpenes:<br>Limonene, $\beta$ -Cubebene,<br>$\beta$ -Caryophyllene,<br>Spathulenol,<br>Caryophyllene oxide |
|--------------------|----------------------------------|-----------------------------------|------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

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### 1.8.1. Antibacterial Activity

The antibacterial activities of *Annona* species have been reported in many studies, for example, both methanolic and ethanolic leaf extracts of *A. muricata* exhibited antimicrobial activity against *Staphylococcus aureus*, and this activity was attributed due to the presence of flavonoids, alkaloids and steroids in the extract [170, 171]. In contrast, an aqueous extract of the peel of *A. muricata* did not show any activity [170, 171]. The root of *A. reticulata* has also been investigated for its antibacterial activity against Gram-positive *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis*, and Gram-negative *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi* [59]. The root extract was found to possess pronounced activity against *Bacillus cereus* as well as notable inhibition against all tested strains [59]. Moreover, the leaves of *A. cherimola* have been reported for antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* with growth inhibition zone diameters of 11 mm and 14 mm, respectively [40]. The aqueous and methanolic seed extracts of *A. squamosa* have reported activity against *Staphylococcus aureus* with Minimum Inhibitory Concentrations (MIC) of 50 mg/mL and Minimum Bactericidal Concentrations (MBC) of 100 mg/mL [172]. The activity of the isolated compounds from *Annona* species has been reported in various studies; for instance, the fatty alcohol 11-hydroxy-16-hentriacontanone isolated from leaves of *A. squamosa* has a reported activity against Gram-positive and Gram-negative bacterial strains, with MIC values of 25–50 µg/mL [173]. Additionally, the alkaloids liriodenine, annonaine, asimilobine, reticuline and cleistopholine isolated from *A. salzmannii* demonstrated activity against a range of Gram-positive bacteria, including *Kocuria rhizophila*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis* with MIC values from 25 to 500 µg/mL [38]. Notably, annonaine and asimilobine had activity equal to or better than the control chloramphenicol (MIC 50 µg/mL) against many of the species tested [38].

### 1.8.2. Anticancer and Antiproliferative Activity

Various studies have reported the anticancer activity of either crude extracts or isolated compounds from *Annona* species. For example, the leaves extract of both *A. squamosa* and *A. reticulata* exhibited potent antiproliferative effects against two human T-lymphotropic virus type = 1 infected cell lines (MT-1 and MT-2) with EC<sub>50</sub> values from 0.1 to 1 µg/mL [174, 175]. In in vitro studies, the ethanolic extract of *A. muricata* leaves was reported for its cytotoxicity against promonocytic leukemic cells (U-937) with an LC<sub>50</sub> = 7.8 µg/mL [176]. Isocoreximine isolated from *A. cherimola* demonstrated cytotoxicity against multiple cancer cell lines. At a concentration of 50 µg/mL, isocoreximine inhibited cell viability of the breast cancer cell line (MCF-7) by 85.76%, human colorectal carcinoma cell line (HCT-15) by 63.05%, human prostate tumor cell line (PC-3) by 78.71%, human astrocytoma cell line (U-251) by 65.23% and human leukemia cell line (K-562) by 94.15% [117].

The antiproliferative activity of methanolic extracts from the leaves and seeds of *A. coriacea* was tested in vitro against a range of human tumor cell lines; including melanoma (UACC-62), non-small cell lung cancer cells (NCI-H460), colon cancer cell line (HT29), breast cancer (MCF-7) and leukemia (K-562). The seed extracts displayed potent antitumor activity with GI<sub>50</sub> values between 0.02 and 3.83 µg/mL, and the leaf extracts exhibited anticancer activity at concentrations ranging from 0.02 to 0.08 µg/mL [37, 177]. The cytotoxicity of annonacin, found in many *Annona* species, has been reported against various cell lines derived from cervical cancer (HeLa and HeLa S3) with IC<sub>50</sub> 0.219 and 0.426 µg/mL, and ovarian cancer (PA-1 and SKOV3) with IC<sub>50</sub>s of 0.452 and 0.411 µg/mL [178]. The cytotoxicity of annonacin has also been demonstrated against bladder cancer (T24), breast cancer (MCH7) and skin cancer (BCC-1) with IC<sub>50</sub>s 0.324, 0.433 and 0.427 µg/mL, respectively [178]. The cytotoxicity of five other acetogenins (squamocin M, annofolin, isolongimicin B, glaucanisin, and

annotacin) isolated from *A. cornifolia* against human breast cancer (MCF-7) was reported, with  $IC_{50}$ s of approximately 0.3  $\mu$ M [179]. In an in vitro study, the annaceous acetogenins laherradurin and cherimolin-2 isolated from *A. diversifolia* were shown to have  $ED_{50}$ s of 0.015 and 0.05  $\mu$ g/mL, respectively, against the cervical cancer cell line (HeLa) [180].

In clinical studies, the anticancer activity of *A. muricata* has been reported in a small number of studies. A patient diagnosed with breast cancer has maintained stable disease activity with no reported side effects after using an aqueous extract of *A. muricata* leaves for more than five years [181]. Another patient with metastatic ovarian cancer experienced disease stability after starting to take a complementary medication containing *A. muricata* as a tablet [182]. Finally, the effect of *A. muricata* leaves extract revealed higher cytotoxicity in the supplemented group with colorectal cancer compared with the placebo group in a randomized controlled trial [183].

### 1.8.3. Antidiabetic and Antilipidemic Activity

Multiple studies have investigated the antidiabetic activity of various extracts from *Annona* plants such as *A. cherimola*, *A. squamosa*, *A. muricata* and *A. reticulata*. The ethanolic leaf extract of *A. cherimola* (300 mg/kg) was administered to alloxan-induced type 2 diabetic rats, and four hours later, blood glucose level had decreased from 331.5 mg/dl to 149.2 mg/dL [63]. The young leaves of *A. squamosa*, often in combination with black pepper (*Piper nigrum*), have been used in northern Indian traditional medicine as an anti-diabetic, and are still in use today. Administration of aqueous *A. squamosa* leaf extract to streptozotocin-nicotinamide type-2 diabetic rats resulted in decreased blood glucose and increased levels of serum insulin [184]. Another traditional Indian medicine used as an antidiabetic and anti-lipidemic is a polyherbal formulation of *A. squamosa* fruits and *Nigella sativa* seeds. The polyherbal formulation administered over a one-month period, dose-dependently decreased blood glucose

and increased insulin in streptozotocin-induced diabetic rats, with a dose of 200 mg/kg showing similar to the effects of a dose of 250 mg/kg tolbutamide [185]. A single dose of 100 or 200 mg/kg of aqueous leaf extract of *A. muricata* did not inhibit blood glucose levels in normal rats; however, the same doses administered to the diabetic rats effectively lowered blood glucose levels by 31.77% and 45.77%, respectively [186]. Finally, a dose of 100 mg/kg of both methanolic extract and the residual fractions of *A. reticulata* leaves decreased blood glucose levels from 432.33 to 371.67 mg/dL and 417.83 to 402.50 mg/dl, respectively, in streptozotocin-induced diabetic rats [187]. *A. cherimola* leaf extract was also found to decrease HbA1c by 7% and lead to a significant decrease in urine glucose over a 28-day subchronic study in streptozotocin-induced diabetic mice [188]. These studies support the traditional use of *Annona* species as antidiabetics, suggesting that further identification of the active constituent (s) with antidiabetic properties and clinical studies of a longer duration are warranted.

Limited studies have also investigated the antilipidemic activity of some *Annona* species. The polyherbal formulation of *A. squamosa* fruits and *Nigella sativa* seeds (200 mg/kg) administered to streptozotocin-induced diabetic rats for one month also resulted in significant inhibition of the formation of both lipid peroxide and tissue lipids [185]. Administration of an extract of *A. muricata* extracts resulted in reductions in the serum total cholesterol, low-density lipoprotein cholesterol and triglycerides in diabetic rats [189]. The tea infusion of leaves from *A. cherimola* (1.5 g) also elicited a reduction in the total cholesterol, triglycerides, and low-density lipoprotein by 15.4, 21.9 and 63.2%, respectively, in streptozotocin-induced diabetic rats [188].

#### 1.8.4. Anti-Inflammatory Activity

The anti-inflammatory effects of *Annona* plants have been reported in many studies; for instance, after one day of orally administered doses of 200 and 400 mg/kg of *A. squamosa* root extract in an acute carrageenan-induced rat paw edema model, significant inhibition was produced with 24% and 47% inhibition respectively compared to diclofenac sodium inhibiting inflammation by 72% [190]. In an in vitro study, the chloroform extract of *A. muricata* leaves significantly inhibited activity of phospholipase A<sub>2</sub> [191]. With doses of 0.2–0.6 mg/mL, the enzyme activity was inhibited by 23.91%–43.48% [191]. In the same study, the chloroform extract of *A. muricata* leaves at 0.5 and 1.0 mg/mL also inhibited prostaglandin synthase activity by 87.46% and 82.92%, respectively, compared to the positive control indomethacin at 1 mg/mL which reduced enzyme activity by 87.46% [191].

Extracts of *A. senegalensis* roots were assessed for anti-inflammatory activity through in vitro inhibition of protein denaturation, hyaluronidase and xanthine oxidase. The ethyl acetate fraction was found to have the greatest activity inhibiting protein denaturation (70.6%), hyaluronidase (72.2%) and xanthine oxidase (78.7%) at a concentration of 100 µg/mL [192]. The ethanolic extract of *A. muricata* leaves exhibited anti-inflammatory effects in the carrageenan-induced rat paw acute edema model. Paw edema was reduced after orally administered doses of 200 and 400 mg/kg with (23.16% and 29.33%) and (29.50% and 37.33%), respectively, after 60 and 90 min of treatment [193]. Additionally, *A. muricata* fruit has been shown to exert an anti-inflammatory effect in a xylene-induced ear edema test [194]. Additional information regards pharmacological activities of *Annona* species, see (Table 1.7).

The lyophilized fruit extract at 50 mg/kg and 100 mg/kg inhibited the xylene-induced ear edema by 82.35% and 76.47%, respectively, compared to prednisolone, which reduced ear edema by 47.06% [194]. At intraperitoneal doses of 25, 50 and 100 mg/kg, the ethanolic extract

of *A. vepretorum* leaves, inhibited carrageenan-induced leukocyte migration to the peritoneal cavity by 62%, 76% and 98%, compared to dexamethasone (2 mg/kg, i.p.). which reduced leukocyte migration by 89% [109]. The flavonoids, quercetin and kaempferol isolated from leaves of *A. dioica* exhibited potent dose- and time-dependent anti-inflammatory activity in a carrageenan-induced paw oedema model with IC<sub>50</sub>s of 8.53 and 10.57 µg/mL, respectively. The crude methanolic extract of *A. dioica* also reduced myeloperoxidase activity 6 h after the induction of paw oedema with a maximal inhibition of 51% at a dose of 300 mg/kg. [195]. Finally, hinesol, z-caryophyllene and beta-maaliene isolated from leaves of *A. sylvatica* also inhibited leukocyte migration at concentrations from 36.04 to 45.37 µg/mL in both carrageenan- and complete Freund's adjuvant-induced mouse paw edema [35].

#### **1.8.5. Antioxidant Activity**

An extract of *A. coriacea* seeds was investigated for its antioxidant activity using free radical 2,2-diphenyl-1-picrilhydrazil (DPPH) and bleaching of β-carotene, and a moderate antioxidant effect was reported of 31.53% in the DPPH test and 51.59% for the β-carotene bleaching test [196]. The pulp of *A. coriacea* fruit displayed a weaker antioxidant activity compared to the seeds, with 13.49% for the DPPH test and 32.32% for the β-carotene assay [196]. Additionally, various parts of *A. muricata*, including bark, leaves and stem, exhibited antioxidant activity using DPPH assay and the EC<sub>50</sub> value was recorded as 90 mg/g for bark, 290 mg/g for leaves, and 116 mg/g for the stem, compared to ascorbic acid with 157.5 mg/g [48]. Finally, an ethanolic extract of *A. squamosa* leaves was also reported as having antioxidant activity when evaluating DPPH, nitric oxide and superoxide radical assays. The activity was reported as 75.12%, 34.69% and 10.29%, respectively, at a concentration of 100 µg/mL [197].

### 1.8.6. Antileishmanial Activity

Many extracts and pure compounds from *Annona* plants have been tested against *Leishmania*, such as methanolic seed and leaf extracts of *A. squamosa*, for activity against *L. amazonensis*, with resulting showing IC<sub>50s</sub> of 46.54 µg/mL and 28.32 µg/mL, respectively [198]. Alkaloids and acetogenins isolated from both leaves and seeds of *A. squamosa* were also reported for their activity against promastigote forms of *L. chagasi*, with the EC<sub>50</sub> value reported as 23.3 µg/mL for alkaloids and from 25.9–37.6 µg/mL for acetogenins [198]. Alkaloids like liriodenin isolated from the leaves of *A. mucosa* exhibited antileishmanial activity against promastigote forms of *L. braziliensis*, *L. guyanensis* and *L. amazonensis* with IC<sub>50s</sub> of 55.92 µg/mL, 0.84 µg/mL 1.43 µg/mL respectively [199]. Finally, O-methylarmepavine isolated from leaves *A. squamosa* displayed antileishmanial activity against both promastigote and amastigote forms of *L. chagasi* with EC<sub>50s</sub> of 23.3 µg/mL and 25.3 µg/mL, respectively [49].

### 1.8.7. Antiviral Activity

The antiviral activity of various *Annona* species was reported in several studies using either whole extract or pure compounds. For instance, 16β,17-dihydroxy-*ent*-kauran-19-oic acid was isolated from the fruits of *A. squamosa* and showed significant activity against human immunodeficiency virus (HIV) replication using H9 lymphocyte cell assay with EC<sub>50</sub> value of 0.8 µg/mL [200]. The ethanolic extract of *A. squamosa* seeds at 0.15 µg/mL, 0.25 µg/mL and 0.35 µg/mL also exhibited dose-dependent antiviral activity against the Avian influenza virus with the percentage of antiviral activity at 33.33%, 43.06% and 59.72%, respectively [201]. The leaves of *A. squamosa* extract were also tested against dengue virus type-2 (DENV-2) in Vero cells using Viral ToxGLO™ assay. At a concentration of 6.25 µg/mL, DENV-2 replication was reduced with IC<sub>50</sub> 73.78 µg/mL in Vero cells [202]. The methanolic extracts from the peels of *A. squamosa* and *A. reticulata* demonstrated antiviral activity against human

immunodeficiency virus 1 (HIV-1) using a non-radioactive immune/colorimetric assay. Both *A. squamosa* and *A. reticulata* revealed high antiviral activity by inhibition of HIV-1 reverse transcriptase with values of 96.45% and 78.63% [203]. Moreover, *A. cherimola* was also evaluated for its antiviral activity against herpes simplex virus type 2 (HSV-2) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The leaf extract inhibited HSV-2 replication and showed antiherpetic activity with a therapeutic index 8.40 [204]. Finally, the ethanolic stem extraction of *A. muricata* demonstrated antiviral activity against herpes simplex virus type 1 (HSV-1) with a minimum inhibitory concentration of 1 mg/mL [205].

### 1.9. Pharmacological Activity of Isolated Compounds from *Annona* Species and their Mechanism of Action

The in vitro and in vivo biological activity of compounds that have been isolated from various parts of *Annona* plants will be discussed. Squamins C–F were isolated from the seeds of *A. globifera* and tested in vitro against trophozoites of *Acanthamoeba* spp. strains such as *A. castellanii* Neff, *A. polyphaga*, *A. griffin* and *A. quina* (Table 1.8) [206]. All tested compounds exhibited antiamoeboid activity against the strains by inducing programmed cell death [206]. The same compounds were also tested for their cytotoxicity against murine macrophage cell line J774A.1 (ATCCTIB-67) and showed no cytotoxicity effect with CC<sub>50</sub> values greater than 200  $\mu$ M [206].

Table 1.8. Antiamoebic activity of squamins C-F versus *Acanthamoeba* spp. Strains.

| Compound  | <i>A. castellanii</i> Neff<br>IC <sub>50</sub> ( $\mu$ M) | <i>A. polyphaga</i><br>IC <sub>50</sub> ( $\mu$ M) | <i>A. griffin</i><br>IC <sub>50</sub> ( $\mu$ M) | <i>A. quina</i><br>IC <sub>50</sub> ( $\mu$ M) |
|-----------|-----------------------------------------------------------|----------------------------------------------------|--------------------------------------------------|------------------------------------------------|
| Squamin C | 20.77 $\pm$ 3.48                                          | 71.78 $\pm$ 0.41                                   | 38.81 $\pm$ 7.34                                 | 24.28 $\pm$ 0.64                               |
| Squamin D | 18.38 $\pm$ 1.14                                          | 71.57 $\pm$ 0.14                                   | 39.53 $\pm$ 5.90                                 | 26.52 $\pm$ 0.87                               |
| Squamin E | 21.00 $\pm$ 0.86                                          | 62.19 $\pm$ 15.52                                  | 44.75 $\pm$ 2.06                                 | 25.82 $\pm$ 0.99                               |
| Squamin F | 18.02 $\pm$ 3.28                                          | 64.08 $\pm$ 12.42                                  | 50.49 $\pm$ 6.92                                 | 30.320.27                                      |



Rollinicin and rolliniastatin-1 isolated from the seed of *A. mucosa* were also reported for their larvicidal effect against *Aedes aegypti* and *Aedes albopictus* larvae [207]. Rolliniastatin-1 exhibited the best larvicidal effect against both *Aedes aegypti* and *Aedes albopictus* with LC50s of 0.43 and 0.20  $\mu\text{g/mL}^{-1}$ , respectively. Rollinicin displayed similar activity against *Aedes aegypti* and *Aedes albopictus* with LC50s of 0.78  $\mu\text{g/mL}$  and 1.128  $\mu\text{g/mL}$ , respectively [207]. However, the larvicidal mechanism action of these compounds was not reported. Annonacin isolated from the seed of *A. muricata* was evaluated for its larvicidal activity on *Aedes aegypti* and *Aedes albopictus* larvae [208]. The greater larvicidal activity was reported against *Aedes aegypti* with a LC50 of 2.65  $\mu\text{g/mL}$  compared to *Aedes albopictus* with LC50 of 8.34  $\mu\text{g/mL}$ . The mechanism of action was reported as being inhibition of their metabolic enzymes, particularly proteases and amylases that are important for the development of *Aedes* spp. larvae [208]. Twelve acetogenins isolated from the seed of *A. cornifolia* were tested for their antioxidant activity against DPPH [95]. These acetogenins were identified as 9-hydroxyfolianin, 4-desoxylongimicin, squamocin M, squamocin L, folianin A, folianin B, annofolin, isolongimicina B, bullatacin, asimicin, cornifolin and anotacin, and showed a strong DPPH radical scavenging with IC<sub>50</sub>s ranging from  $0.99 \pm 0.18$  to  $1.95 \pm 0.34$   $\mu\text{g/mL}$  compared to ascorbic acid with an IC<sub>50</sub>  $1.62 \pm 0.35$   $\mu\text{g/mL}$  [95]. It has been suggested that the antiradical activity of acetogenins may be related to the  $\alpha,\beta$ -unsaturated lactone ring moiety, which is also present in ascorbic acid [95]. Furthermore, the antioxidant activity of pure compounds from the bark of *A. salzmanni* after isolation of five alkaloids identified as liriodenine, anonaine, asimilobine, reticuline and cleistopholine [209]. The antioxidant activity was assessed through the Oxygen Radical Absorbance Capacity (ORAC) assay and asimilobine was found to be the most active alkaloid with ORAC value of 2.09 [209]. The rest of the compounds exhibited antioxidant activity with ORAC values ranging from 0.25 to 0.85 [209]. These same compounds were also examined for their antimicrobial activity against *Kocuria rhizophila*,

*Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis* with MIC values from 25 to 100 µg/mL [209]. In an in vitro study, the antimicrobial activity of isolated alkaloids from aerial parts of *A. senegalensis* was assessed in a microdilution assay [159]. These alkaloids were identified as anonaine and asimilobine, and demonstrated against *Streptococcus mutans* with MIC values of 0.12 and 0.25 mg/mL, respectively [159]. For trypanocidal activity, three alkaloids liriodenine, annomontine, and O-methylmoschatoline were isolated from the branch of *A. foetida* and tested against both epimastigote and trypomastigote forms of *Trypanosoma cruzi* [106]. A potent trypanocidal effect was demonstrated against epimastigote forms with IC<sub>50</sub>s ranging from 92.0 ± 18.4 to 198.0 ± 4.2 µg/mL, and from 3.8 ± 1.8 to 4.2 ± 1.9 µg/mL for trypomastigote forms [106]. Additionally, N-hydroxyannomontine isolated from the bark of *A. foetida*, demonstrated antileishmanial activity versus *Leishmania. braziliensis* and *L. guyanensis* with IC<sub>50</sub> values of 252.7 ± 2.2 and 437.5 ± 2.5 µM, respectively [210].

Many compounds isolated from various *Annona* species have demonstrated cytotoxicity against different cancer cell lines (Table 1.9). Three alkaloids were isolated from leaves of *A. crassiflora* identified as crassiflorine, xylopine and stephalagine and tested for their activity against colon carcinoma cells (HCT-116) using MTT assay [210]. The cytotoxicity activity for the tested compounds was reported with IC<sub>50</sub> values of 143.4 µM, 30.2 µM and 48.5 µM, respectively [210]. Muricin J–K isolated from the fruit of *A. muricata* exhibited anticancer activity against human prostate cancer cell lines (PC-3) through inhibition of the mitochondrial complex I in an in vitro study [211]. The anticancer activity was also reported for the alkaloid coclaurin isolated from aerial parts of *A. squamosa*. Cytotoxicity studies against human breast cancer cells (MCF-7), human colon cancer cells (HCT116) and human liver cancer cells (HEPG-2) reported IC<sub>50</sub> values of 15.345 µg/mL, 8.233 µg/mL and 1.674 µg/mL, respectively [212]. Bullatacin isolated from *A. cherimola* demonstrated inhibition of tumor growth at a dose

of 15 µg/kg in mice bearing HepS and S180 xenografts and tumor growth was reduced by 63.4% and 65.8%, respectively [213]. In the same study, annonacin administrated orally (10 mg/kg) to hybrid mice (BDF-1) models significantly reduced lung cancer by 57.9% [213]. However, the mechanism of action of these acetogenins was not described in this study. Finally, stephalagin, an alkaloid isolated from the peel of *A. crassiflora* fruit, was reported as pancreatic lipase inhibitor with an  $IC_{50}$  of  $8.35 \mu\text{g/mL}^{-1}$  in vitro study [214].

Table 1.9. Anticancer activity of isolated compounds from *Annona* species and their mode of action.

| <b><i>Annona</i> Species</b> | <b>Plant Part</b> | <b>Isolated Components</b>                    | <b>Cell Line or Animal Model</b>                                                               | <b>Mechanism of Action</b>                                               | <b>References</b> |
|------------------------------|-------------------|-----------------------------------------------|------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|-------------------|
| <i>A. cherimola</i>          | Seeds             | Annomolin and Annocherimolin                  | Prostate tumor cell line (PC-3), breast (MCF-7) and colon (HT-29) cancer cell lines            | Exhibited potent cytotoxicity                                            | [215]             |
|                              | Leaves            | Asimilobine                                   | Acute myeloid leukemia cell line                                                               | Upregulation of Bax, downregulation of Bcl2, and cleavage of PARP        | [216]             |
| <i>A. crassiflor</i>         | Crude extract     | Catechin                                      | Cervical cancer cell                                                                           | Apoptosis via intrinsic pathway                                          | [217]             |
| <i>A. glabra</i>             | Fruits            | Annoglabasin H                                | Lung adenocarcinoma cell line (LU-1), human breast carcinoma (MCF-7), human melanoma (SK-Mel2) | Exhibited significant cytotoxic activity                                 | [218]             |
|                              |                   | Annoglabayin                                  | Human liver cancer cell line (Hep G2)                                                          | Apoptosis via mitochondrial pathway                                      | [137]             |
|                              |                   | Cunabic acid and ent-kauran-19-al-17-oic acid | Liver cancer (HLC) cell line SMMC-7721                                                         | Apoptosis via down-regulation of BCL-2 gene and upregulation of bax gene | [219]             |
|                              | Leaves            | Asinicin                                      | Human monocytic leukemia cells (CRL-12253)                                                     | Mitochondria mediated anticancer and antiproliferative effects           | [220]             |
|                              |                   | Annoglacin A and B                            | Human breast carcinoma (MCF-7) and Pancreatic carcinoma (PACA-2) cell lines                    | Suppressed proliferation                                                 | [221]             |

|                      |        |                              |                                                                     |                                                                                                         |       |
|----------------------|--------|------------------------------|---------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------|-------|
|                      |        | Icariside D2                 | Human leukemia cell line (HL-60)                                    | Induced apoptosis and decreased phosphorylation of AKT in cells                                         | [222] |
| <i>A. muricata</i>   | Leaves | Annomuricin                  | Breast cancer cell                                                  | Suppressed breast cancer proliferation and induced apoptosis                                            | [223] |
|                      |        | Muricoreacin, Murihexocin    | Colon cancer cell (HT-29, HCT-116)                                  | Up-regulation of Bax, downregulation of Bcl-2 proteins and activated initiator and executioner caspases | [224] |
|                      |        | Annomuricine, Muricapentocin | Pancreatic carcinoma (PACA-2) and colon adenocarcinoma (HT-29) cell | Exhibited repressive effect                                                                             | [225] |
|                      |        | Muricatocins A and B         | Lung tumor cell line (A-549)                                        | Enhanced cytotoxic activity                                                                             | [151] |
|                      | Fruits | Muricin M and Muricin N      | Prostate cancer (PC-3) cells                                        | Exhibited cytotoxicity                                                                                  | [226] |
| <i>A. purpurea</i>   | Roots  | Annopurpuricins A–D          | HeLa and HepG2 cells                                                | Mitochondrial membrane depolarization and apoptosis                                                     | [227] |
| <i>A. reticulata</i> | Fruits | Catechin                     | Breast cancer cell line (MCF-7)                                     | Inhibition via apoptosis                                                                                | [228] |
|                      | Seeds  | Annonacin                    | T24 bladder cancer cells                                            | Bax expression was induced, caspase-3 activity enhanced and caused apoptosis                            | [178] |
|                      |        | Bullatacin                   | Leukemia cell line (K562) and breast cancer cell line (MCF-7)       | Cell death via apoptosis                                                                                | [229] |

|                        |        |                                                          |                                                                                            |                                                                                                                                    |       |
|------------------------|--------|----------------------------------------------------------|--------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------|-------|
|                        | Leaves | Annomonicin                                              | Colon cancer (HCT15), human lung cancer (Hop65) and human hepatoma (HEPG2) cell lines      | Exhibited cytotoxic effect                                                                                                         | [230] |
|                        |        | Rolliniastatin                                           | Breast cancer cell (T-47D)                                                                 | Caspases dependent apoptosis                                                                                                       | [231] |
| <i>A. senegalensis</i> | Leaves | (–) Roemerine                                            | Breast cancer MDA-MB-231 cells                                                             | Exhibited dose-dependent cytotoxicity via targeting the ribosomal protein eL42 and arresting the crosslinking reaction with tRNAox | [232] |
|                        | Bark   | Kaurenoic acid                                           | Pancreatic tumor (PANC-1) cell lines and Henrietta Lacks' cervical cancer cell line (HeLa) | Exhibited significant cytotoxic activity                                                                                           | [233] |
|                        | Stem   | Ent-kaurenoids                                           | Breast cancer (MCF-7) cells, prostate cancer (PC-3) cells                                  | Exhibited significant cytotoxic activity                                                                                           | [234] |
| <i>A. squamosa</i>     | Leaves | Annoreticuin                                             | Breast cancer cell (MCF-7)                                                                 | Induced Apoptosis                                                                                                                  | [235] |
|                        | Seeds  | Dieporeticenin B, Squamocin, Annosquatin III Asimilobine | Nasopharyngeal cancer (KB) cells, breast cancer (MCF-7) cells                              | Exerted inhibitory activity                                                                                                        | [236] |
|                        |        |                                                          | Human colon cancer cell (WiDr)                                                             | Increased expression of caspase-3                                                                                                  | [237] |
|                        |        | Annosquatin A, B                                         | Human leukemia cell line (K-562), human colon carcinoma (COLO-205)                         | Reduced intracellular glutathione levels and regulation of Bcl-2 and PS externalization                                            | [238] |
|                        |        | Annosquacins A-D, Annosquatin A, B                       | Human breast cancer cell line (MCF-7), human lung                                          | Exhibited cytotoxic activity                                                                                                       | [239] |

|                                  |        |                                                                                            |                                        |                                                                                                                      |       |
|----------------------------------|--------|--------------------------------------------------------------------------------------------|----------------------------------------|----------------------------------------------------------------------------------------------------------------------|-------|
|                                  |        |                                                                                            | adenocarcinoma cell line<br>(A-549)    |                                                                                                                      |       |
|                                  |        | (-)-Anonaine                                                                               | H22 solid tumor cell                   | Inhibition of IL-6/Jak/Stat3<br>pathway                                                                              | [240] |
|                                  | Bark   | Coclaurine                                                                                 | DMBA painted hamsters                  | Enhanced lipid peroxidation                                                                                          | [241] |
|                                  | Fruits | (-)-Ent-kaur-16-en-19<br>oic acid, 16 $\alpha$ ,17<br>dihydroxy-ent-kauran-<br>19-oic acid | Dalton's lymphoma cells,<br>HeLa cells | Exhibited cytotoxic activity                                                                                         | [242] |
| A. <i>sylvatica</i><br>A.St.-Hil | Leaves | Quercetin<br>Kaempferol                                                                    | Anti-inflammatory                      | Leukocytes migration was<br>significantly reduced at IC <sub>50</sub><br>8.53 and 10.57 $\mu$ g/mL,<br>respectively. | [195] |
| A. <i>vepretorum</i><br>Mart.    | Leaves | Bicyclogermacrene                                                                          | Antimicrobial                          | Against <i>Candida tropicalis</i><br>with a MIC value of 100 $\mu$ g<br>$\cdot$ mL <sup>-1</sup> .                   | [243] |

## 1.10. Toxicity and Interactions

Generally, the safety of natural medicines can be assessed according to their effects and drug-drug interactions. An epidemiological study has reported that consumption of fruits of Annonaceae led to the prevalence of atypical parkinsonism in Guadeloupe due to the presence of acetogenins in the plant fruits [244]. According to Champy et al. 2005, the amount of annonacin per a single fruit is approximately 15 mg, and an adult who consumes a daily intake of one fruit for one year is equivalent to the amount of annonacin injected into rats, which induced the brain lesions [245]. It has been suggested that the toxicity might be related to the capacity of the tetrahydrofuran ring to chelate calcium ions [37]. Moreover, the fruit of *A. squamosa* has been analysed for its quantity of squamocin using HPLC-MS and reported 13.5–36.4 mg of squamocin for each fruit, and that long-term consumption of *A. squamosa* fruit may be a risk factor in the development of neurodegenerative disorders [246]. Additionally, the use of a dietary supplement sold in the USA containing an extract of *A. muricata* has been found to exhibit neurotoxic effects in human neuron cultures [247].

The interactions of *Annona* species with other drugs have been reported in other studies; for instance, administration of capsules of *A. muricata* leaves in combination with glibenclamide resulted in improved glycaemic control compared to patients who received only glibenclamide [248]. An additional study reported that a combination of aqueous custard apple leaf extract and glipizide enabled a decrease in the dose of glipizide by up to half in rats with type-2 diabetes and reduced the risk of requiring insulin therapy [249]. These outcomes suggest the potential use of certain *Annona* species in conjunction with antidiabetic medications to maximize the efficacy of a lower therapeutic dose.



### 1.11. Conclusion

This review provides a comprehensive summary of the botanical features, ethnomedical uses, pharmacology and phytochemistry of the main species of Annonaceae family and, in particular, the *Annona* genera used in traditional medicine practices. Of the many members of the Annonaceae family, the *Annona* species is heavily used in traditional medicines across the world. Among the 30 reviewed *Annona* species, six species *A. squamosa*, *A. muricata*, *A. cherimola*, *A. senegalensis*, *A. reticulata* and *A. coriacea* are the most widely studied for their pharmacological activities and phytochemical profiles of their bark, leaves, fruits and seeds. Various pharmacological properties have been reported, including antidiabetic, hepatoprotective, anti-inflammatory, antiprotozoal, antitumor, antioxidant, antimicrobial and anticonvulsant activity.

With regard to the phytochemistry of *Annona* species, the main classes of constituents identified to date are acetogenins, alkaloids, phenols and essential oils. The alkaloids are mainly present in the leaves, whereas acetogenins are present in the seeds and found in smaller quantities in the pulp and leaves of *Annona* species. The chemical profiles of the acetogenins present in different species have been extensively studied and their anticancer activity investigated, with low concentrations exhibiting chemotoxicity against several cancer cell lines. These preclinical results, along with the reported case studies, suggest that further clinical studies evaluating the role of acetogenins in the treatment of various types of cancers are warranted. Importantly, formulations, including the parts of the *Annona* species used, agricultural practices and the extraction methods vary considerably, leading to likely variations in the phytochemical and pharmacological profiles. In this respect, further characterization of standardized formulations of *Annona* species is required to predict likely clinical effects. Additional interesting results on the antidiabetic effects of fruits from *Annona* species also warrant further investigation as nutraceuticals to assist in the therapy of diabetes.

## CHAPTER 2

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# **The phytochemical constituents and pharmacological activities of Annona atemoya: A systematic review.**

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## Abstract

*Annona atemoya* also known as the custard apple is a hybrid between two Annonaceae species: Cherimoya (*Annona cherimola*) and the sugar apple (*Annona squamosa*). It is widely cultivated in tropical and subtropical continents including north and south America, Asia, Africa and Australia. Despite becoming an increasingly important commercial fruit plant due to its' creamy succulent flesh, compared to other Annonaceae species relatively few studies have investigated the phytochemistry and bioactivities of *A. atemoya*. Studies that evaluated *A. atemoya* extracts and its constituents were searched through the databases Scopus, PubMed and Embase from inception to June 2020. Constituents of *A. atemoya* include alkaloids, flavonoids, terpenes and acetogenins. The results indicate that the constituents of *A. atemoya* possess cytotoxic, anti-angiogenic, hypolipidemic, antioxidant, anti-inflammatory and neuroprotective activities. However, many of these studies are currently limited in quality and further phytochemical and pharmacological studies are required.

**Keywords:** *Annona atemoya*; Custard apple; Nutraceutical; Phytochemistry; Bioactivity; Pharmacological activity.

## 2.1. Introduction

*Annona atemoya* is a commercially important fruiting plant belonging to the Annonaceae family [250]. *A. atemoya*, is widely cultivated in tropical and subtropical continents including southern and northern America, Asia, Africa and Australia [113], Spain and Israel [251]. *A. atemoya* is a hybrid between two Annonaceae species: Cherimoya (*Annona cherimola*) and the sugar apple (*Annona squamosa*). Hybridization was conducted by P. J. Webster in Florida (USA) between 1907 and 1908 [113, 251]. However, natural hybridization is also believed to have occurred between 1850 and 1930 in Australia and Palestine [251]. The cultivars vary between countries e.g. United States of America (Bradley, Page, Keller, Priestly, Stremer and Caves) [113]; Brazil (African Pride, Pink's Mammoth, Thompson and Gefner) [252]; Australia (Island Gem, Nielsen, Hillary White, Maroochy Gold, Pink's Mammoth and African Pride) [113, 253]; Israel (Jennifer, Kabri, Malalai, African Pride and Gefner) [113] and Thailand (Golden Flesh and Pet Pakchong) [253].

*A. atemoya* grows to approximately 7.5 - 10 m, closely resembling the plant of *A. cherimoya* and it is characterised by its short-bunked and low drooping branches [113, 251]. The leaves of *A. atemoya* are alternate, leathery, deciduous and less hairy than those of the parent cherimola, averaging 15 cm in length [113]. The flowers are triangular, long stalked and yellow in colour, approximately 6 cm long and 4-5 cm in width. The fruit is fleshy, pale to bluish green, and variable shape from heart-shaped to round (2-2.5 kg), with a bumpy skin and its surface covered with prominent angular areoles that have some protuberances [113, 251]. The fruits are also characterized by a pleasant flavour, the flesh is white and the pulp can be easily separable from the seeds which are smooth and brown to black in colour [250, 251, 253, 254]. In Australia, this hybrid is commonly known as the "Custard apple" and the increasingly popular fruit is available in retail food outlets [251, 255]. Various *Annona* species such as

*Annona muricata* have documented indications for use in traditional and complementary medicine practice [256]. As a relatively recent hybrid, traditional uses of *A. atemoya* are lacking, with some limited anecdotal evidence that the leaves of *A. atemoya* are purchased from growers for making teas. Several phytochemical constituents have been isolated from different parts of the *A. atemoya* plant and assessed for their biological potential in both *vivo* and *vitro* studies [257-260]. The leaves, fruits and seeds of *A. atemoya* are the most widely studied for their chemical and pharmacological properties. Although *A. atemoya* is a hybrid of *A. squamosa* and *A. cherimola* and both species have reported uses in traditional and folkloric medicine [11, 258, 261, 262], to date only limited studies have investigated the phytochemistry and bioactivities of *A. atemoya*. The aim of this review was to systematically search the literature to identify studies reporting on the phytochemical constituents and bioactivity of *A. atemoya*.

## 2.2. Methodology

### 2.2.1. Search strategy

A systematic search of the literature published from inception to 9 June 2020 was performed by using the three electronic databases Scopus, PubMed and Embase using the search terms: “*Annona atemoya*” OR “custard apple” OR acetogenins OR bullatacin AND phytochemistry OR “phytochemical constituents” AND “pharmacological effects” OR “biological activities” OR anti-cancer OR anti-bacterial OR antioxidant OR anti-angiogenic AND toxicity OR toxicology.

### 2.2.2. Inclusion and exclusion criteria

Studies reporting on the pharmacological properties of isolated compounds or whole extracts

of *A. atemoya*, and/or studies which examined the phytochemical constituents of any parts of the plant (aerial part, leave, fruit, seed, stem and root) were considered for inclusion in this review. Finally, regards language, both English and non-English language (one Portuguese paper) were included. Articles that discussed isolation or biological activity of acetogenins, synthetic acetogenins and bullatacin were not included unless they had been isolated from *A. atemoya*.

### **2.2.3. Data extraction**

Data extracted included information relating to the pharmacological and phytochemistry of *A. atemoya*: method of extraction, solvent of extraction, analytical techniques used for identifying pure compounds, structure of pure compounds, pharmacological activity of both crude extracts and pure compounds, animal tested, cell line tested, dosage range, route of administration, duration of treatment, *in vivo* or *in vitro* study, and the reported outcomes.

## **2.3. Results**

### **2.3.1. Eligible studies**

As presented in figure 2.1, a total of 1054 abstracts were identified in the initial search. After title and abstract screening and removal of duplicates, 114 studies were further evaluated for key terms and relevance to the aims of the review. Of the 114 abstracts identified, a further 72 articles were removed due to not meeting the inclusion criteria. A total 42 full text articles were evaluated resulting in a further 10 articles being removed due to not reporting pharmacological activity or phytochemical constituents or focusing on an *Annona* species other than *A. atemoya*. A total of 32 articles were included in this review. Of the 32 selected articles, 18 studies examined the phytochemical constituents of various parts of *A. atemoya* and 14 studies investigated the biological activities *in vivo* ( $n = 5$ ), *in vitro* ( $n = 7$ ) and two studies discussed its *in vitro* toxicity.

The quality assessment of animal studies was undertaken using the Animal Research: Reporting of In Vivo Experiments (ARRIVE) quality assessment criteria including ethical statement, study design, experimental procedure, experimental animals, housing and husbandry, sample size, allocating animals to experimental groups, experimental outcomes, statistical methods, baseline data, number of analysed, outcomes and estimation and adverse events [263]. Each ARRIVE quality assessment criteria was assessed as being met or not. Two authors agreed on the inclusion, exclusion criteria and search strategy. This systematic review was conducted by one author and reviewed by two authors.

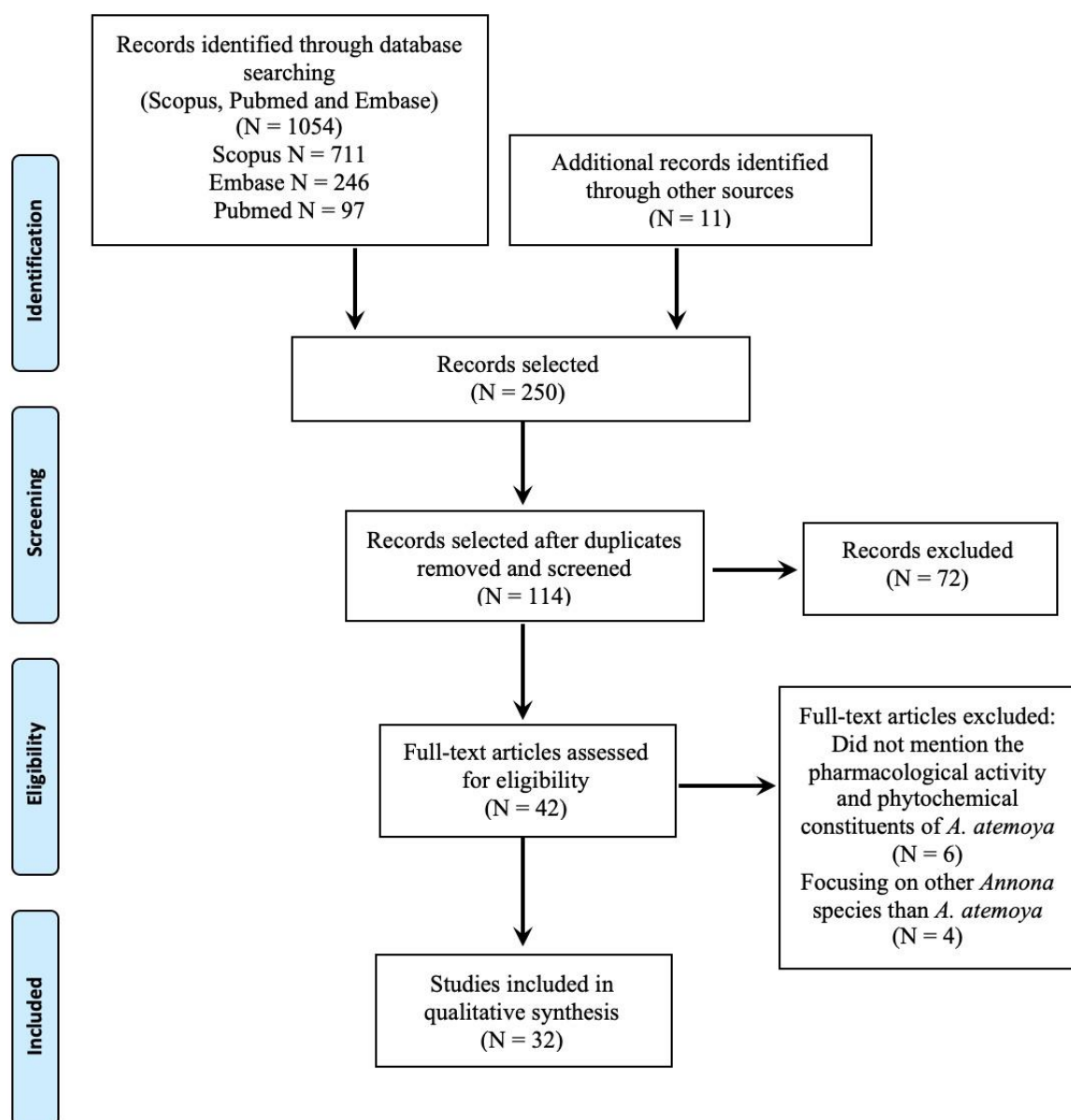


Figure 2.1. Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart of literature search strategy and results.

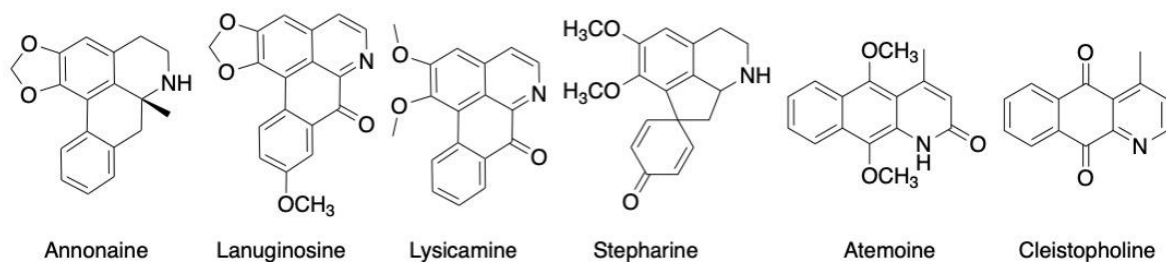
### 2.3.2. *Phytochemicals in A. atemoya*

As presented in appendix B, an extensive number of phytochemicals have been isolated from the parts of *A. atemoya*, the structures of some representative phytochemicals from *A. atemoya* are shown in (Figure 2.2). The fruit has been reported to contain a wide range of volatile oils and phenolic compounds [264-266]. The seeds contain alkaloids and annonaceous acetogenins,

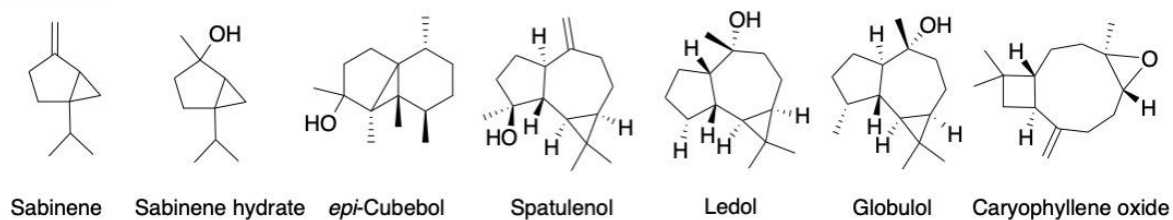


and the leaves contain alkaloids and phenolic compounds [267-273]. *A. atemoya* is rich in the minerals iron, calcium, magnesium, zinc, and vitamins including thiamine, riboflavin, niacin,  $\alpha$ -carotene,  $\beta$ -carotene, cryptoxanthin and ascorbic acid [266, 273-275].

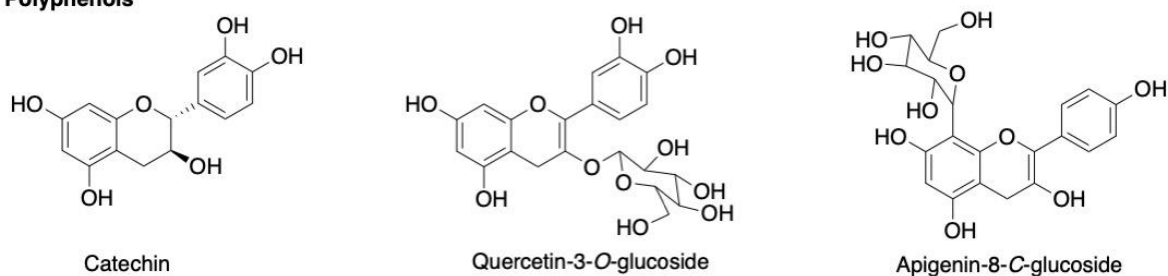
### Alkaloids



### Terpenoids



### Polyphenols



### Acetogenins

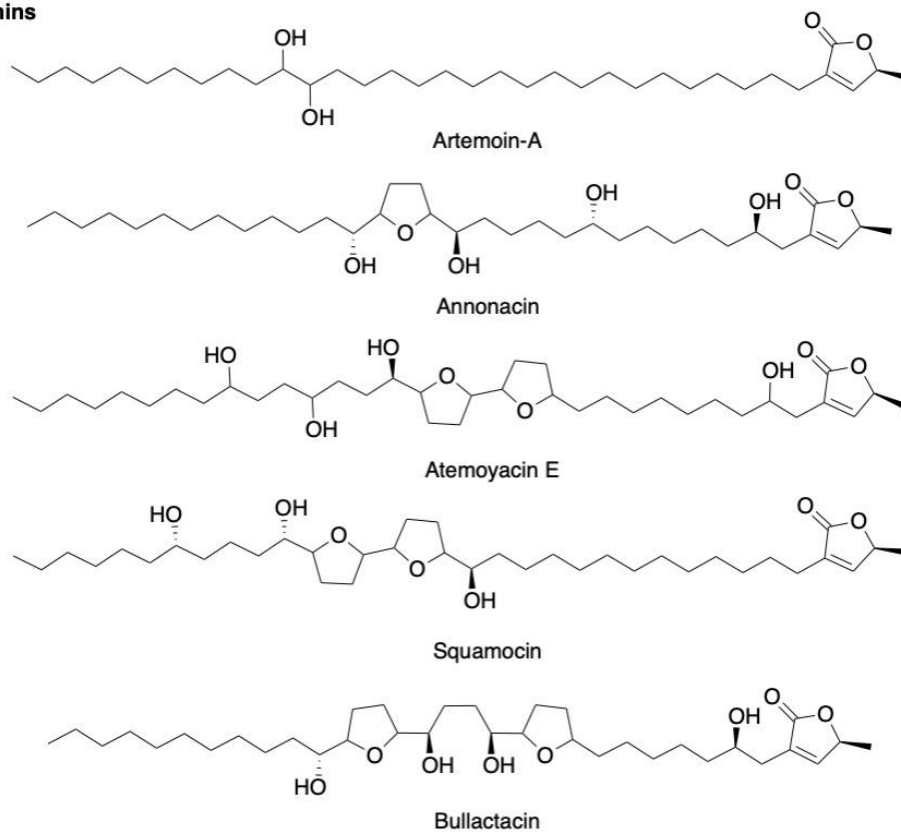


Figure 2.2. Structures of some representative phytochemical constituents of *A. atemoya*.

### 2.3.2.1. Fruits

As the fruit of *A. atemoya* has a unique sweet and custard like aroma when ripe, the volatile constituents of *A. atemoya* have been investigated in many studies and the fruit is found to be the richer in volatiles compared to other parts of the plant [264, 266]. Gas chromatography/mass spectrometry (GCMS) analysis of the essential oils from the mature, fresh and ripe fruits collected in South East Queensland Australia and stored at 25 °C for five days identified 40 phytochemical components [264]. They reported that 94% of the GC peak area contained esters, with methyl butanoate, ethyl butanoate and methyl hexanoate being the predominant constituents. The main terpenes from a total of 25 identified, were  $\alpha$ -pinene,  $\beta$ -pinene, (E)-ocimene and germacrene D [264]. The volatile constituents of *A. atemoya* were also examined in various studies and suggested that both mono and sesquiterpene hydrocarbons were the main class of aromatic volatiles with their proportion being between 77% and 80% of the total volatiles, and the main compounds identified in these studies were,  $\alpha$ -pinene (70.4 ppm), limonene (44.2 ppm),  $\beta$ -pinene (27.3 ppm), germacrene D (20.0 ppm) and bornyl acetate (14.6 ppm) [254, 255, 264]. Additionally, another study reported the volatile constituents isolated from immature fruits of *A. atemoya* by various drying methods including solar drying and oven drying at 30 °C and at 50 °C [265]. The results of this study showed some similarities to the previously mentioned studies. Oven drying at 50 °C led to more volatile compounds being identified compared to the other methods. Both mono and sesquiterpene hydrocarbons were the largest group of phytochemicals identified [265].

Using liquid chromatography mass spectrometry/mass spectrometry (LC -MS/MS), the fruit of *A. atemoya* was shown to contain phenolic compounds including epicatechin and catechin at concentrations of 211  $\mu\text{g/g-dw}$  and 38.6  $\mu\text{g/g-dw}$ , respectively [274]. Other compounds were identified at minor concentrations: 3,4-dihydroxybenzoic acid ( $7.75 \pm 0.18 \mu\text{g/g-dw}$ ), chlorogenic acid (1.79  $\mu\text{g/g-dw}$ ) and *p*-coumaric acid (1.77  $\mu\text{g/g-dw}$ ). Caffeic acid and ferulic

acid, rutin and quercetin were not reported in the samples tested in this study [274].

One study on the antitumour effects of bullatacin states that bullatacin is isolated from the fruit of *A. atemoya* [276], yet reference cited the paper by Chang et al (1999) reporting bullatacin as being isolated from the seeds [267]. Given that acetogenins have not been reported as being isolated from *A. atemoya* fruit, it is most likely that the bullatacin was isolated from the seeds of the fruit.

#### 2.3.2.2. Leaves

The alkaloid constituents isolated by extraction with hexane and methanol from the leaves of *A. atemoya* collected in Petrolina in Brazil have been reported [277]. The phytochemical investigation of the methanol extract led to the identification of seven alkaloids: anonaine, asimilobine, lanuginosine, liriodenine, lysicamine, pronuciferine and stepharine were identified by mass spectrometry (MS) and nuclear magnetic resonance (NMR). This was the first time that these alkaloids have been identified in the leaves of *A. atemoya* [277]. A recent study also reported similar compounds isolated from an 3:1 ethanol:water extract of the leaves of a commercial cultivar of *A. atemoya* grown in Sicily, however, the alkaloids anonaine and asimilobine, were not detected which may be because both anonaine and asimilobine are not soluble in ethanol: water solution [60]. *A. atemoya* was also found to have comparable amounts of both oxoaporphine (liriodenine and lanuginoside) and proaporphine (stepharine and pronuciferine) alkaloids in the leaves [60]. In the same study, a number of phenolic compounds were isolated from the leaves of *A. atemoya* such as quercetin-3-*O*-rutinoside-7-*O*-glucoside, quercetin-3-*O*-rutinoside-7-*O*-pentoside, quercetin-3-*O*-rutinoside, kaempferol-3-galactoside-7-rhamnoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, luteolin-3-galactoside-7-rhamnoside, luteolin-3-glucoside-7-rhamnoside, apigenin-8-*C*-glucoside, catechin and

epicatechin [60].

### 2.3.2.3. *Seeds*

Few investigations have been conducted on the chemical constituents of *A. atemoya* seeds. According to many studies, the annonaceous acetogenins are the main phytochemical components of seeds compared to both phenolic and alkaloid constituents [267-272, 278, 279]. The seeds of *A. atemoya* collected in Taiwan, were crushed and extracted with EtOAc and then partitioned to yield aqueous and chloroform (CHCl<sub>3</sub>) extracts [267]. The CHCl<sub>3</sub> layer was concentrated and partitioned between *n*-hexane and MeOH. A waxy residue (140 g) was collected from the MeOH extract and acetogenins identified using Kedde's reagent [267]. Purification by chromatography and recrystallisation led to the identification of 17 annonaceous acetogenins present in the seeds (12,15-*cis*-squamosatin-D, 12,15-*cis*-squamosatin-A, squamosatin-A, squamosatin-D, neoannonin, artemoin-A, artemoin-B, artemoin-C, artemoin-D, squamocin, bullatacin, bullatanocin, bullatalicin, 12,15-*cis*-bullatanocin, 12,15-*cis*-bullatalicin, desacetyluvaricin and isodesacetyluvaricin) and their structures were established [267].

A study of *A. atemoya* seeds collected in China and the seeds were initially extracted with 95% ethanol (EtOH) [278]. The resulting residue partitioned between H<sub>2</sub>O and EtOAc, leading to the isolation of a waxy residue (12.5g) from the organic phase. Purification of the waxy material by column chromatography led to one fraction which was further purified via preparative thin layer chromatography afforded a whitish wax identified as atemoyacin A [278].

Additionally, four studies have examined the phytochemical constituents of seeds collected in Australia [268-272]. A total of 22 compounds were identified (annonisin, parviflorin, asimicin,

cherimolin-1 and -2, mortilin, molvizarin, rolliniastatin-1, annonacin, almunequin, atemoyin, desacetylutaricin, squamocin, rolliniastatin-2, neoannonin, isodesacetylutaricin, uvariamicin-III, annotemoyin-1 and 2, reticulatin-1, bulladecin and atemotetrolin) and their structures determined spectroscopically [268-272]. Finally, chromatographic purification of the ethanolic extracts of the seeds of *A. atemoya* afforded atemoyacin E [279].

Comparing the phytochemical constituents of *A. atemoya* with those identified and isolated from both parent species, *Annona cherimola* and *Annona squamosa*, demonstrates that all three species have some similarities in their constituents regardless of the parts of the plant from which the phytochemicals were isolated from. For instant, various annonaceous acetogenins, alkaloids and volatile components have been isolated from *A. atemoya* and *A. squamosa* such as 12,15-cis-squamostatin-A, bullatacin, anonaine,  $\alpha$ -pinene, camphene,  $\beta$ -pinene, myrcene, spathulenol, germacrene D, squamostatin D,  $\beta$ -caryophyllene, uvariamicin-III, squamocin-G, squamocin-H, squamocin-J, squamocin-K, squamocin-L, squamocin-M; squamostatin-A, squamocin, annotemoyin-1, annotemoyin-2, liriodenine, annonacin, squamocin and molvizarin [280-286]. Anonaine, asimilobine, lysicamine, liriodenine, stepharine,  $\alpha$ -pinene, camphene,  $\beta$ -pinene, myrcene, germacrene D, molvizarin, rolliniastatin-2, squamocin were also isolated from both *A. atemoya* and *A. cherimola* [261, 287-289].

Both the fruits and seeds of *A. atemoya* contain a greater range and concentration of phytochemical constituents, and have been studied more extensively compared to the leaves. However, this should be interpreted within the context that there are fewer studies evaluating the *A. atemoya* leaves. The volatile constituents are the major components of the fruit, whereas the annonaceous acetogenins are the main components of the seed. Although the annonaceous acetogenins have been isolated from the seeds, no studies have reported the isolation of annonaceous acetogenins from the fruits or leaves. Alkaloids have been identified in both

leaves and seeds, and phenolic components have been isolated from fruits and seeds. Therefore, it can be deduced from the studies included in this review, that the seed is the most diverse and richest source of phytochemicals including annonaceous acetogenins, alkaloids and phenolic compounds.

Despite the fact that many pure compounds have been isolated from *A. atemoya* and various extracts of different plant parts have been examined for their activity, knowledge about the pharmacological activities of *A. atemoya* remains relatively limited. Only fourteen studies included in this review evaluated the pharmacological activities of *A. atemoya*. Of the fourteen studies, only two studies reported the cytotoxic activities of pure compounds against various cancer cell lines [267, 290], These compounds were isolated from the seeds and identified as annonaceous acetogenins which have been reported in other *Annona* species [267]. The remaining twelve studies described below, discuss the biological activity of crude extracts of different plant parts such as seed, leave, stem and fruit.

### **2.3.3. Pharmacological properties of *A. atemoya***

Different extracts of *A. atemoya* and the phytochemical constituents isolated from several parts of the plant exhibit pharmacological activities such as anti-inflammatory and antinociceptive activity [291], antioxidant [60, 259, 292, 293], antibacterial [292], anti-angiogenic [260], neuroprotective and anti-Alzheimer's [257, 259], anticancer [60, 267, 290], hypolipidemic [294] and antiobesity activity [295], in both *in vivo* and *in vitro* studies (Table 2.1).

Table 2.1. Plant part, extraction methods and biological activities and results.

| Plant part | Extraction method/type | Bioactivity      | Model                                                             | Main results                                                                                                                            | Ref.  |
|------------|------------------------|------------------|-------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------|-------|
| Leaves     | Ethanolic extract      | Neuroprotective  | <i>In vitro</i> : anti-oxidant activity                           | Dose-dependently (6.25 - 100 mg/mL) enhanced scavenging activity against ABTS and DPPH radicals                                         | [257] |
|            |                        | Neuroprotective  | <i>In vitro</i> : HT22 neuronal cell death                        | Extract significantly reversed H <sub>2</sub> O <sub>2</sub> -induced neuronal cell death at 25 or 50 µg/mL                             | [257] |
|            |                        | Neuroprotective  | <i>In vivo</i> : A $\beta$ -injected AD like mouse-model          | Increased expression of NeuN and BDNF in hippocampus reversing the effects of intracerebroventricular injection of A $\beta$ aggregates | [257] |
|            |                        | Neuroprotective  | <i>In vivo</i> : A $\beta$ -injected AD like mouse-model          | Reduced the A $\beta$ -mediated phosphorylation of EGFR and GRK2                                                                        | [257] |
|            |                        | Anti-Alzheimer's | <i>In vitro</i> : A $\beta$ aggregation                           | Dose-dependently inhibited A $\beta$ aggregation by 91.35% at 100 mg/mL                                                                 | [257] |
|            |                        | Anti-Alzheimer's | <i>In vivo</i> : A $\beta$ -injected AD like mouse-model          | At 100 µg/ml extract significantly attenuated the effects of A $\beta$ aggregation in the passive avoidance task and Y-maze test        | [257] |
|            |                        | Neuroprotective  | <i>In vivo</i> : SCO-induced hippocampal neuronal damage          | Prevented scopolamine-induced neuron damage in SCO-mediated memory deficit mice as shown by cresyl violet staining                      | [259] |
|            |                        | Neuroprotective  | <i>In vivo</i> : cholinergic function in scopolamine-treated Mice | Increased acetylcholine content, choline acetyltransferase, and acetylcholinesterase activity in the hippocampus of SCO-treated mice    | [259] |



|                    |                          |                                                                                                             |                                                                                                                              |       |
|--------------------|--------------------------|-------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------|-------|
| Methanolic extract | Neuroprotective          | <i>In vivo</i> : oxidative Stress in scopolamine-treated Mice                                               | Attenuated the SCO-induced increase in reactive oxygen species (ROS) levels in the hippocampus                               | [259] |
|                    | Neuroprotective          | <i>In vivo</i> : neuronal apoptosis in SCO-treated mice                                                     | Significantly decreased apoptotic activation in hippocampus of SCO-treated mice                                              | [259] |
|                    | Anti-Alzheimer's         | <i>In vivo</i> : SCO-induced cognitive deficit mouse model                                                  | Significantly attenuated the memory deficits from scopolamine treatment in passive avoidance task and Y-maze test            | [259] |
|                    | Antioxidant              | <i>In vitro</i> : ABTS and DPPH free radical scavenging assays                                              | At 100 µg/ml, AALE dose-dependently enhanced scavenging activity against ABTS and DPPH radicals by 97% and 82% respectively. | [259] |
|                    | Antioxidant              | <i>In vitro</i> : ABTS, DPPH and FRAP free radical scavenging assays                                        | ABTS 5.01 TE g <sup>-1</sup><br>DPPH 13.51 TE g <sup>-1</sup><br>14.79 TE g <sup>-1</sup>                                    | [60]  |
|                    | Anticancer               | <i>In vitro</i> : cytotoxicity HeLa, HepG2 cells                                                            | GI <sub>50</sub> ~ 2 µg/mL                                                                                                   | [60]  |
|                    | Antinociceptive activity | <i>In vivo</i> : acetic acid-induced writhing and formalin mouse models                                     | AAIW 100 mg/kg inhibited writhing 63.48%<br>FMM 100 mg/kg inhibited pain response 63.48%                                     | [291] |
|                    | Anti-inflammatory        | <i>In vivo</i> : air pouch mouse model<br><br><i>In vivo</i> : carrageenan-induced peritonitis mouse models | 100 mg/kg inhibited leukocyte migration in to air-pouch by 73.16%<br>100 mg/kg inhibited leukocyte migration by 63.85%       | [291] |
|                    | Antibacterial            | <i>In vitro</i> : against strains of <i>S. epidermidis</i> , <i>B. cereus</i> ,                             | MBC range 3,125 to 12,500 µg/ml.                                                                                             | [292] |

|        |                   |                      |                                                                                                                                                                         |                                                                                                    |            |
|--------|-------------------|----------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------|------------|
|        | Hexane extract    | Antioxidant          | methicillin-resistant <i>S. aureus</i> , <i>K. pneumoniae</i> and <i>S. aureus</i> .<br><i>In vitro</i> : inhibition of $\beta$ -carotene-linoleic acid bleaching assay | 41.12 $\pm$ 4.35% inhibition                                                                       | [292]      |
| Seeds  | Ethanolic extract | Anti-angiogenic      | <i>In vitro</i> and <i>in vivo</i> models, involving cell proliferation, HUVEC and tumour-induced angiogenesis.                                                         | EEAA dose-dependently inhibited HUVEC proliferation at conc. $\geq$ 100 $\mu$ g/ml.                | [260]      |
|        |                   | Anticancer           | <i>In vitro</i> : cytotoxicity Hep G <sub>2</sub> , Hep 2,2,15, KB, CCM <sub>2</sub> and CEM cells                                                                      | Isolated acetogenins ED <sub>50</sub> from 2.2x10 <sup>-4</sup> to > 500 $\mu$ g/mL                | [267, 290] |
|        |                   | Neurotoxicity        | <i>In vitro</i> : LUHMES cells                                                                                                                                          | 0.1 $\mu$ g/mL reduced cell viability to 4.0% $\pm$ 0.8%                                           | [247]      |
|        | Methanolic        | Antioxidant          | ABTS and DPPH free radical scavenging assays                                                                                                                            | 46.14 $\pm$ 1.25 and 4.82 $\pm$ 0.32 $\mu$ mol TE g <sup>-1</sup>                                  | [293]      |
|        |                   | Larvicidal           | <i>In vitro</i> : <i>Trichoplusia ni</i>                                                                                                                                | Topical LC <sub>50</sub> 197.7 $\mu$ g/larva<br>Oral LC <sub>50</sub> 382.4 ppm                    | [296]      |
| Stem   | Ethanolic extract | Antioxidant          | <i>In vitro</i> : ABTS and DPPH free radical scavenging assays                                                                                                          | DPPH; IC <sub>50</sub> = 10.44 $\pm$ 1.25 $\mu$ g/mL<br>ABTS; IC <sub>50</sub> = 24.81 $\pm$ 0.49% | [292]      |
|        |                   | Antibacterial        | <i>In vitro</i> : against <i>S. epidermidis</i> , <i>B. cereus</i> , methicillin-resistant <i>S. aureus</i> , <i>K. pneumoniae</i> , <i>S. aureus</i> .                 | MBC range 781-6,250 $\mu$ g/ml.                                                                    | [292]      |
| Fruits | Ethanolic extract | Hypolipidemic Effect | <i>In vivo</i> : oral administration of extracts to Female KKAY mice (5                                                                                                 | Significantly lowered the plasma triglyceride (TG) concentration at doses of 125 and 500 mg/kg.    | [294]      |

|                               |                       |                                                                                   |                                                               |
|-------------------------------|-----------------------|-----------------------------------------------------------------------------------|---------------------------------------------------------------|
| Ethanolic and hexane extracts | Anti-Obesity Activity | weeks of age) fed a high fat diet for 4 wks<br><i>In vitro</i> : 3T3-L1 cell line | 50% or more inhibition of adipogenesis in 3T3-L1 cells. [295] |
|                               | Neurotoxicity         | <i>In vitro</i> : LUHMES cells                                                    | 10 µg/mL decreased cell viability to 12.7% ± 3.7% [247]       |

AAIW acetic acid induced writing; A $\beta$   $\beta$ -amyloid; ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; AD Alzheimer's disease; AALE *A. atemoya* leaf extract; BDNF brain derived neurotropic factor; DPPH 2,2-diphenyl-1-picrylhydrazyl; EEAA ethanol extract *A. atemoya*; EGFR epidermal growth factor receptor; FMM formalin mouse model; FRAP ferric reducing antioxidant power; GRK2 G protein-coupled receptor kinase 2; HUVEC: human umbilical vascular endothelial cells; MBC minimum bactericidal concentration; NeuN neuronal nuclear protein; ROS reactive oxygen species; SCO scopolamine.

## 2.3.3.1. Cytotoxic

Annonaceous acetogenins have attracted considerable attention due to their broad biological activities. Compounds isolated from the seeds of *A. atemoya* exhibit potent cytotoxicity against various cancer cell lines such as Hep G2/2.2.15, CCM2, CEM, KB and Hep G2 [267]. These compounds were identified as 12,15-*cis*-squamostatin-D, squamostatin-D, squamocin, neoannonin, bullatacin and desacetyluvaricin. Their activity on different cancer cells was assessed using a methylene blue colorimetric assay [267]. The cytotoxic activity of these compounds is presented in (Table 2.2).

Table 2.2. Isolated pure compounds from *A. atemoya* and their cytotoxic activities [267].

| Compound                          | (ED <sub>50</sub> µg/ml) |                       |                       |                       |     |
|-----------------------------------|--------------------------|-----------------------|-----------------------|-----------------------|-----|
|                                   | Hep G2                   | Hep 2,2,15            | KB                    | CCM2                  | CEM |
| 12,15- <i>cis</i> -Squamostatin-D | $2.20 \times 10^{-4}$    | $3.10 \times 10^{-3}$ | $4.05 \times 10^{-4}$ | --                    | --  |
| Squamostatin-D                    | $1.50 \times 10^{-4}$    | $1.50 \times 10^{-3}$ | $3.90 \times 10^{-4}$ | --                    | --  |
| Squamocin                         | $8.80 \times 10^{-4}$    | $1.50 \times 10^{-3}$ | $2.70 \times 10^{-1}$ | $1.60 \times 10^{-2}$ | 149 |
| Neoannonin                        | $1.10 \times 10^{-4}$    | $1.26 \times 10^{-4}$ | $1.46 \times 10^{-4}$ | 10.9                  | 520 |
| Bullatacin                        | $9.70 \times 10^{-5}$    | $1.11 \times 10^{-4}$ | $1.17 \times 10^{-4}$ | $1.41 \times 10^{-1}$ | 169 |
| Desacetyluvaricin                 | $1.02 \times 10^{-4}$    | $1.18 \times 10^{-4}$ | $1.35 \times 10^{-4}$ | 23.5                  | 100 |

Hep G2 = Human liver cancer cell line, Hep 2,2,15 = Human hepatoblastoma cell line. KB = KERATIN-forming tumor cell line, CCM2 = Cerebral cavernous malformations 2, CEM = T lymphoblast cell line

Bullatacin isolated from *A. atemoya* demonstrated a potent cytotoxic activity inhibiting the proliferation of Hep G2/2.2.15 cells by inducing apoptosis [276], via a reduction of intracellular cAMP and cGMP levels [290]. Using the methylene blue assay, a significant inhibition in Hep G2/2.2.15 cell proliferation was recorded after treatment with bullatacin ( $10^{-1}$  to  $10^{-5}$  µM), ED<sub>50</sub> values were recorded as  $7.8 \pm 2.5$  nM for 24 hours [290],

An aqueous-ethanolic extract of the leaves of *A. atemoya* was shown to be cytotoxic to both HeLa and HepG2 cancer cells. The anti-cancer activity was not found to correlate to either polyphenol content, antioxidant properties or total alkaloid content. However, there was a

moderate correlation between antiproliferative activity and aporphine-type alkaloid content [60].

#### 2.3.3.2. Anti-angiogenic activity

The ethanolic extract of *A. atemoya* seeds was examined for its anti-angiogenic activity in human umbilical vein endothelial cells (HUVECs). The ethanolic seed extract of *A. atemoya* elicited a significant dose-dependent inhibition of HUVEC mobility at concentration  $\geq 100$   $\mu\text{g/ml}$  without decreasing cell viability [260]. Using the matrigel plug assay, the ethanol extract at concentration  $\geq 25$   $\mu\text{g/ml}$  was also shown to inhibit the formation of new blood vessels *in vivo*, in a dose-dependent manner. Further investigation indicated that under hypoxic conditions the extract down-regulated the expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF-1 $\alpha$ /2 $\alpha$ ) [260]. These results indicate that the extract modulated angiogenesis both directly through the endothelial cells and indirectly through modulating the tumour-induced angiogenic pathways [260].

#### 2.3.3.3. Hypolipidemic effect

The ethanolic fruit extract of *A. atemoya* was investigated for its hypolipidemic effects using female KKAY mice at one month of age [294]. For four weeks, the female KKAY mice were fed a high fat diet. Oral administration of ethanolic fruit extract of *A. atemoya* at doses of 125 and 500 mg/kg to the female KKAY mice led to a significant inhibition of plasma triglyceride concentrations and the hypolipidemic effect might be through diminished fatty acid mobilization [294]. The hypolipidemic mechanism of the ethanolic fruit extract is not reported, although the authors claim that in a previous study [295], both the hexane and ethanolic extracts of *A. atemoya* fruit (along with many other popular Okinawa food stuffs) inhibited adipogenesis activity in 3T3-L1 cells by 50% or more. However, this study provides only limited results and does not include the extraction methodology or the concentrations of the extracts used, making it difficult to understand if this is a likely mechanism for the anti-obesity actions of *A. atemoya*.

[295]. While interesting, the dose of the crude extract used in the hypolipidemic study is high, translating into a dose of approximately 9g/per day for an average adult. However, the significant issues faced by many western populations due to obesity and related health issues, mean that the hypolipidemic activities warrant further study. In particular, further purification of the extract and isolation of the hypolipidemic constituent(s) is required.

#### *2.3.3.4. Antioxidant activity*

In an *in vitro* study, the methanolic seed extract of *A. atemoya* was examined for antioxidant potential [293]. Both 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and oxygen radical absorbance capacity (ORAC) assays were employed. For the ORAC and DPPH assays, the Trolox equivalent antioxidant activity of the methanolic seed extract was recorded at  $46.14 \pm 1.25$  and  $4.82 \pm 0.32 \mu\text{mol TE g}^{-1}$ , respectively [293].

Other studies have reported the free radical scavenging activity of ethanolic extracts of stems and the hexane extract of leaves using DPPH, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and antioxidant activity  $\beta$ -carotene/linoleic acid assays [292]. The ethanolic extract of stems exhibited the most effective antioxidant in DPPH and ABTS ( $\text{IC}_{50} = 10.44 \pm 1.25 \mu\text{g/mL}$ ) and ( $24.81 \pm 0.49\%$ ), respectively. The hexane leaf extract of *A. atemoya* has also demonstrated antioxidant activity in the  $\beta$ -carotene linoleic acid assay with approximately ( $41.12 \pm 4.35\%$ ) [292]. The aqueous ethanol leaf extracts of Sicilian *A. atemoya* also showed strong antioxidant activity in the ABTS ( $5.01 \text{ TE g}^{-1}$ ), DPPH ( $13.51 \text{ TE g}^{-1}$ ) and metal reducing properties in the ferric reducing antioxidant power (FRAP) assay ( $14.79 \text{ TE g}^{-1}$ ) [60].

#### *2.3.3.5. Antibacterial activity*

The antimicrobial potential of the hexane and methanolic extract of leaves, and the ethanolic extract of the stems of *A. atemoya* has been investigated against both Gram-positive and Gram-negative bacteria using a serial dilution protocol [292]. All strains were resistant to the hexane

extract. However, both the leaf methanolic and stem ethanolic extracts exhibited activity against gram positive *Staphylococcus epidermidis*, *Bacillus cereus*, *Staphylococcus aureus* and a clinical isolate of *methicillin-resistant Staphylococcus aureus* (MRSA), with *Klebsiella pneumoniae* the only Gram-negative strain sensitive to the extract. The (MBC) values recorded for the ethanolic stem extract ranged from 781 µg/mL against MRSA to 6,250 µg/mL against *K. pneumoniae* and *S. aureus*. The methanolic leaf extract showed weaker activity with MBC values ranging from 3,125 against *S. epidermidis* to 12,500 µg/mL (*S. aureus*, *K. pneumoniae* and *B. cereus*) [292]. Although, the MBC was determined, the (MIC) was not determined for any of the strains. Therefore, it is not possible to determine whether the mechanism of action is bactericidal or bacteriostatic. From the reported total polyphenol content of these extracts it is suggest that flavonoids are responsible for the activity antibacterial action of both the ethanol stem extract and the methanol leaf extract [292].

#### 2.3.3.6. Antinociceptive activities

An *in vivo* study evaluating the antinociceptive activity of *A. atemoya* was conducted using the acetic acid-induced writhing and formalin mouse models [291]. The ethanolic extract of leaves (25, 50 and 100 mg/kg) inhibited acetic acid-induced writhing by 42.14, 48.88 and 63.48%, correspondingly. The same doses of the ethanolic extract of leaves also exhibited antinociceptive activity with 56.71, 64.35 and 50.09% inhibition, respectively using the formalin test [291].

#### 2.3.3.7. Anti-inflammatory activities

The anti-inflammatory activity of *A. atemoya* was reported in a single study with the evaluation carried out using the air pouch and carrageenan-induced peritonitis models in an *in vivo* study [291]. In the carrageenan-induced peritonitis model the ethanolic extract of leaves (25, 50 and 100 mg/kg) inhibited leukocyte migration by 35.40, 46.20 and 63.85%, respectively. In the air

pouch assay, the leaf extract (25, 50 and 100 mg/kg) also reduced leukocyte migration into the air pouch by 56.17, 62.04 and 73.16% respectively [291]. As this study used only the crude the ethanolic extract it is not possible to identify which constituents may be responsible for the antinociceptive and anti-inflammatory activities.

#### *2.3.3.8. Neurological activities*

Oral administration of an ethanolic extract of *A. atemoya* was found to reduce scopolamine-induced memory impairment in the mouse Y-maze and passive avoidance tests [257, 259]. Investigation into the mechanism of action indicated that *A. atemoya* extract inhibits memory impairment by preventing cholinergic dysfunction and decreasing cell death associated with stress induced oxidation [257]. The crude ethanolic leaf extract and to a lesser degree the fruit extract but not the seed extract was also shown to prevent amyloid- $\beta$  (A $\beta$ ) aggregation and increase free radical scavenging activity, preventing H<sub>2</sub>O<sub>2</sub> induced damage to hippocampal HT22 cells in vitro [257]. Immunohistochemical and western blotting studies indicated that the extract inhibited A $\beta$ -mediated loss of brain derived neurotrophic factor (BDNF) expression and decreased A $\beta$ -mediated phosphorylation of epidermal growth factor receptor (EGFR) and G Protein-Coupled Receptor Kinase 2 (GRK2) [257]. Further fractionation into hexane, butanol and water led to all fractions inhibiting A $\beta$  aggregation to some degree, with the ethyl acetate fraction demonstrating the greatest inhibition. However, the majority of the neuroprotective activity was found in the butanol fraction with the hexane and ethyl acetate fractions (the fractions most likely to contain the lipophilic acetogenins) being cytotoxic to the cultured neurons. Phytochemical analysis of the butanol extract suggested that the flavonoids rutin and isoquercitrin were predominantly responsible for the reported bioactivity [257].

However, one study has reported the neurotoxicity of ethyl acetate extracts of the pulp and seeds in an *in vitro* study using Lund human mesencephalic (LUHMES) cells [247]. This



suggests that constituents of the seeds and pulp are neurotoxic as has been found for other members of the *Annona* family [113]. There have been suggestions that a high dietary consumption of *A. muricata* may be linked a Parkinson disease-like syndrome possibly due to the annonacin content [297]. The studies investigating the neurotoxicity of *Annona* species have used ethyl acetate extracts which would predominantly contain the acetogenins, or purified acetogenins such as annonacin. Acetogenins have been identified as highly potent inhibitors of mitochondrial complex I [298], and this has been suggested as the mechanism of neurotoxicity. The neuroprotective effect of crude ethanolic extracts of leaves and fruit of *A. atemoya*, containing both acetogenins and flavonoids, suggests that the presence of the flavonoids may offer protection from the neurotoxic effects of the acetogenins.

#### 2.3.3.9. Toxicity

The neurotoxicity of the ethyl acetate extracts of a range of Brazilian *A. atemoya* pulp and seeds in LUHMES cells was assessed using both MTT assay to determine cell viability and LDH assay to determine cell death [247]. The pulp extract of *A. atemoya* had little effect on cell viability of the LUHMES cells at 1 µg/mL, but at 10 µg/mL decreased cell viability to 12.7% ± 3.7%. The seed extract of *A. atemoya* exhibited significantly higher toxicity with cell viability reduced to 4.0% ± 0.8% at a concentration of 0.1 µg/mL. LDH levels were increased to 52.8% ± 3.9% by treatment of the LUHMES cells with concentration of 10 µg/mL of the *A. atemoya* pulp extract and to 60.8% ± 4.3% when treated with 0.1 µg/mL of seed extract [247]. When compared to the fruit pulp extract of other *Annona* species in the study *A. atemoya* had a lesser effect on cell viability than *A. squamosa* but resulted in a similar increase in LDH indicating a similar effect on cell death. This is despite *A. atemoya* containing a similar amount of annonacin to *A. squamosa* (3.8 of 2.2 µg/g dry weight) and approximately a 100-fold greater amount of squamocin [247]. The ethyl acetate extract used in this study will contain a complex

mixture of lipophilic compounds, including many acetogenins, and the neurotoxicity cannot be attributed only to annonacin. It is possible that *A. atemoya* may also contain lipophilic neuroprotective compounds.

A second study reported the toxicity of *A. atemoya* on *Trichoplusia ni* (cabbage looper larva) using topical and oral administration. The methanolic seed extract was applied to *T. ni* larvae and the LC<sub>50</sub> was recorded at 197.7 µg/larva [296]. Finally, the methanolic seed extract also showed toxicity to *T. ni* larvae when administered orally with the LC<sub>50</sub> recorded as 382.4 ppm [296].

#### 2.3.4. Quality analysis

As presented in (Table 2.3), the quality of the *in vivo* studies none of the studies met all of the ARRIVE criteria [263]. In particular, no studies reported baseline animal data or adverse events. With regards allocating animals to experimental groups, this was reported by only one group [257]. These factors limit the reliability of our findings in terms of the *in vivo* studies.

Table 2.3. Methodological quality assessment of animal studies using ARRIVE quality assessment criteria.

| Study | *ARRIVE QUALITY ITEMS |   |   |   |   |   |   |   |   |    |    |    |    |
|-------|-----------------------|---|---|---|---|---|---|---|---|----|----|----|----|
|       | 1                     | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| [257] | Y                     | Y | Y | Y | Y | Y | Y | Y | Y | N  | N  | Y  | N  |
| [259] | Y                     | Y | Y | Y | Y | Y | N | Y | Y | Y  | N  | Y  | N  |
| [260] | Y                     | Y | Y | Y | N | N | N | Y | N | N  | N  | Y  | N  |
| [294] | N                     | Y | Y | Y | Y | N | N | Y | N | N  | N  | Y  | N  |
| [291] | Y                     | Y | Y | Y | Y | Y | N | Y | Y | Y  | N  | Y  | N  |

**\*ARRIVE Quality items:** 1 Ethical statement, 2 Study design, 3 Experimental procedure, 4 Experimental animals, 5 Housing and husbandry, 6 Sample size, 7 Allocating animals to experimental groups, 8 Experimental outcomes, 9 Statistical methods, 10 Number of analysed, 11 Baseline data, 12 Outcomes and estimation, 13 Adverse events.

## Conclusion

To our knowledge, this is the first systematic review to summarize the available literature reporting the phytochemical constituents and pharmacological effects of *A. atemoya*. The systematic review of the included studies affords a comprehensive report of the currently available data regarding both the phytochemical constituents and biological activities of *A. atemoya*. The selected studies in this systematic review were judged to be of medium quality as there are limited animal studies and no human studies reported. The phytochemical components of *A. atemoya* have been examined as extracts and pure compounds in both *in vivo* and *in vitro*. However, many studies used only crude ethanolic or methanolic extracts and did not attempt to purify these further in order to identify which constituents may be responsible for the activities. In regards to the phytochemical screening of *A. atemoya* plant, several studies have reported a range of constituents isolated from various parts including the leaves, fruits and seeds. However, very few of the studies attempted to profile or quantify the bioactive constituents. Amongst the selected studies, only three studies reported the cultivar of *A. atemoya* (African Pride) one of the most common varieties [266, 268, 269]. It is also of interest to note that at the current time no acetogenins have been isolated from the fruit or leaves of *A. atemoya*, unlike the many acetogenins isolated from the fruit and leaves of *A. cherimola* and *A. squamosa*.

Although, there is no solid evidence of medicinal or nutraceutical uses of *A. atemoya* there is some limited anecdotal evidence reporting the use of leaves for making teas in a traditional manner, or possibly using the leaves as a supplement. As an increasingly important commercial fruit crop with nutraceutical potential, further studies are recommended in order to isolate and investigate the pure components, and identify the bioactive compounds responsible for the effects identified.

# CHAPTER 3

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## **Annonaceous Acetogenins and Alkaloids Isolated and Identified from the Leaves of Annona Atemoya Cultivars**

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Chapter 3 and 4 have been published in the *International Journal of Molecular Science*, 2023, 24(3), 2294, with the title ' Identification of Annonaceous Acetogenins and Alkaloids from the Leaves, Pulp, and Seeds of Annona atemoya'. Authors: Al Kazman, B.S.M.; Harnett, J.E.; Hanrahan, J.R.

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## Abstract

Annonaceae is a large family composed of more than 119 genera and more than 2500 species that are distributed in both tropical and subtropical areas. *Annona* genus is a member of Annonaceae family that encompassing about 175 species and most of them are native of Brazil and tropical America. This plant is commonly found in tropical and subtropical continents such as Asia, Africa, southern and northern America and Australia. The phytochemical investigations of *A. atemoya* leaves have been reported in limited studies. The results of this study indicated the presence of annonaceous acetogenins in the leaves of *A. atemoya* for the first time. Fifteen compounds were reported, eleven were acetogenins and four were alkaloids. Additionally, two compounds were isolated, and their structures and absolute configuration were determined by interpretation of the spectroscopic data and previous reported studies. The concentration of acetogenins in the hexane extract was very low compared to the ethyl acetate extract.

**Keywords:** *Annona atemoya*; leaves; extract; annonaceous acetogenins; alkaloids

### 3.1. Introduction

The phytochemical constituents of *Annona atemoya* leaves have been reported in only a limited number of studies. Two studies reported the isolation of alkaloids such as lanuginosine, liriodenine, lysicamine, pronuciferine, anonaine, asimilobine and stepharine [60, 299], and a recent study reported the isolation of additional alkaloids such as dehydroanomuricine-*N*-oxide, anomuricine, reticuline, scoulerine, nornuciferine and norisocorydine [300]. The leaves of *A. atemoya* have been found to have a number of phenolic compounds including quercetin-3-O-rutinoside-7-O-glucoside, quercetin-3-O-rutinoside, quercetin-3-O-glucoside, luteolin-3-galactoside-7-rhamnoside, apigenin-8-C-glucoside, quercetin-3-O-rutinoside-7-O-pentoside, kaempferol-3-galactoside-7-rhamnoside, luteolin-3-glucoside-7-rhamnoside, catechin and epicatechin [60]. Even though many *Annona* species have been extensively studied for their phytochemical constituents, limited studies have investigated the phytochemical constituents of *A. atemoya*. Therefore, the objective of the present study was to screen for the phytochemical constituents of *A. atemoya* leaf in general and more specifically for annonaceous acetogenins. These compounds were only recently identified in the leaf part of *A. atemoya* by our group and reported for the first time [301].

### 3.2. Methodology

#### 3.2.1. Plant material collection and preparation

The fully matured fresh leaves of both *A. atemoya* cultivars African Pride (AP) and Pink's Mammoth (KJ) were collected in July 2020 from a local farm in southeast Queensland. The leaves were washed with running tap water to remove dirt and debris, and then spread out on the bench for air drying at room temperature in a well-ventilated area for one week. The leaves were then crushed into small pieces and powdered via an electric blender (Multifunctional crusher). The final weight of the (AP) and (KJ) leaves powder was 222.4 g and 180.9 g,

respectively.

### ***3.2.2. Preparation of plant extracts***

The dried and powdered leaves of both (AP) 210 g and (KJ) 170 g were macerated initially with hexane (AP: 2.5 L) and (KJ: 2 L) and filtered. The residues were then, extracted with ethyl acetate, and filtered. Finally, the residue was extracted with ethanol. All extractions were carried out for once at room temperature with stirring for 72 hours and filtrated using vacuum filtration (Figure 3.1).

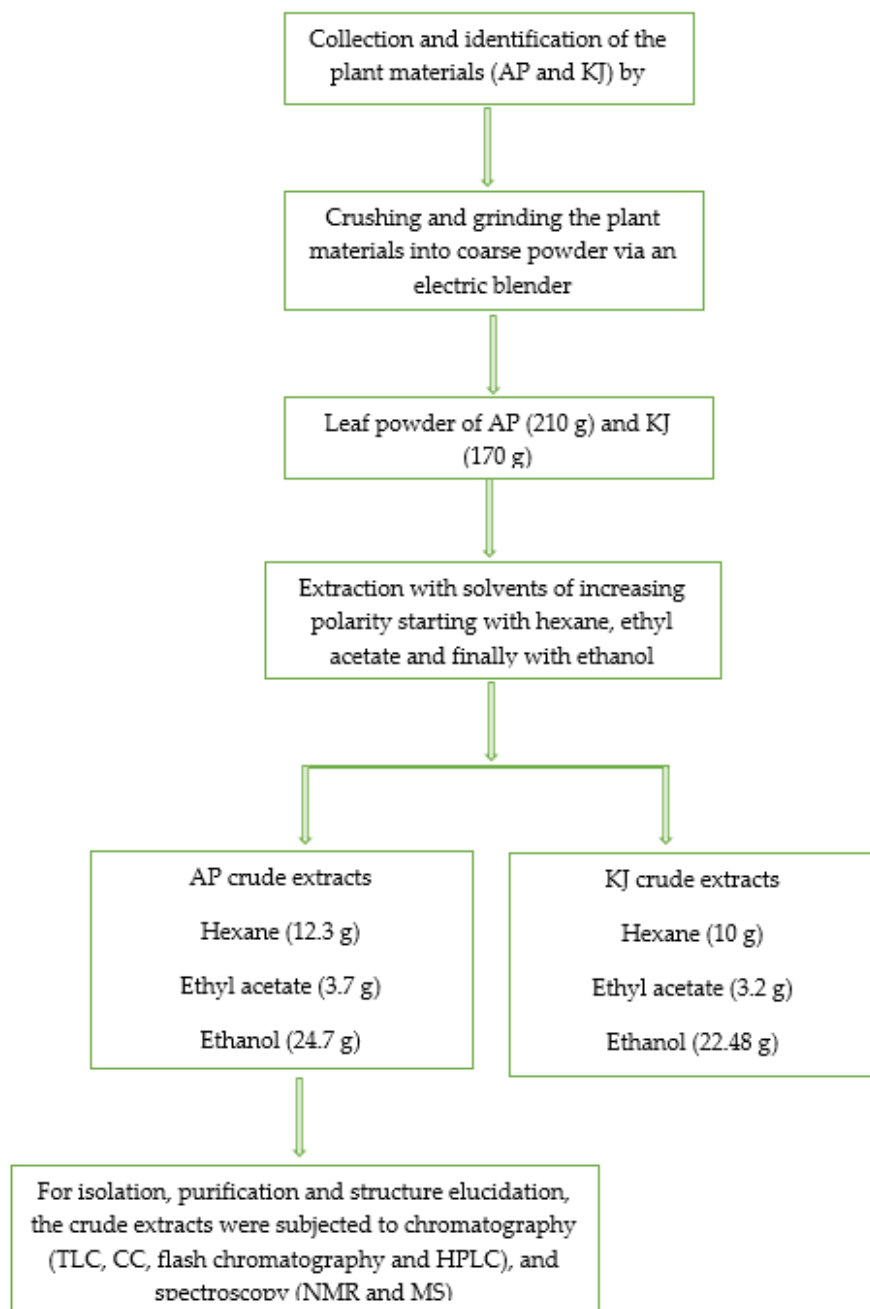


Figure 3.1. Schematic presentation of extraction, isolation, and characterization of compounds from *A. atemoya* cultivars (AP and KJ).

After each extraction solvents were evaporated under reduced pressure affording the yields shown in Table 3.1.



Table 3.1. The final weight of the leaf crude extract of *A. atemoya* cultivars.

| Extraction solvent | African Pride (g) | Pink's Mammoth (KJ) (g) |
|--------------------|-------------------|-------------------------|
| Hexane             | 12.3              | 10.0                    |
| Ethyl acetate      | 3.7               | 3.2                     |
| Ethanol            | 24.7              | 22.8                    |

### 3.2.3. Chemical and reagents

Laboratory grade hexane, dichloromethane (DCM), ethyl acetate (EtOAc), ethanol (EtOH) and methanol (MeOH) were obtained from the School of Chemistry, University of Sydney. HPLC grade methanol, acetonitrile, LCMS grade methanol and deuterated solvents ( $\text{CD}_3\text{OD}$  (99.8% D) and  $\text{CDCl}_3$  (99.8% D) were purchased from Sigma-Aldrich (Castle Hill, New South Wales, Australia).

### 3.2.4. Phytochemical screening

For each extract, a small quantity of crude extracts was qualitatively tested using Thin Layer Chromatography (TLC) to disclose the existence of both annonaceous acetogenins and alkaloids. These phytochemical constituents were identified according to the characteristic colour changes utilizing various reagents such as Kedde's and Dragendorff's reagents.

### 3.2.5. Thin Layer Chromatography (TLC) and Column Chromatography (CC)

Analytical plates for TLC were Silica Gel 60 F<sub>254</sub>, Aluminium sheets, 200um, (20 x 20 cm) and Merck silica gel 60, particle size (0.040 – 0.063 mm) (Merck KGaA, Darmstadt, Germany). A few milligrams of both (AP) and (KJ) leaves powder were spotted on the TLC plate using methanol: dichloromethane (10: 90) as a mobile phase. As the compounds of interest were not detected under the ultraviolet light (UV) at (254nm, 365nm), and in order to visualize these compounds various reagents were prepared for instance, Kedde's, vanillin and Dragendorff's reagents [302].

The Kedde's reagents were prepared as solutions of 2% (2 g) of 3,5-dinitrobenzoic acid in 100 ml of ethanol and 5% (5 g) of KOH in 100 ml of ethanol.

Dragendorff's reagent was prepared as follows:

Solution A: 1.7 g of bismuth nitrate was dissolving in 100 ml water: acetic acid (4:1).

Solution B: 40 g of potassium iodide was dissolving in 100 ml of water.

Both solvent A and B were combined as follows: 5 ml A + 5 ml B + 20 ml acetic acid + 70 ml water. Finally, the vanillin reagent was prepared by adding 15 g of vanillin powder in 250 ml of ethanol.

### ***3.2.6. Flash chromatography***

All experiments for flash chromatography were performed on a Reveleris iES system utilizing the Reveleris® Navigator™ software. This instrument consists of a binary pump with four solvent selection, fraction collector and evaporating light scattering detector (ELSD). Reveleris® flash cartridges packed silica.

### ***3.2.7. Nuclear Magnetic Resonance (NMR)***

All the fractions obtained, and the pure compounds were characterized by <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectroscopy. <sup>1</sup>H and <sup>13</sup>C spectra were recorded at 400MHz and 100MHz, respectively using a Varian Gemini 400-MR automated spectrometer at room temperature, using CDCl<sub>3</sub> as solvent referenced to the residual signal set at 7.26 ppm for <sup>1</sup>H and to 77.0 ppm for <sup>13</sup>C.

### ***3.2.8. Mass Spectrometry (MS) and Tandem mass spectrometry (MS/MS)***

Mass spectra were carried out on a Thermo Scientific TSQ series Quantum Access Max liquid chromatography-mass spectrometry LC-MS/MS system (Thermo Fisher Scientific, Massachusetts, USA), using heated electrospray ionization in positive ionization mode. High purity nitrogen (BOC, Sydney, Australia) at 60 psi was used as the sheer gas and ultra-high

purity argon (BOC) at 2 mTorr was used as collision gas. The mass spectroscopy was conducted at a voltage of 4500 V. The heated capillary and vaporizer were heated at 380 °C and 350 °C, respectively. The mass spectra were acquired at a scan range of 100 – 1000 m/z. Data was analysed by XCalibur 4.1.50 (Thermo Fisher Scientific, Massachusetts) software. For (MS/MS) experiment, solution of crude extracts was prepared at a concentration of 1 mg/ml in MeOH, and the injection volume was 5 µl. The optimal collision energy was 70 eV and Lithium iodide (2mM in MeOH) was added to each sample before analysis.

### 3.3. Results and Discussion

#### 3.3.1. TLC and CC

Each crude extract of AP and KJ leaves was analysed by TLC to investigate their phytochemical constituents. 2 mg of each extract was dissolved in 1 ml of solvent and spotted on the TLC plate which was subsequently developed in CAMAG 20x10cm twin-through glass developing chambers using (MeOH: DCM) (10: 90 v/v) as mobile phase. The TLC plate was then examined under UV light at (254nm, 365nm), then the TLC plate was further visualized using Kedde's reagent. Firstly, the TLC plate was dipped in 2% of 3,5-dinitrobenzoic acid in 100 ml of ethanol and left to dry for five minutes and then sprayed with 5% of KOH in 100 ml of ethanol. Based on TLC results, all six extracts of both AP and KJ leaves indicated the presence of acetogenins as pink bands after reacting with Kedde's reagents (Figure 3.2, A). As AP and KJ known to be cultivars of *A. atemoya*, both cultivars indicated some similarities in their phytochemical components as they displayed similar bands on the TLC plate (Figure 3.2, B). For instance, the ethanolic extracts of both AP and KJ leaves exhibited the same bands of the most polar acetogenins at  $R_f$  0.25. Additionally, the ethyl acetate extractions of both cultivars illustrated the same bands of the polar acetogenins at (0.25), however, the spots were

less concentrated compared to the ethanolic extractions. Moreover, the ethyl acetate extracts of AP and KJ were the richest in an abundance of acetogenins and contained approximately three separated bands in comparison to the ethanolic and hexane extracts. With regards to the hexane extracts, the concentration of acetogenins was very low and demonstrated the lowest polar acetogenins. As both cultivars showed similarities in their composition when analysed via TLC only AP cultivar was further purified.

For further purification, both hexane and ethanolic extracts of AP leaves were submitted to CC using solvents of increasing polarity; hexane, DCM, EtOAc and MeOH. For the hexane extract, 6.57 g of the dried extract was eluted with a gradient from hexane over DCM to MeOH. A total of 163 fractions were collected (10 ml), and fractions 1H (110 -129) (145.2 mg) were collected in one flask as they indicated the lowest polarity acetogenins in the extract along with other constituents. Fraction 2H (130 - 131) (33.3 mg) indicated the presence of acetogenins (Kedde's reagent) containing some contaminants visible under UV light. Finally, six fractions from 138 to 143 (24 mg) also indicated the presence of very polar acetogenins and combined in one flask and namely as fraction 3H. The TLC plate did not display a clear spot of acetogenins due to the low concentration of acetogenins in leaves hexane extract.

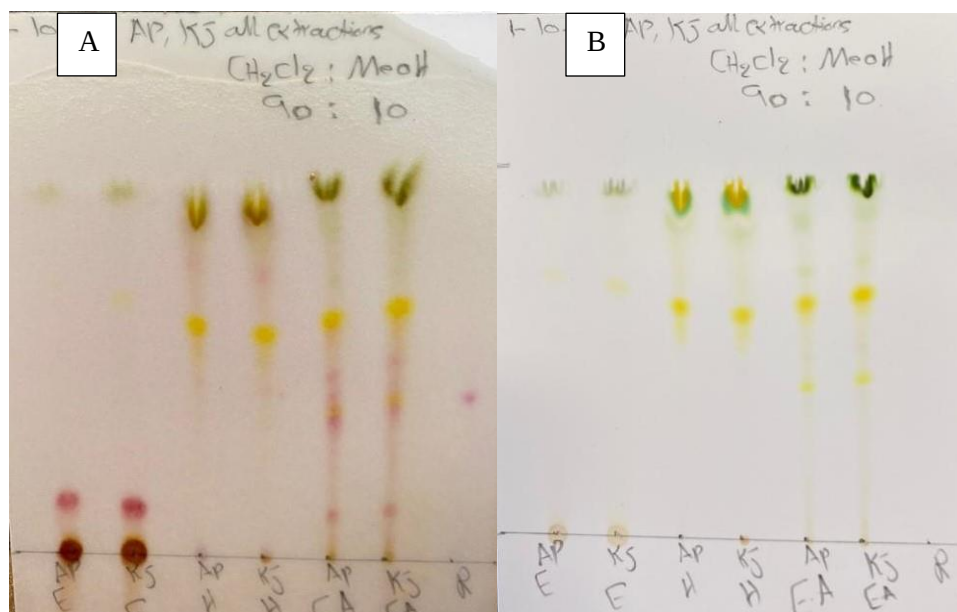


Figure 3.2. TLC profile of total *A. atemoya* cultivars (AP and KJ), from left to right AP and KJ ethanolic extractions, then, AP and KJ hexanolic extractions, AP and KJ ethyl acetate extractions and (R) (annonacin) after spraying with Kedde's reagents.

The ethanol extract (12.6 g) of the sample was eluted with (DCM: MeOH) (100: 0 v/v) to (DCM: MeOH) (0: 100 v/v). A total of 276 fractions were collected and separation resulted in five fractions namely as A (240 mg), B (80 mg), C (2.27 g), D (5.9 g) and E (2.60 g). Of the five fractions, only fractions A and B exhibited concentrated pink bands with  $R_f$  at (0.21) (Figure 3.3, A), as well as four orange spots indicating alkaloids with  $R_f$  ranging from 0.42 to 0.85 after spraying with Dragendorff's reagent (Figure 3.3, B). The remaining fractions C, D and E also indicated the presence of pink spot with major contaminants. Due to small amount of AP ethyl acetate crude extract, it was submitted directly to flash chromatography for further purification.

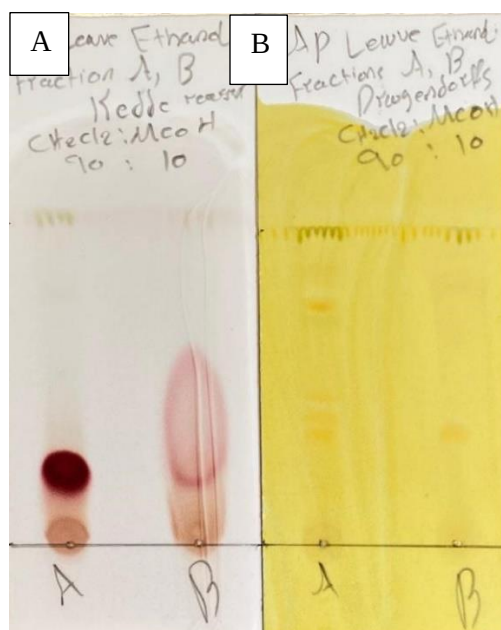


Figure 3.3. TLC plates for AP leaves ethanolic fraction (A) and (B) indicated pink spot after spraying with Kedde's reagents, and three orange spots indicating alkaloids after spraying with Dragendorff's reagent.

### 3.3.2. Flash chromatography

The three fractions of the AP hexane extract were further purified via flash chromatography. Each fraction was dissolved in a small amount of EtOAc and then adsorbed onto a small amount of silica gel. The mixture was evaporated to dryness under reduced pressure and loaded on prepacked Flash Grace Reveleris® silica cartridge of 12 g. For fractions 1H (110 - 129) (145.2 mg), the first purification was run using (DCM: MeOH) (100: 0 v/v) to (DCM: MeOH) (0: 100 v/v), and 61 subfractions (25 ml) were collected and analysed by TLC. Sub-fractions 1Ha (44 – 46) (39.8 mg) indicated the presence of acetogenins with some contaminants and were then combined and subjected to the second purification by CC with (Hexane: EtOAc) (100: 0 v/v) to (Hexane: EtOAc) (0: 100 v/v). A total of 55 (25 ml) sub-fractions were collected and four sub-fractions named as sub-fractions 1Hb (43 – 46) indicated acetogenins with  $R_f$  at (0.85). Each subfraction was kept separate, and the final weight was calculated as: sub-fractions 43 (1.5 mg), 44 (3.5 mg), 45 (4.3 mg) and 46 (0.8 mg) (Figure 3.4, A).

The columns for both fractions 2H (130 - 131) (33.3 mg), and fractions 3H (138 - 143) (24 mg) were eluted with the same gradient combinations of (DCM: EtOH) (100: 0 v/v) to (DCM: EtOH) (0: 100 v/v). A total of 53 (25 ml) subfractions were collected and four sub-fractions 2Ha (18 – 21) (3 mg) indicated the presence of acetogenins with some minor contamination for fractions 2H (130 - 131); and 56 subfractions (25 ml) were collected for fractions 3H (138 - 143) and four sub-fractions (35 – 38) (3.4 mg) indicated the presence of acetogenins with some contamination and namely as Sub-fractions 3Ha. The concentration of acetogenins in these fractions is low making it difficult to identify them using TLC and NMR. Hence, they were selected for further analysis by mass spectrometry. With regards to AP ethyl acetate extract, it was submitted directly to flash chromatography experimentation due to the small amount of the crude extract. The crude extract (2.991 g) was dissolved in DCM and purified using CC from (DCM: MeOH) (100: 0 v/v) to (DCM: MeOH) (0: 100 v/v) as ideal eluting system. 158 fraction (25 ml) were collected and then analysed by TLC and similar fractions were combined as follow: fractions 1E (46 – 66) (121.4 mg) indicated the most polar acetogenins present in the ethanolic extract, fractions 2E (67 - 79) (66.7 mg) illustrated the lowest polar acetogenins, fractions 3E (80 – 100) (119.4 mg), fractions 4E (101 - 132) (164.2 mg) and fractions 5E (133 - 144) (190.9 mg) (Figure 3.4, B).

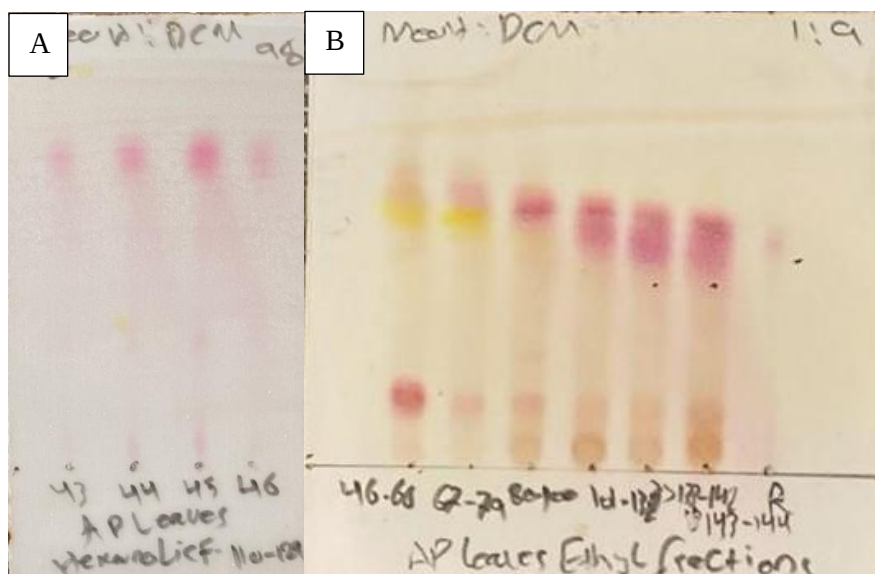


Figure 3.4. TLC plates for AP leaves hexane fractions 1H (110 - 129) sub-fractions 1Hb (43 – 46) (A), and AP leaves ethyl acetate fractions (46 - 144) (B) after spraying with Kedde's reagent.

The first purification for fractions 1E (46 – 66) (121.4 mg) was run using (DCM: MeOH) (100: 0 v/v) to (DCM: MeOH) (0: 100 v/v). 72 fractions were collected and sub-fractions from 37 to 55 (25 ml) indicated the presence of acetogenins with major contamination and they divided into two groups as follow: sub-fractions 1Ea (37 - 44) (12.2 mg), and sub-fractions 1Eb (45 – 55) (6 mg). The sub-fractions 1Ea (37 - 44) (12.2 mg) was further purified and the column was eluted with gradient combination of solvents involving (DCM: EtOAc) (100: 0 v/v) to (DCM: EtOAc) (0: 100 v/v). Seventy-five subfractions (25 ml) were collected and two sub-fractions 1Ec (71 – 72) (9 mg) indicated the presence of acetogenins, and some contamination. Due to the small amount, they were subject to MS.

For the second fractions 2E (67 - 79) (66.7 mg), it was firstly subjected to column using (DCM: EtOAc) (100: 0 v/v) to (DCM: EtOAc) (0: 100 v/v) as eluting system. 45 subfractions (25 ml) were collected and only one sub-fraction 25 (44.3 mg) indicated the presence of acetogenins with major contamination. Finally, this subfraction was submitted for another experiment using only (100% EtOAc) and 22 subfractions (25 ml) were collected and sub-fractions 2Eb (6 – 9)



(11.2 mg) indicated the spot for acetogenins with  $R_f$  value (0.79) (Figure 3.5). For the rest fractions of AP leaves ethyl acetate and to avoid repetition, the whole experiments for flash chromatography were briefly summarised in (Table 3.2).



Figure 3.5. TLC plate for AP leaves ethyl acetate fractions 2E (67 - 79) sub-fractions 2Eb (6 – 9) after spraying with Kedde's reagents.

Table 3.2. AP extracts were submitted for flash chromatography experiments using different eluting systems.

| Extraction      | Fractions indicated<br>acetogenins with major<br>contamination | Eluting system                                       | Subfractions indicated<br>acetogenins                                         | Eluting system                                               | Subfractions indicated<br>acetogenins                                   |
|-----------------|----------------------------------------------------------------|------------------------------------------------------|-------------------------------------------------------------------------------|--------------------------------------------------------------|-------------------------------------------------------------------------|
| Hexane extract  | Fraction 1H (110 - 129) (145.2 mg)                             | (DCM: MeOH) (100: 0 v/v) to (DCM: MeOH) (0: 100 v/v) | Sub-fractions 1Ha (44 – 46) (39.8 mg)                                         | (Hexane: EtOAc) (100: 0 v/v) to (Hexane: EtOAc) (0: 100 v/v) | Sub-fractions 1Hb 43 (1.5 mg), 44 (3.5 mg), 45 (4.3 mg) and 46 (0.8 mg) |
|                 | Fraction 2H (130 - 131) (33.3 mg)                              | (DCM: EtOH) (100: 0 v/v) to (DCM: EtOH) (0: 100 v/v) | Sub-fractions 2Ha (18 – 21) (3 mg)                                            | Due to the small amount, sample submitted for MS analysis    |                                                                         |
|                 | Fraction 3H (138 - 143) (24 mg)                                | (DCM: EtOH) (100: 0 v/v) to (DCM: EtOH) (0: 100 v/v) | Sub-fractions 3Ha (35 – 38) (3.4 mg)                                          |                                                              |                                                                         |
| Ethanol extract | Fraction B (80 mg)                                             | (DCM: MeOH) (100: 0 v/v) to (DCM: MeOH) (0: 100 v/v) | Subfractions 37 to 43 (18 mg)                                                 | (DCM: EtOAc) (100: 0 v/v) to (DCM: EtOAc) (0: 100 v/v)       | from 16 to 28 (1.4 mg) and from 30 to 36 (1.8 mg)                       |
|                 | Fraction 1E (46 – 66) (121.4 mg)                               | (DCM: MeOH) (100: 0 v/v) to (DCM: MeOH) (0: 100 v/v) | Sub-fractions 1Ea (37 - 44) (12.2 mg), and sub-fractions 1Eb (45 – 55) (6 mg) | (DCM: EtOAc) (100: 0 v/v) to (DCM: EtOAc) (0: 100 v/v)       | Sub-fractions 1Ec (71 – 72) (9 mg)                                      |
|                 | Fraction 2E (67 - 79) (66.7 mg)                                | (DCM: EtOAc) (100: 0 v/v) to                         | Sub-fractions 2Ea (25) (44.3 mg)                                              | (100% EtOAc)                                                 | Sub-fractions 2Eb (6 – 9) (11.2 mg)                                     |

|                          |                                    |                                                               |                                          |                                                                 |                                                                                      |
|--------------------------|------------------------------------|---------------------------------------------------------------|------------------------------------------|-----------------------------------------------------------------|--------------------------------------------------------------------------------------|
| Ethyl acetate<br>extract |                                    | (DCM: EtOAc)<br>(0: 100 v/v)                                  |                                          |                                                                 |                                                                                      |
|                          | Fraction 3E (80 – 100) (119.4 mg)  | (DCM: MeOH)<br>(100: 0 v/v) to<br>(DCM: MeOH)<br>(0: 100 v/v) | Sub-fractions 3Ea<br>(32 – 37) (73.9 mg) | (100% EtOAc)                                                    | Sub-fractions 3Eb<br>(26 – 30) (9 mg) and<br>Sub-fractions 3Ec<br>(32 – 36) (4.7 mg) |
|                          | Fraction 4E (101 - 132) (164.2 mg) | (DCM: MeOH)<br>(100: 0 v/v) to<br>(DCM: MeOH)<br>(0: 100 v/v) | Sub-fractions 4Ea<br>(31 – 38) (65.4 mg) | (DCM: EtOAc)<br>(100: 0 v/v) to<br>(DCM: EtOAc)<br>(0: 100 v/v) | Sub-fractions 4Eb<br>(42 – 47) (30 mg)                                               |
|                          | Fraction 5E (133 - 144) (190.9 mg) | (DCM: MeOH)<br>(100: 0 v/v) to<br>(DCM: MeOH)<br>(0: 100 v/v) | Sub-fractions 5Ea<br>(27 – 39) (73 mg)   | (DCM: EtOAc)<br>(100: 0 v/v) to<br>(DCM: EtOAc)<br>(0: 100 v/v) | Sub-fractions 5Eb<br>(31 – 32) (8.6 mg)                                              |

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### 3.3.3. NMR and MS analysis

The concentration of acetogenins in the hexane extract was extremely low compared to the ethyl acetate extract. Four compounds were identified in common to both extracts according to the NMR and MS results. Both NMR and MS indicated the presence of the same acetogenins in hexane and ethyl acetate extracts as the TLC also confirmed very weak pink spot. The fragmentation pathway used to identify acetogenins in this study was previously illustrated in two studies [303, 304] (Figure 3.6). The peak appears at 6.98 ppm and the presence of an epoxy ring was suggested by  $^1\text{H}$ -NMR resonances at  $\delta$  2.90 and 2.93 ppm, and in MS a molecular mass was determined as 530 by ESI-MS ( $[\text{M}+\text{H}]^+$   $m/z$  = 531) leading to the molecular formula  $\text{C}_{35}\text{H}_{62}\text{O}_3$  indicated epoxymurin-A (1). The MSMS suggested the existence of product ion spectrum of  $[\text{M} + \text{Li}]^+$  ( $m/z$  = 537) and intense fragments at  $m/z$  313, 301, 285 and 271 allowing localization of epoxy ring at C-15/C-16 with the double bond location at C-19/C-20. The concentration of this compound was less in the ethyl acetate extract compared to the hexane as both peaks in NMR and MS showed a small peak. Both NMR and MS results are consistent with the only two studies reported this compound in the pulp and stem bark of *A. muricata* [143, 305]. Another two epoxy acetogenins were identified as a mixture of two isomeric compounds in both hexane and ethyl acetate extracts are diepomuricanin A and B (2+3) and showed a peak at 6.96 ppm and molecular mass with 564  $m/z$  corresponding to the molecular formula  $\text{C}_{35}\text{H}_{62}\text{O}_4$ . The MSMS indicated the existence of product ion spectrum of  $[\text{M} + \text{Li}]^+$  ( $m/z$  = 553), and the location of the epoxy rings in the alkyl chain for diepomuricanin A at the C-15/C-16 and C-19/C-20 followed from the presence of two pairs of peaks at  $m/z$  383, 371 and  $m/z$  313, 301, and for diepomuricanin B the epoxy rings were observed at the C-13/C-14 and C-17/C-18 due to the presence of two pairs of peaks at  $m/z$  355, 343 and  $m/z$  285, 273. These compounds were identified via comparison with spectral data from the only study that have reported these compounds from hexane fraction of the methanolic extract of *A.*

*muricata* seeds [306]. Moreover, annotemoyin-1 & 2 (4+5) were reported in the hexane and ethyl acetate extracts and showed peaks for NMR at 6.98 ppm, and in MS a molecular mass with 564  $m/z$ . The MSMS analysis revealed that intense fragments at  $m/z$  277, 247 and 205 suggested the localization of mono-THF ring at C-18/C-21. These mono tetrahydrofuran acetogenins were identified from the seed of *A. atemoya* (African Pride cultivars) [307].

The ethyl acetate extract was also investigated, and four compounds were identified of which two of them were isolated and characterised using NMR to confirm their structures. The isolation and identification of acetogenins from *A. atemoya* leaf has not been reported to date. Since the ethyl acetate extract is the most concentrated and richest in presence of acetogenins compared to other extracts, it is the only crude extract from which the isolated compounds can be reported. The ethyl acetate crude extract (2.991g) was purified as described and the structures elucidated based on NMR experiments which were consistent previously reported spectra for two adjacent bis-THF acetogenins, affording squamocin G (6) (11.2 mg) and squamocin C (7) (9 mg) [284, 308, 309].

Compound 6 ( $C_{37}H_{66}O_7Na$ ;  $m/z$  645.4  $[M + Na]^+$ , ESI-MS) was determined to possess an adjacent bis-THF ring system that located between C-15 and C-24 according to both MS and NMR data. The presence of  $\alpha$ ,  $\beta$ -unsaturated  $\gamma$ -lactone was suggested by  $^1H$ -NMR data at 7.18 (1H, d, H-35), 5.06 (1H, qd, H-36), 3.85 (1H, m, H-4), 2.50 (1H, m, H-3a), 2.40 (1H, m, H-3b), and 1.44 (3H, d, H-37) and  $^{13}C$ -NMR data at 174.33 (C-1), 151.77 (C-35), 131.12 (C-2), 77.96 (C-36), 69.99 (C-4), 33.2 (C-3), and 19.05 (C-37).

### **Squamocin G (6)**

White powder  $^1H$ -NMR (400 MHz,  $CDCl_3$ ) 0.87 (3H, t,  $J=6.9$  Hz, H-34), 1.44 (3H, d,  $J=6.7$  Hz, H-37), 2.40 (1H, dd,  $J=15.0, 8.2$  Hz, H-3a), 2.50 (1H, br d,  $J=15.0$  Hz, H-3b), 3.39 (1H, m, H-15), 3.84-3.93 (3H, m, H-16, -23, -24), 3.85 (1H, m, H-4), 3.84-3.90 (2H, m, H-19, 20),

5.06 (1H, qq,  $J=6.8, 1.4$  Hz, H-36), 7.18 (1H, br s, H-35),  $^{13}\text{C}$ -NMR (400 MHz,  $\text{CDCl}_3$ ) 14.10 (C-34), 19.10 (C-37), 22.67 (C-33), 24.49 (C-22), 25.54 (C-6 or C-13), 25.61 (C-13 or C-6), 26.03 (C-26), 28.34 (C-17), 28.88-28.93 (C-18, -21), 29.10-29.67 (C-7, -8, -9, -10, -11, -12, -27, -28, -29, -30, -31), 31.89 (C-32), 32.42 (C-25), 33.2 (C-3), 33.32 (C-14), 37.39 (C-5), 69.99 (C-4), 71.33 (C-24), 74.04 (C-15), 77.97 (C-36), 82.26 (C-19 or C-20), 82.51 (C-20 or C-19), 82.77 (C-23), 83.2 (C-16), 131.18 (C-2), 151.77 (C-35), 174.33 (C-1) (Table 3.3).

Table 3.3.  $^{13}\text{C}$  and  $^1\text{H}$  NMR [ $\text{CDCl}_3$ , 400 MHz], spectral data for compound 6

| No | $^{13}\text{C}$ | $^1\text{H}$ | No | $^{13}\text{C}$ | $^1\text{H}$ |
|----|-----------------|--------------|----|-----------------|--------------|
| 1  | 174.33          |              | 20 | 82.26           | 3.84         |
| 2  | 131.18          |              | 21 | 28.93           | 1.97-1.63    |
| 3  | 33.2            | 2.40, 2.50   | 22 | 24.49           | 1.92-1.90    |
| 4  | 69.99           | 3.85         | 23 | 82.77           | 3.93         |
| 5  | 37.39           | 1.42         | 24 | 71.33           | 3.84         |
| 6  | 25.54           | 1.25         | 25 | 32.42           | 1.33-1.56    |
| 7  | 29.10-29.67     | 1.25         | 26 | 26.03           | 1.25         |
| 8  | 29.10-29.67     | 1.25         | 27 | 29.10-29.67     | 1.25         |
| 9  | 29.10-29.67     | 1.25         | 28 | 29.10-29.67     | 1.25         |
| 10 | 29.10-29.67     | 1.25         | 29 | 29.10-29.67     | 1.25         |
| 11 | 29.10-29.67     | 1.25         | 30 | 29.10-29.67     | 1.25         |
| 12 | 29.10-29.67     | 1.25         | 31 | 29.10-29.67     | 1.25         |
| 13 | 25.61           | 1.25         | 32 | 31.89           | 1.25         |
| 14 | 33.32           | 1.33-1.56    | 33 | 22.67           | 1.25         |
| 15 | 74.04           | 3.39         | 34 | 14.10           | 0.87         |
| 16 | 83.2            | 3.84         | 35 | 151.77          | 7.18         |
| 17 | 28.34           | 1.97-1.63    | 36 | 77.97           | 5.06         |
| 18 | 28.88           | 1.97-1.63    | 37 | 19.10           | 1.44         |
| 19 | 82.51           | 3.90         |    |                 |              |

Compound 7 ( $\text{C}_{37}\text{H}_{66}\text{O}_7\text{Na}$ ;  $m/z$  645.4  $[\text{M} + \text{Na}]^+$ , ESI-MS) was determined to possess an adjacent bis-THF ring system that located between C-15 and C-24 according to both MS and NMR data.  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ). The occurrence of  $\alpha$ ,  $\beta$ -unsaturated  $\gamma$ -lactone was suggested by  $^1\text{H}$ -NMR data at 6.98 (1H, q, H-35), 4.99 (1H, qd, H-36), 2.25 (2H, t, H-3) and 1.40 (3H, d, H-37). Additionally, the spectrum also revealed the presence of a terminal methyl proton as a triplet at 0.87 (C-34).  $^{13}\text{C}$ -NMR data on the other hand, confirmed the existence of  $\alpha$ ,  $\beta$ -unsaturated  $\gamma$ -lactone by indicating the presence of seven oxygenated carbons at 83.30 (C-

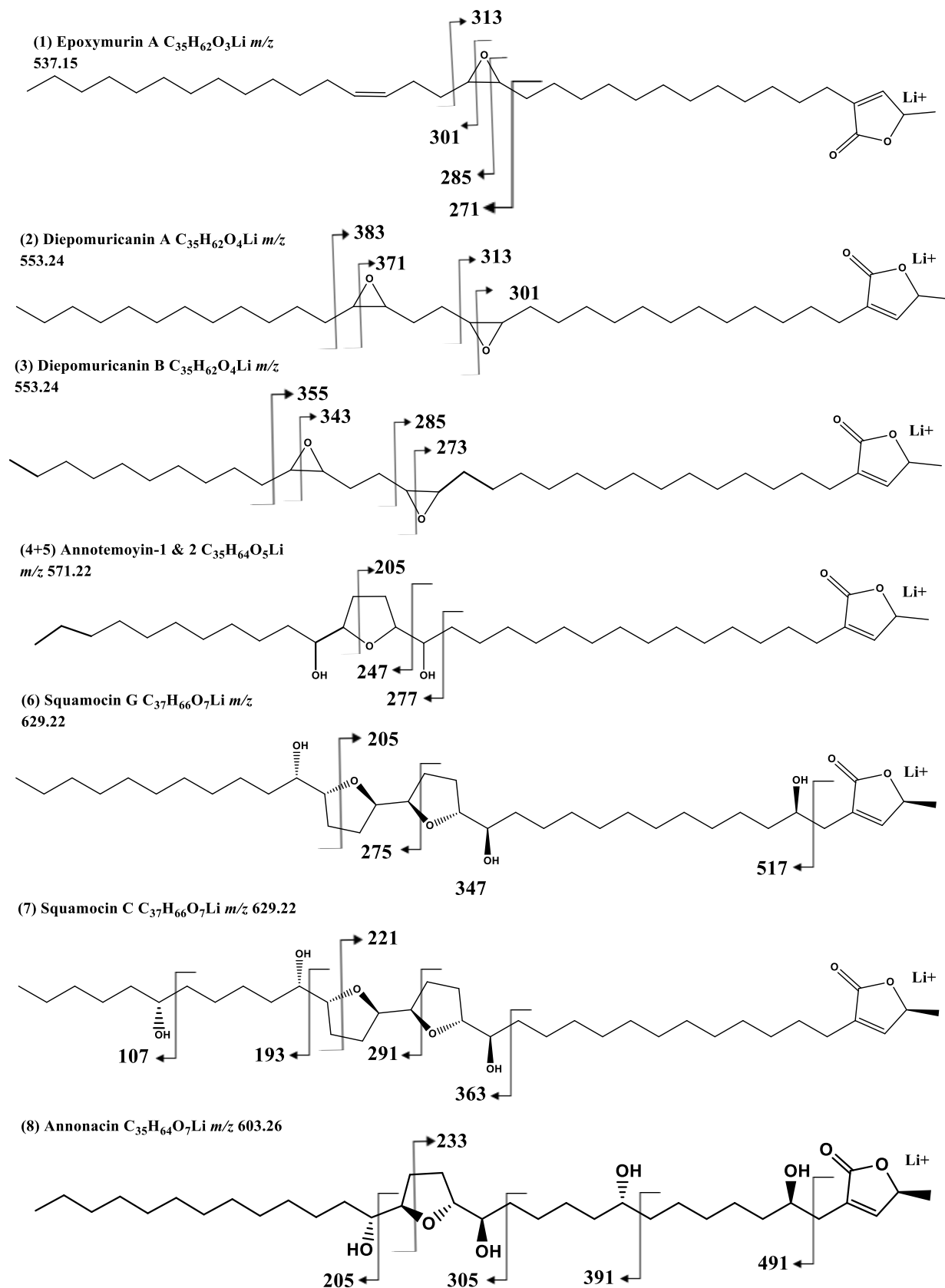
16), 82.21 (C-19), 82.52 (C-20) and 82.78 (C-23). The remaining three hydroxylated carbons (C-15), (C-24) and (C-29) indicated their resonance at 74.12, 71.39 and 71.77 respectively.

### Squamocin C (7)

White powder  $^1\text{H}$ -NMR (400 MHz,  $\text{CDCl}_3$ ) 0.87 (3H, t,  $J=6.7$  Hz, H-34), 1.40 (3H, d,  $J=7.1$  Hz, H-37), 2.25 (2H, t,  $J=7.7$  Hz, H-3), 3.40 (1H, m, H-15), 3.59 (1H, m, H-29), 3.82-3.96 (5H, m, H-16, -19, -20, -23, -24), 4.99 (1H, q,  $J=6.9$  Hz, H-36), 6.98 (1H, s, H-35),  $^{13}\text{C}$ -NMR (400 MHz,  $\text{CDCl}_3$ ) 14.1 (C-34), 19.2 (C-37), 22.0 (C-26), 22.6 (C-33), 24.80 (C-22), 25.15 (C-3), 25.31 (C-31), 25.64 (C-13, -27), 27.37 (C-4), 28.36 (C-17), 28.9 (C-18, -21), 29.16 (C-5), 29-30 (C-6, -7, -8, -9, -10, -11, -12), 31.9 (C-32), 32.45 (C-25), 33.28 (C-14), 37.23 (C-30), 37.48 (C-28), 71.39 (C-24), 71.77 (C-29), 74.12 (C-15), 77.4 (C-36), 82.21 (C-19), 82.52 (C-20), 82.78 (C-23), 83.3 (C-16), 134.3 (C-2), 148.87 (C-35), 173.9 (C-1). (Table 3.4).

Table 3.4.  $^{13}\text{C}$  and  $^1\text{H}$  NMR [ $\text{CDCl}_3$ , 400 MHz], spectral data for compound 7

| No | $^{13}\text{C}$ | $^1\text{H}$ | No | $^{13}\text{C}$ | $^1\text{H}$ |
|----|-----------------|--------------|----|-----------------|--------------|
| 1  | 173.9           |              | 20 | 82.52           | 3.82-3.96    |
| 2  | 134.3           |              | 21 | 28.9            | 1.52-1.9     |
| 3  | 25.15           | 2.25         | 22 | 24.80           | 1.52-1.9     |
| 4  | 27.37           | 1.52         | 23 | 82.78           | 3.82-3.96    |
| 5  | 29.16           | 1.2-1.7      | 24 | 71.39           | 3.82-3.96    |
| 6  | 29-30           | 1.2-1.7      | 25 | 32.45           | 1.25-1.4     |
| 7  | 29-30           | 1.2-1.7      | 26 | 22.0            | 1.25-1.4     |
| 8  | 29-30           | 1.2-1.7      | 27 | 25.64           | 1.25-1.4     |
| 9  | 29-30           | 1.2-1.7      | 28 | 37.48           | 1.25-1.4     |
| 10 | 29-30           | 1.2-1.7      | 29 | 71.77           | 3.59         |
| 11 | 29-30           | 1.2-1.7      | 30 | 37.23           | 1.25-1.4     |
| 12 | 29-30           | 1.2-1.7      | 31 | 25.31           | 1.25-1.4     |
| 13 | 25.64           | 1.2-1.7      | 32 | 31.9            | 1.25-1.4     |
| 14 | 33.28           | 1.2-1.7      | 33 | 22.6            | 1.25-1.4     |
| 15 | 74.12           | 3.40         | 34 | 14.1            | 0.78         |
| 16 | 83.3            | 3.82-3.96    | 35 | 148.87          | 6.98         |
| 17 | 28.36           | 1.52-1.9     | 36 | 77.4            | 4.99         |
| 18 | 28.9            | 1.52-1.9     | 37 | 19.2            | 1.40         |
| 19 | 82.21           | 3.82-3.96    |    |                 |              |





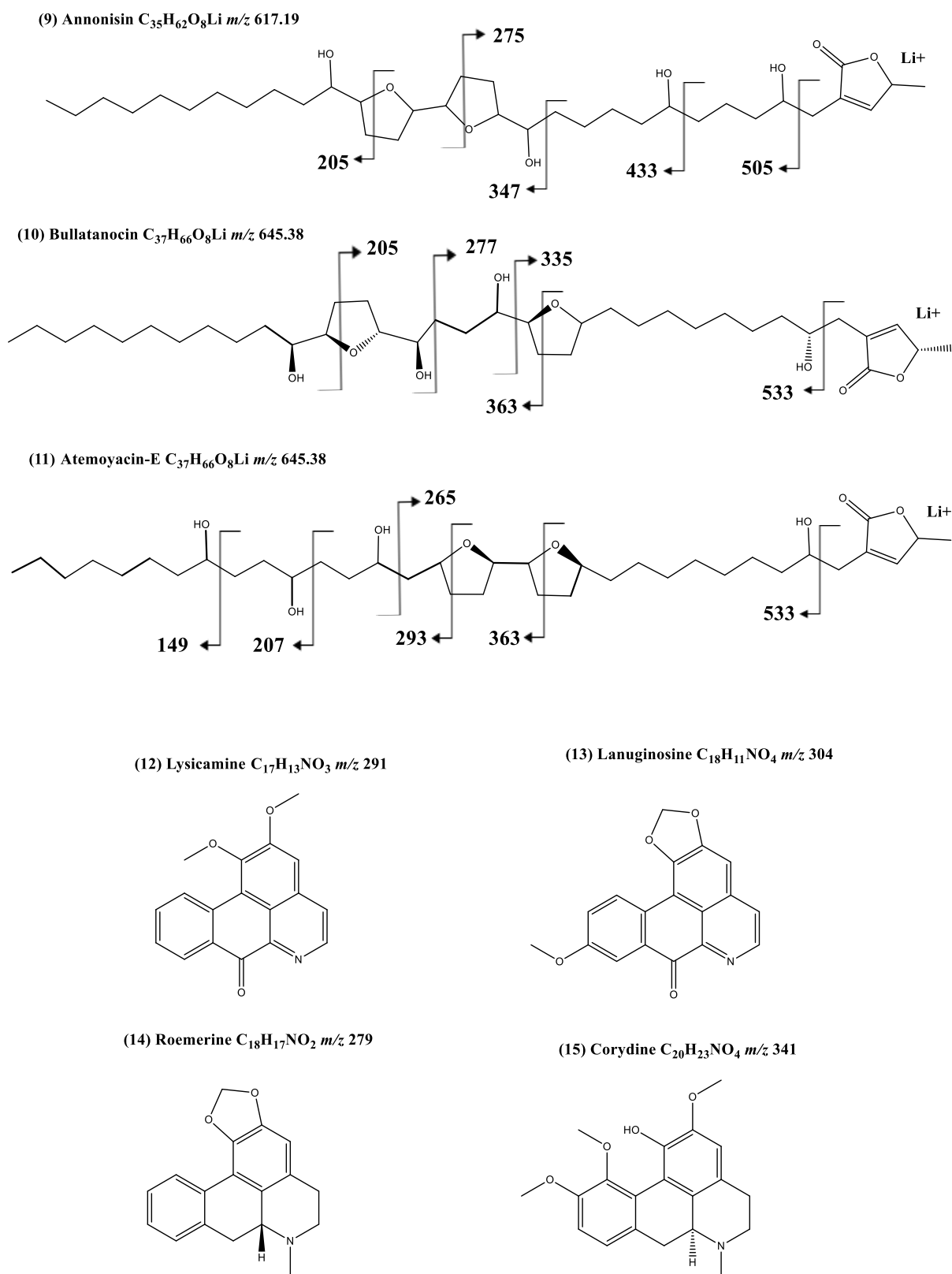


Figure 3.6. Structures and fragmentation patterns for the  $[M + Li]^+$  adducts of acetogenins and alkaloids identified in the leaves of *A. atemoya*.

Additionally, annonacin (8) and annonisin (9) were also reported in the ethyl acetate extract of

the leaves and the MSMS suggested the existence of a product ion spectrum of  $[M + Li]^+$  ( $m/z = 603$  and  $617$ ) respectively. Both compounds (8) and (9) revealed intense fragments at  $m/z$  491, 391, 305, 233 and 205, and 505, 433, 347, 275 and 205 respectively, and have been reported in the *A. atemoya* seeds [268]. Finally, the most concentrated acetogenins reported in the leaves of *A. atemoya* was reported at  $[M + Li]^+$  ( $m/z = 645$ ) leading to the molecular formula  $C_{37}H_{66}O_8$  indicated bullatanocin (10) and atemoyacin-E (11). A series of fragments were reported in MSMS suggested the presence of compounds (10) and (11) at  $m/z$  533, 363, 335, 277 and 205, and 533, 363, 293, 265, 207 and 149, respectively and both compounds have been reported in the *A. atemoya* seeds [267, 279].

For compound (10), the presence of  $\alpha$ ,  $\beta$ -unsaturated  $\gamma$ -lactone was suggested by  $^1H$ -NMR data at 7.18 (1H, q, H-35), 5.06 (1H, qq, H-36), 3.86 (1H, m, H-4), 2.54 (1H, m, H-3a), 2.40 (1H, m, H-3b), and 1.42 (3H, d, H-37) and  $^{13}C$ -NMR data at 174.46 (C-1), 151.90 (C-35), 131.11 (C-2), 78.01 (C-36), 69.88 (C-4), and 19.07 (C-37), and the occurrence of two nonadjacent THF rings was indicated by proton resonances at 3.86 (H-12) and 3.81 (H-15, 20, 23). Amongst of the identified acetogenins, only two compounds (1 and 8) were reported in the leaves of KJ cultivar.

Other chemical constituents that identified from leaves of *A. atemoya* (AP cultivar) were alkaloids and the TLC and MS confirmed their presence in the ethanolic extract. For instance, positive ESI-MS  $m/z$ : 291  $[M + H]^+$  indicated lysicamine (12) and the MS data are in agreement with the literature [299, 310]. Moreover, other oxoaporphine alkaloids reported in both ethyl acetate and ethanol extracts was lanuginosine (13) and the positive ESI-MS reported at  $m/z$ : 304  $[M + H]^+$ . The MS data are in agreement with the literature and both compound (12 and 13) have been reported in the leaves of *A. atemoya* [299]. Roemerine (14) an aporphine alkaloids was also reported in the ethanol extract at ESI-MS  $m/z$ : 279  $[M + H]^+$  and this compound was reported in the leaves of *A. squamosa* and stem of *A. cherimola* [281, 311, 312].

Finally, other aporphine alkaloids corydine (15) was also confirmed by ESI-MS  $m/z$ : 341  $[M + H]^+$  and this compound was reported in the leaves of *A. squamosa* [281]. For all NMR and MS analysis for both cultivars see appendix C and D.

### 3.4. Conclusion

To our knowledge, this is the first quantitative determination of acetogenins in the leaves of *A. atemoya*. All acetogenins (1-11) were identified putatively for the first time in the leaves of *A. atemoya* (AP cultivar). Two compounds (6 and 7) were isolated, and the rest were identified using NMR and MSMS. Both hexane and ethyl acetate extracts indicated the presence of acetogenins with high concentration in the ethyl acetate extract. Four acetogenins (1-4) were common to both extracts, whereas the ethanolic extract indicated four alkaloids (12-15). Among of the reported acetogenins three were epoxy type with mono-epoxy compound (1) and bis-epoxy compounds (2 and 3). Furthermore, three compounds (4, 5 and 8) were reported for having a mono-THF, four compounds with adjacent bis-THF compounds (6, 7, 9 and 11), and one with non-adjacent bis-THF compound (10). Finally, among of the eleven acetogenins identified, only two compounds (1 and 8) were reported in the leaves of KJ cultivar.

# CHAPTER 4

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## **MS/MS Analysis of Annonaceous Acetogenins and Alkaloids from the fruit's Pulp and Seeds of Annona Atemoya Cultivars**

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[doi.org/10.3390/ijms24032294](https://doi.org/10.3390/ijms24032294)

## Abstract

The genus of *Annona* is one of 120 genera of the Annonaceae family and contains more than 119 species of trees and shrubs. This plant is widely cultivated in tropical and subtropical continents including north and south America, Asia, Africa and Australia. The phytochemical investigations of *A. atemoya* fruit and seeds have been reported in limited studies include alkaloids, flavonoids, terpenes and acetogenins. The results of this study indicated the presence of annonaceous acetogenins in the fruit's pulp of *A. atemoya* for the first time. Eighteen compounds were identified in both cultivars, sixteen acetogenins and two alkaloids. The concentration of acetogenins in the pulp was very low compared to the seeds, and all three extracts of seeds illustrated almost the same acetogenins with more compounds reported in the ethyl acetate extract.

**Keywords:** *Annona atemoya*; fruit's pulp; seeds; annonaceous acetogenins; alkaloids

## 4.1. Introduction

The plant of *Annona atemoya* (*A. atemoya*) originated from the hybridization of two *Annona* species *Annona squamosa* and *Annona cherimola* in Florida between 1907 and 1908 [113, 313]. It was also reported that natural hybridization occurred in Australia and Palestine from 1850 to 1930 [113, 313]. *A. atemoya* has various cultivars for instance, in Australia it is known as Island Gem, Nielsen, Hillary White, Maroochy Gold, Pink's Mammoth and African Pride; in Brazil it is known as African Pride, Pink's Mammoth, Thompson and Gefner; and in the United States of America *A. atemoya* is known as Bradley, Page, Keller, Priestly, Stremer and Caves [113, 252, 253]. *A. atemoya* grows from 7.5 to 10 m, with low drooping branches [113, 313]. It produces a delicious fruit that is conical or heart shaped and typically weighs between from 2 to 2.5 Kg. [313]. The pulp can be eaten either fresh or made into juice, and can be separated from the seeds [313]. The seeds are characterized as having a cylindrical shape, and are smooth and dark brown to black in colour [313]. An extensive number of phytochemicals have been isolated from the fruits and seeds of *A. atemoya*. The fruits of *A. atemoya* have been reported to comprise a wide range of essential oils and the main compounds identified to date are  $\alpha$ -pinene,  $\beta$ -pinene, germacrene D and (*E*)-ocimene [254, 255, 264, 314]. In addition, the fruit of *A. atemoya* has also been shown to contain phenolic compounds like epicatechin, catechin, 3,4-dihydroxybenzoic acid, chlorogenic acid and *p*-coumaric acid [274]. The phytochemical constituents of the seeds were also investigated in a limited number of studies and according to these studies annonaceous acetogenins (AC) are the main constituents in comparison to alkaloid, *N*-acyl-tryptamines and phenolic components [267, 268, 278, 279, 315-318]. Approximately 36 acetogenins were isolated and identified from the seed of *A. atemoya* [267-269, 278, 279, 315-317], two alkaloids including atemoine and cleistopholine [275], and four phenolic compounds including chlorogenic acid, ferulic acid, vanillic acid and myricetin

[293]. Due to limited research to date, the aim of this study was to investigate the phytochemical constituents of the seeds and the pulp of *A. atemoya*.

## 4.2. Materials and methods

### 4.2.1. Plant material

The semi-matured fresh Australian grown custard apples of *A. atemoya* cultivars African pride (AP) and KJ pinks (KJ) were collected in July 2020 from a local farm in Queensland, Australia. The fruits were washed with running tap water and separated into pulp and seed portions and then kept on the bench for air drying at room temperature and well-ventilated area for one month. Then, the pulp and seeds were crushed into small pieces and powdered via an electric blender (Multifunctional crusher). The final volume of the pulp and seeds powders is 380 g and 50.6 g for AP cultivar, and 200 g and 34.1 g for KJ cultivar.

### 4.2.2. Preparation of plant extracts

The dried and powdered pulp and the seeds of both cultivars were extracted by sonication using three solvents of increasing polarity (i.e., hexane, ethyl acetate and water) in consecutive steps. All extracts were shaken by using an electronic stirrer and kept for three days, and then filtrated extracts were transferred into glass vials, and solvents were left to evaporate at room temperature. The final crude extracts weight for the pulp and seeds was calculated and listed in (Table: 4.1).

Table 4.1. The final weight of crude extract of pulp and seeds of *A. atemoya* cultivars.

| Extraction solvents | AP cultivar |          | KJ cultivar |          |
|---------------------|-------------|----------|-------------|----------|
|                     | Pulp (g)    | Seed (g) | Pulp (g)    | Seed (g) |
| Hexane              | 2.63        | 10.93    | 1.787       | 6.32     |
| Ethyl acetate       | 0.783       | 1.72     | 0.884       | 1.3      |
| Ethanol             | 25.52       | 1.5      | 30.2        | 1.09     |

#### **4.2.3. Chemical and reagents**

Hexane, dichloromethane (DCM), ethyl acetate (EtOAc), ethanol (EtOH) methanol (MeOH), and all the other solvents were purchased from the School of Chemistry, University of Sydney. LC-MS grade solvents and formic acid were obtained from Sigma Aldrich, Castle Hill, Sydney, Australia.

#### **4.2.4. Phytochemical analysis**

A small amount of each crude extract was qualitatively tested to disclose the presence of acetogenins. Neither flash or column chromatography experiments were run due to the small amount of the crude extracts of both seeds and pulp, and the phytochemical constituents were identified according to the characteristic colour changes on Thin Layer Chromatography (TLC) utilizing Kedde's reagent.

#### **4.2.5. Thin Layer Chromatography (TLC)**

Analytical plates for TLC were Silica Gel 60 F<sub>254</sub>, Aluminium sheets, 200um, (20 x 20 cm) and Merck silica gel 60, particle size (0.040 – 0.063 mm) for normal CC (Merck KGaA, Darmstadt, Germany). A small amount of each extract (1-2 mg) of seeds and pulp was run using TLC plate using MeOH: DCM (1: 9) as a mobile phase. Since AC is not detected under the ultraviolet light (UV) at (254nm, 365nm), Kedde's reagent was used to visualize these compounds.

#### **4.2.6. Nuclear Magnetic Resonance (NMR)**

All the extracts were characterized by <sup>1</sup>H-NMR and <sup>13</sup>C-NMR spectroscopy. <sup>1</sup>H and <sup>13</sup>C spectra were recorded at 400MHz and 100MHz, respectively using a Varian Gemini 400-MR automated spectrometer at room temperature, using CDCl<sub>3</sub> and CD<sub>3</sub>OD as solvents and their residual signals were set to 7.26 ppm for <sup>1</sup>H and to 77.0 ppm for <sup>13</sup>C, and 3.35, 4.78 ppm for <sup>1</sup>H and to 49.3 ppm for <sup>13</sup>C, respectively.



#### ***4.2.7. Mass Spectrometry (MS) and Tandem mass spectrometry (MS/MS)***

Mass spectra were carried out on a Thermo Scientific TSQ series Quantum Access Max liquid chromatography-mass spectrometry LC-MS/MS system (Thermo Fisher Scientific, Massachusetts, USA), using heated electrospray ionization in positive ionization mode. High purity nitrogen (BOC, Sydney, Australia) at 60 psi was used as the sheer gas and ultra-high purity argon (BOC) at 2 mTorr was used as collision gas. The mass spectroscopy was conducted at a voltage of 4500 V. The heated capillary and vaporizer were heated at 380 °C and 350 °C, respectively. The mass spectra were acquired at a scan range of 100 – 1000 m/z. Data was analysed by XCalibur 4.1.50 (Thermo Fisher Scientific, Massachusetts) software. Regards MSMS experiment, 1 mg solution of each crude extracts was dissolved in 1 ml of MeOH, and the injection volume was 5 µl. The optimal collision energy was 70 eV and Lithium iodide (2mM in MeOH) was added to each sample before analysis.

### **4.3. Results and Discussion**

#### ***4.3.1. TLC***

A small amount (2 mg) of each crude extract of pulp and seeds of AP cultivar was dissolved in 1 ml of solvent and then analysed by TLC to investigate their phytochemical constituents. The TLC was developed in CAMAG 20x10cm twin-through glass developing chambers using (MeOH: DCM) (10: 90 v/v) as mobile phase. Since acetogenins compounds cannot be detected using UV light at (254nm, 365nm) and Kedde's reagent were used in order to visualize them. The TLC plate was dipped in 2% of 3,5-dinitrobenzoic acid in 100 ml of ethanol and left to dry for five minutes and then sprayed with 5% of KOH in 100 ml of ethanol. With regards to the pulp and based upon TLC findings, the concentration of the acetogenins was too low and the

ethyl acetate extract illustrated two spots with  $R_f$  0.25 and 0.85. Similarly, the hexane extract was also indicated a very weak spot of non-polar acetogenins with  $R_f$  0.85. Finally, the ethanolic extract of the pulp did not display any spot for acetogenins and was subject to NMR and MS analysis (Figure 4.1 A). To our knowledge, this is the first time the presence of the acetogenins in the pulp of *A. atemoya* have been identified and reported.

For the seeds, all three extracts indicated the presence of acetogenins as pink bands after reacting with Kedde's reagents (Figure 4.1 B). Both hexane and ethanol extracts indicated the same bands of the polar acetogenins at (0.25), however, these spots were less concentrated compared to the ethyl acetate extract. Moreover, the ethyl acetate extract was the richest in an abundance of acetogenins compared to the hexane and ethanol extracts. Due to time shortages and to perform other experiments, KJ cultivar was only submitted for MS analysis.

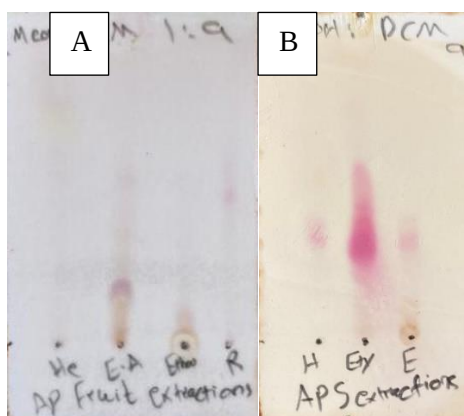


Figure 4.1. TLC profile of total AP cultivar pulp (A) and seed (B), after spraying with Kedde's reagents.

#### 4.3.2. NMR and MS analysis

For AP cultivar, the concentration of acetogenins in the pulp was very low compared to the seeds, and all three extracts (hexane, ethyl acetate and ethanol) reported the presence of the acetogenins according to NMR and MS analysis. In the NMR, the peak appears at 6.98 ppm

and the presence of an epoxy ring with resonances at  $\delta$  2.90 and 2.93 ppm indicated the presence of epoxy acetogenins. In the MS a molecular mass was determined as 530 by ESI-MS ( $[M+H]^+$   $m/z = 531$ ) leading to the molecular formula  $C_{35}H_{62}O_3$  suggested compound (1) (Figure 4.2). The MSMS also confirmed the presence of product ions spectrum of  $[M + Li]^+$  ( $m/z = 537$ ) and intense fragments at  $m/z$  313, 301, 285 and 271. The existence of these product ions was determined the localization of epoxy ring at C-15 and C-16 with the double bond location at C-19 and C-20. This compound was reported in all three extracts and the NMR and MS results consistent with the only two studies reporting this compound in the pulp and stem bark of *A. muricata* [143, 305]. Another compound identified only in the ethyl acetate extract was montanacin A (16) and showed a doublet peak at 7.20 ppm, and at  $[M + Li]^+$  ( $m/z = 647$ ) leading to the molecular formula  $C_{37}H_{68}O_8$  (Figure 4.3). The MSMS analysis revealed fragments at  $m/z$  535, 463, 337, 277 and 205 suggested the localization of mono-THF ring at C-20 and C-23. This compound was reported in *A. montana* seed, and consistent with this current study [319]. To our knowledge, this was the first time identifying and characterizing these compounds in *A. atemoya* and to report the presence of acetogenins in *A. atemoya* fruit.

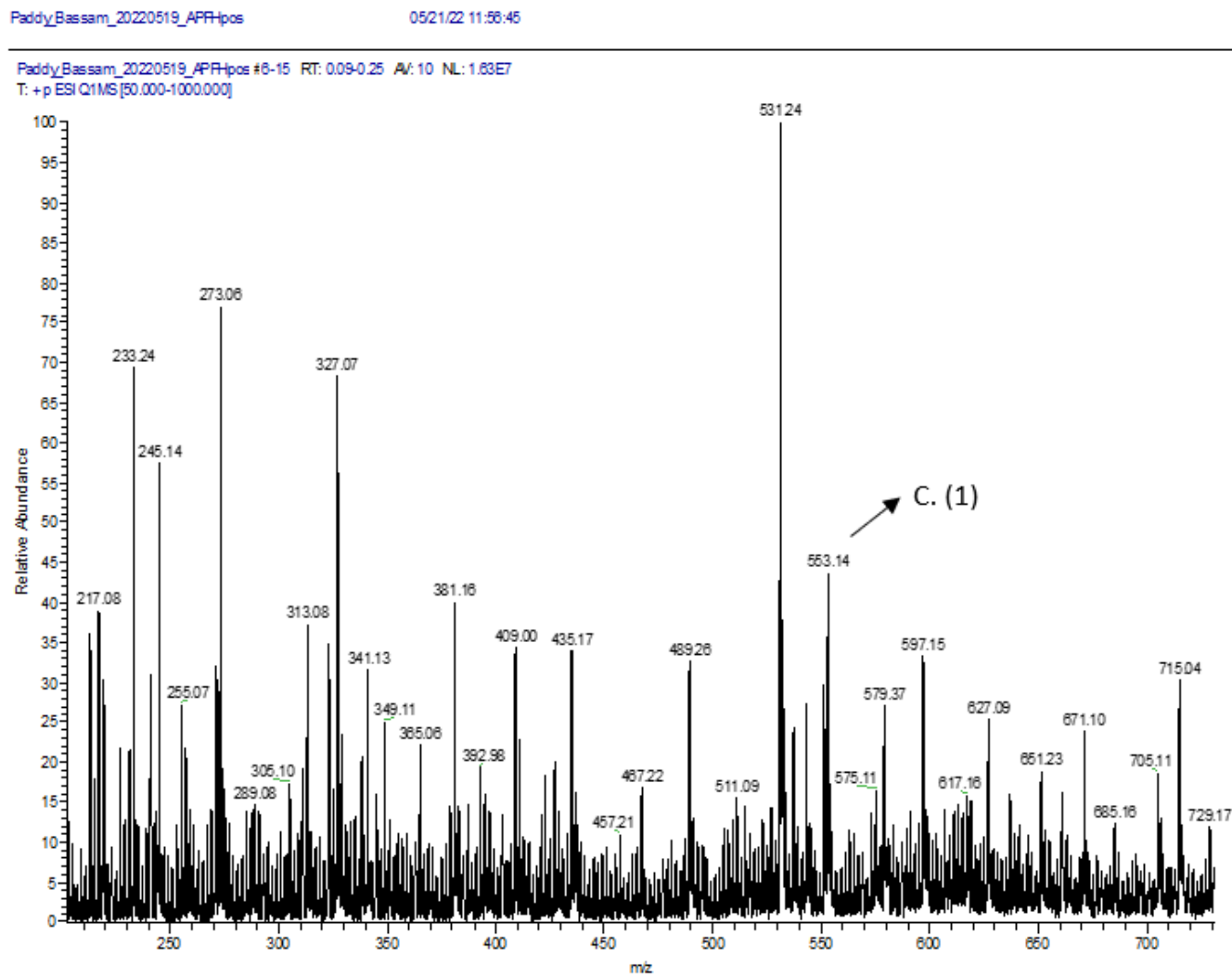


Figure 4.2. MS analysis of *A. atemoya* fruit's pulp indicated the presence of compound (1) in the hexane extract.

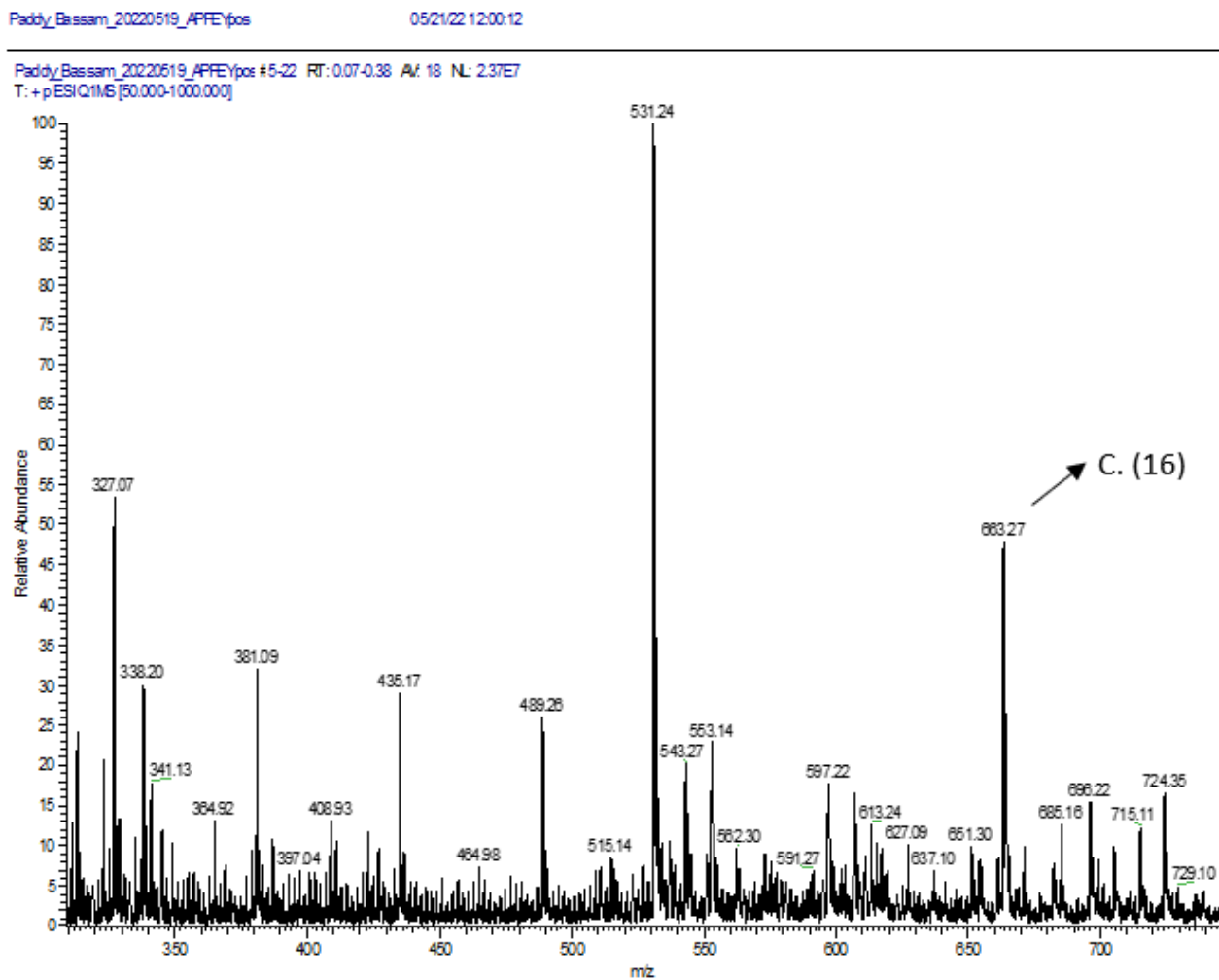


Figure 4.3. MS analysis of *A. atemoya* fruit's pulp indicated the presence of compound (16) in the ethyl acetate extract.

With regards to the seeds, all three extracts illustrated almost the same acetogenins with more compounds reported in the ethyl acetate extract. For instance, compound (1) was also reported only in the hexanolic extract of seed with a molecular mass determined as 530 by ESI-MS ( $[M+H]^+$   $m/z = 531$ ). Other adjacent bis-THF compound was identified solely in the hexane extract was desacetylurvaricin (17) and in MS a molecular mass was determined by ESI-MS ( $[M+H]^+$   $m/z = 606$ ) leading to the molecular formula  $C_{37}H_{62}O_6$ . The MSMS analysis revealed that intense fragments at  $m/z$  347, 275, 205 and 177 suggested the localization of bis-THF ring between C-16 and C-23. This compound was previously reported in the seed of *A. atemoya* [316]. Interestingly, nine compounds were reported in common in the three extracts according to the MS and MSMS analysis. These compounds were identified as diepomuricanin A & B (2 + 3), annotemoyin-1 & 2 (4 + 5), atemoyacin-A (18), squamocin G (6), squamocin C (7), bullatanocin (10) and atemoyacin-E (11).

For compounds (2 + 3), a molecular mass with 546  $m/z$  corresponding to the molecular formula  $C_{35}H_{62}O_4$ , and the MSMS indicated the existence of product ion spectrum of  $[M + Li]^+$  ( $m/z = 553$ ). The location of the epoxy rings in the alkyl chain for compound (2) was detected at C-15/C-16 and C-19/C-20 followed from the presence of two pairs of peaks at  $m/z$  383, 371 and  $m/z$  313, 301, and for compound (3) the epoxy rings was observed at the C-13/C-14 and C-17/C-18 due to the presence of two pairs of peaks at  $m/z$  355, 343 and  $m/z$  285, 273. These compounds were identified via comparison with spectral data from the only study to have reported these compounds from hexane fraction of the methanolic extract of *A. muricata* seeds [306]. With regards to compounds (4 + 5), the MS revealed a peak at 564  $m/z$  and the MSMS analysis showed intense fragments at  $m/z$  277, 247 and 205 indicated the localization of mono-THF ring at C-18/C-21. Both compounds (4 + 5) were previously reported in the seed of *A. atemoya* (African Pride cultivars) [317].

An additional compound reported in three extracts was atemoyacin-A (18) and the molecular weight was indicated by the peak at  $m/z$  595  $[MH^+]$  corresponding to the molecular formula  $C_{35}H_{62}O_7$ . The MSMS revealed a series of fragments at  $m/z$  489, 347, 275, 205 and 177 confirmed the presence of compound (18) in all three extracts, and was previously reported in the seed of *A. atemoya* Hort [278]. Moreover, Compounds (6 + 7) were determined to have a molecular mass with ( $m/z = 645.4 [M + H]^+$ , ESI-MS) corresponding to the molecular formula  $C_{37}H_{66}O_7$ ; and determined to possess an adjacent bis-THF ring system located between C-15 and C-24 according to MS data. The MSMS analysis revealed that intense fragments at  $m/z$  517, 347, 275 and 205 for compound (6), and at  $m/z$  363, 291, 221, 193 and 107 for compound (7).

The second most concentrated acetogenins reported in the seed of *A. atemoya* was reported at  $[M + H]^+$  ( $m/z = 661$ ) leading to the molecular formula  $C_{37}H_{66}O_8$  indicated both compounds (10) and (11). In the MSMS, a series of fragments were reported at  $m/z$  533, 363, 335, 277 and 205, and 533, 363, 293, 265, 207 and 149, respectively and both compounds have been reported in the *A. atemoya* seeds [267, 279].

Other compounds which only reported in the ethyl acetate extract of seed were atemoyin (19), annonisin (9) and robustocin (20). For compound (19), the MS showed peak at  $m/z = 601 [M + Na]^+$ , and the MSMS revealed intense fragments at  $m/z$  347, 275, 205 and 177. The fragmentation pattern observed in the MSMS spectra demonstrated that the two THF rings of compound (19) were located between C- 13 and C- 22. Regards compound (9), a molecular mass at  $m/z = 633 [M + Na]^+$  corresponding to the molecular formula  $C_{35}H_{62}O_8$  and the MSMS indicated the existence of predicted fragments at  $m/z$  505, 433, 347, 275 and 205 allowed placement of the bis-THF part between C- 13 and C- 22. Both compounds 19 and 9 were previously reported from methanolic and ethanolic extracts of *A. atemoya* seeds [268, 316].

Finally, compound (20) was reported for the first time in *A. atemoya* and the MS demonstrated peak at  $m/z = 585$   $[M + Na]^+$  (Figure 4.4), and the MSMS revealed two intense fragments at  $m/z$  261 and 233. The fragmentation pattern confirmed the occurrence of the two THF rings between C- 9 and C- 18, and this compound was previously reported only in the seed of *A. muricata* [320].

Other phytochemicals identified in the pulp and seed of *A. atemoya* were alkaloids and MS confirmed their presence. For instance, lanuginosine (13) was reported in all three pulp's extracts as well as the hexane and ethanol extracts of seed and the positive ESI-MS reported at  $m/z$ : 304  $[M + H]^+$ . The MS data was in agreement with the literature and this compound (13) has been previously been reported to be in the leaves of *A. atemoya* [299]. Anonaine (21) was also reported in the ethanol extract of pulp at ESI-MS  $m/z$ : 265  $[M + H]^+$  and this compound was reported in the leaves of *A. atemoya* previously [299].



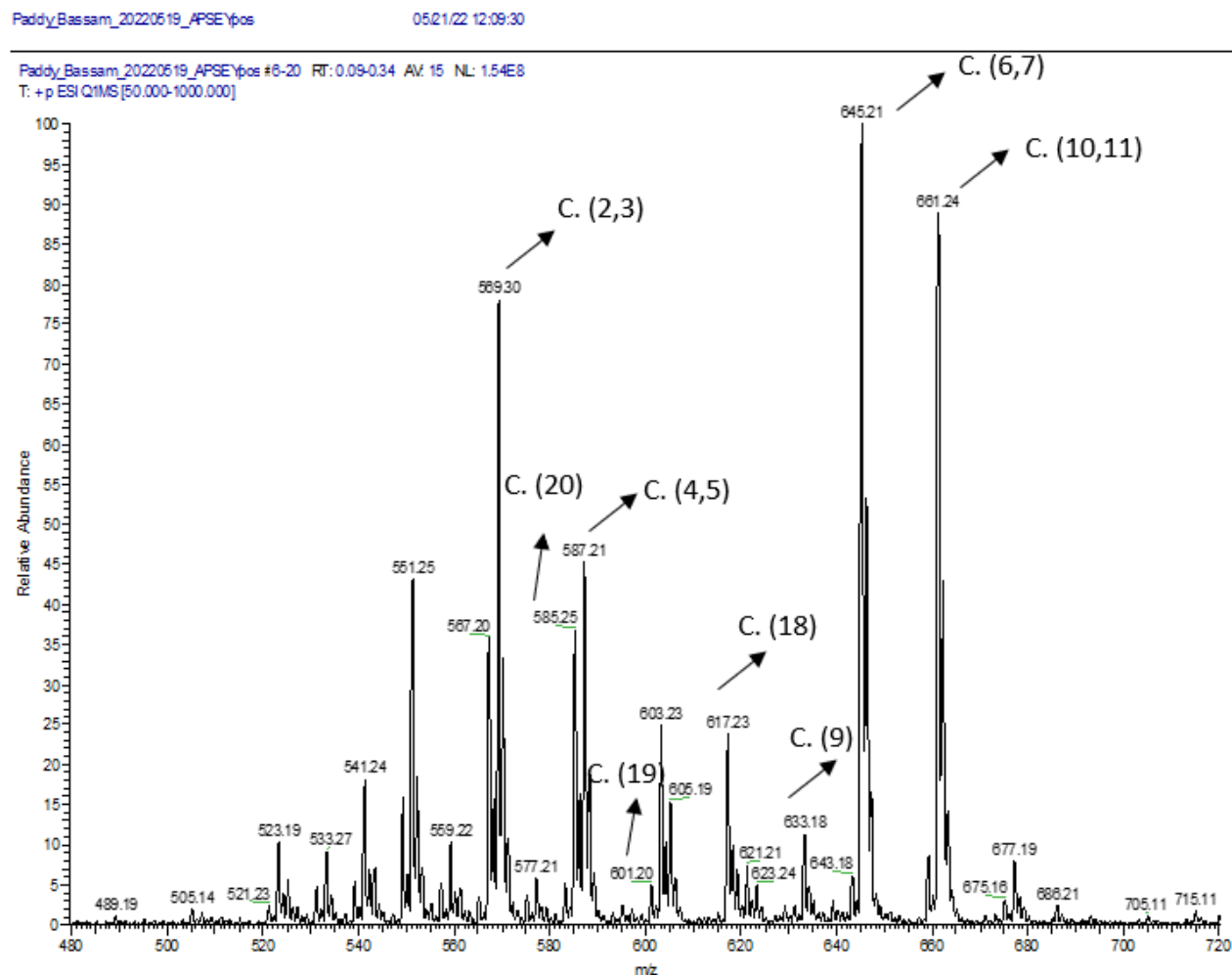


Figure 4.4. MS analysis of *A. atemoya* seeds indicated the presence of compounds (1-7, 9-11 and 17-20) in the hexane, ethyl acetate and ethanol extracts.

For the second cultivar (KJ), it was only analyzed by MS analysis and some similarities were reported between both cultivars. With regards to the fruit's pulp, compound (1) was reported only in the ethanolic fruit extract with a molecular mass was determined as 530 by ESI-MS ( $[M+H]^+$   $m/z = 531$ ) leading to the molecular formula  $C_{35}H_{62}O_3$ , compared to AP cultivar which reported in whole three extracts. Additionally, compound (16) was stated in both hexane and ethyl acetate extracts with a molecular mass was determined by ESI-MS ( $[M+H]^+$   $m/z = 640$ ). However, this compound was solely reported in the ethyl acetate extract of AP cultivar. Remarkably, the ethyl acetate extract indicated the presence of compounds (2 + 3) with a molecular mass 564  $m/z$  corresponding to the molecular formula  $C_{35}H_{62}O_4$ , and Annonacin (8) was described in both hexane and ethanolic extracts of KJ fruit's pulp with molecular weight determined by ESI-MS ( $[M+H]^+$   $m/z = 596$ ). Neither compounds (2 + 3) nor compound (8) reported in the AP fruit's pulp. The fragmentation patterns of each compound reported are shown in (Figures 4.5 to 4.7).

For the seeds, the KJ cultivar revealed the same acetogenins reported in the AP cultivar with less compounds reported in the KJ cultivar. For instance, two compounds (16) and (8) were reported in common in three extracts of KJ cultivar with a molecular mass was determined by ESI-MS ( $[M+H]^+$   $m/z = 640$  and  $596$ ), respectively. Additionally, compounds (2 + 3) were also reported in both hexane and ethyl acetate extracts with a molecular weight determined by ESI-MS ( $[M+H]^+$   $m/z = 564$ ) corresponding to the molecular formula  $C_{35}H_{62}O_4$ . Other resemblances reported in both cultivars was identification of compounds (6 + 7) and (10 + 11) with molecular mass determined by ESI-MS ( $[M+H]^+$   $m/z = 645.4$  and  $661$ ), leading to the molecular formula  $C_{37}H_{66}O_7$  and  $C_{37}H_{66}O_8$  respectively. These compounds (6 + 7) and (10 + 11) were reported only in the KJ ethyl acetate extract of the seed, compared to the AP cultivar which reported these compounds in all three extracts. The NMR and MS spectra are shown in the appendix C and D.

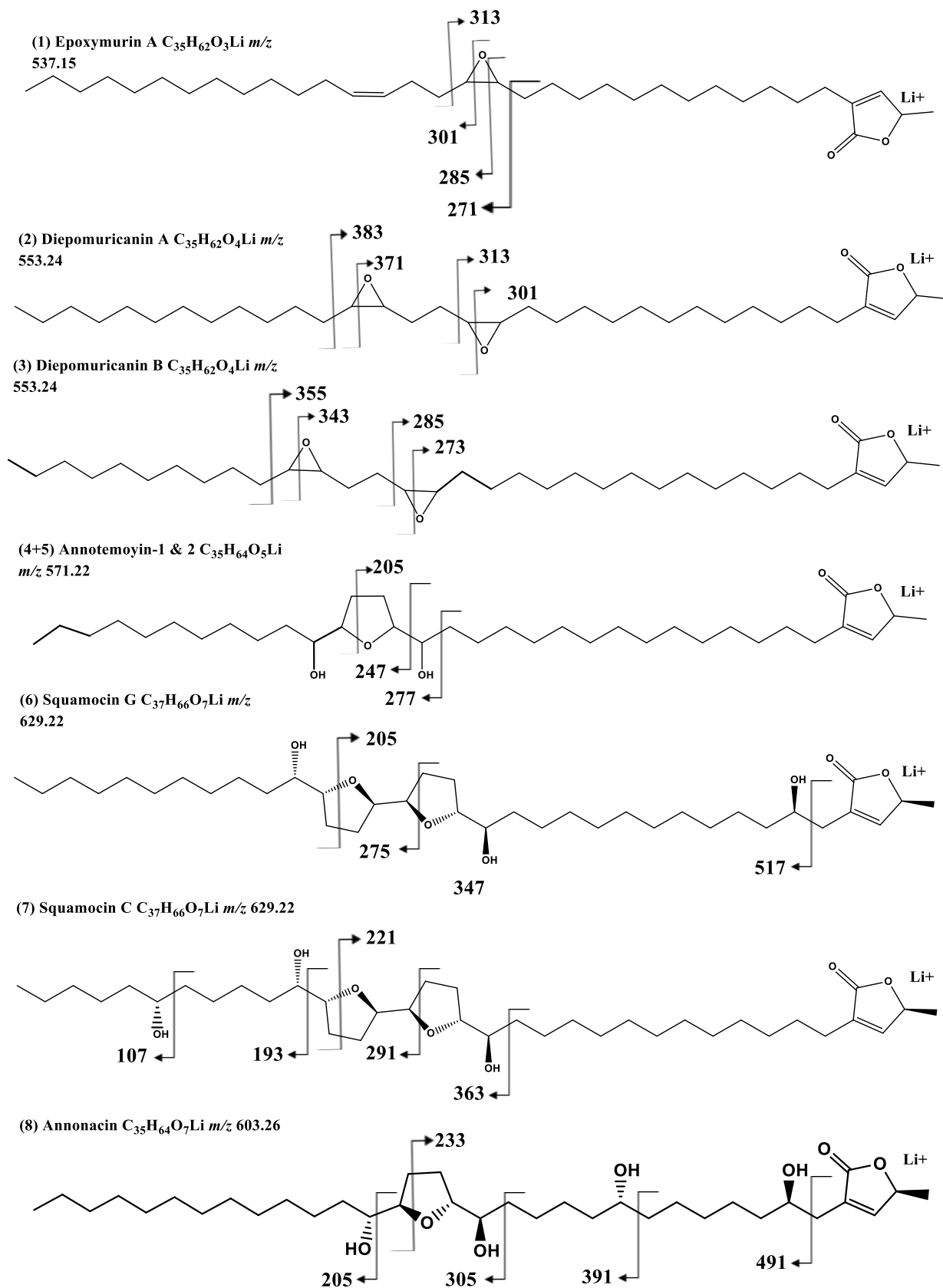
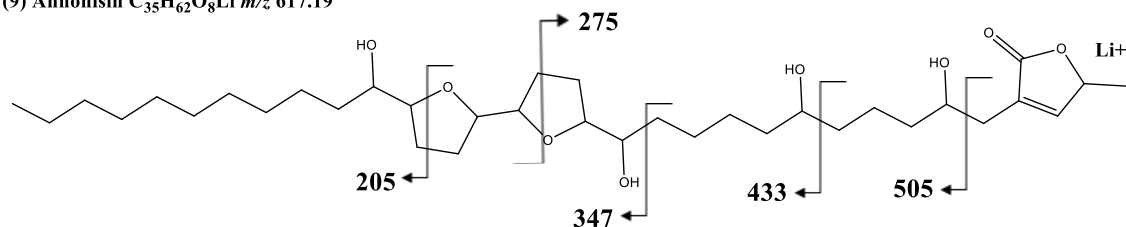
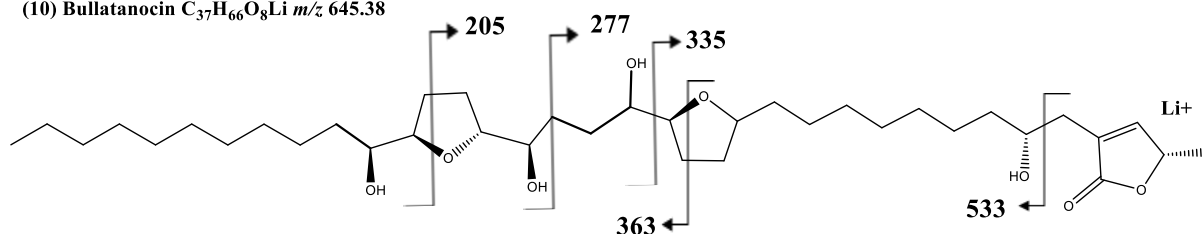


Figure 4.5. Structures and fragmentation patterns for the  $[M + Li]^+$  adducts of acetogenins (1-8) identified in the pulp, and seeds of *A. atemoya*.

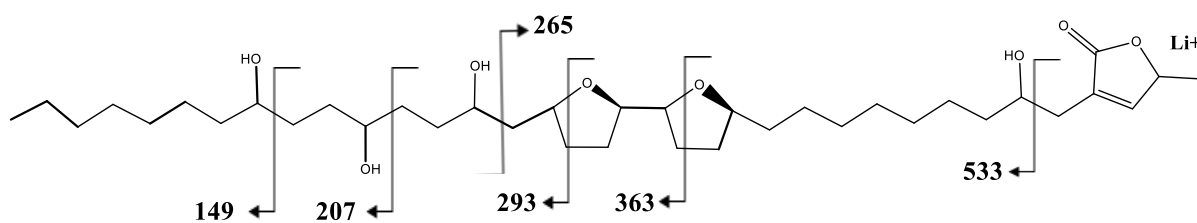
(9) Annonisin  $C_{35}H_{62}O_8Li$   $m/z$  617.19



(10) Bullatanocin  $C_{37}H_{66}O_8Li$   $m/z$  645.38



(11) Atemoyacin-E  $C_{37}H_{66}O_8Li$   $m/z$  645.38



(13) Lanuginosine  $C_{18}H_{11}NO_4$   $m/z$  304

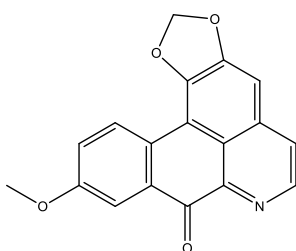
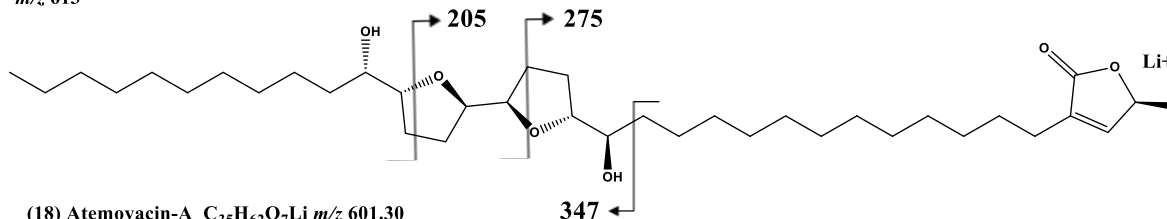


Figure 4.6. Structures and fragmentation patterns for the  $[M + Li]^+$  adducts of acetogenins and alkaloids (9-11 and 13) identified in the pulp, and seeds of *A. atemoya*.

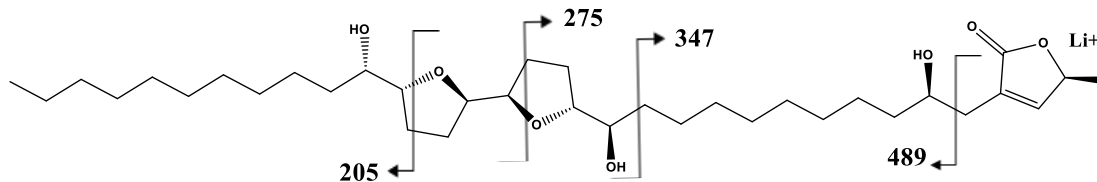
(16) Montanacin A  $C_{37}H_{68}O_8Li$   $m/z$  647.24



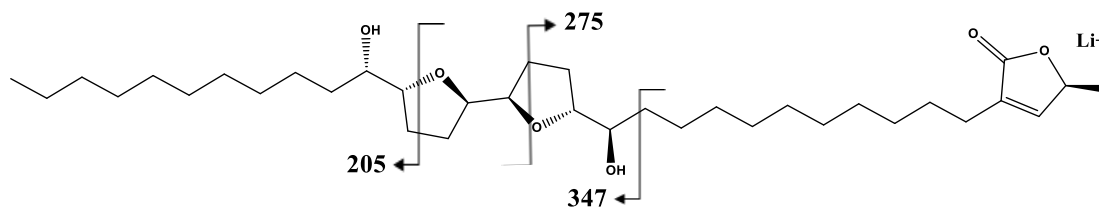
(17) Desacetylurarin A  $C_{37}H_{62}O_6Li$   $m/z$  613



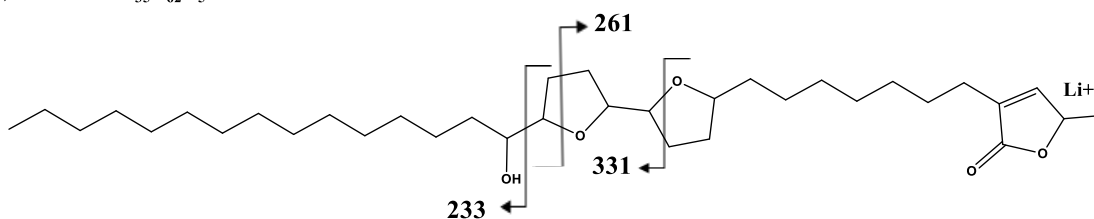
(18) Atemoyacin-A  $C_{35}H_{62}O_7Li$   $m/z$  601.30



(19) Atemoyin  $C_{35}H_{62}O_6Li$   $m/z$  585.22



(20) Robustocin  $C_{35}H_{62}O_5Li$   $m/z$  562.44



(21) Anonaine  $C_{17}H_{15}NO_2$   $m/z$  265

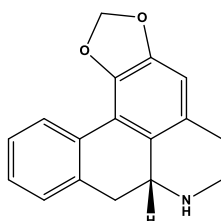


Figure 4.7. Structures and fragmentation patterns for the  $[M + Li]^+$  adducts of

acetogenins and alkaloids (16-21) identified in the pulp, and seeds of *A. atemoya*.

#### 4.4. Conclusion

To conclude, only few studies have discussed and investigated the chemical constituents of *A. atemoya* and some studies indicated the presence of acetogenins only in the *A. atemoya* seeds. In total, eighteen compounds were identified in both cultivars, sixteen acetogenins (1-11 and 16 to 20) and two alkaloids (13 and 21). The seeds of both cultivars were the richest in acetogenins, with sixteen compounds identified (1-11 and 16 to 20), compared with the pulp, which contained five acetogenins (1, 2, 3, 8 and 16). For the first time our data shows the presence of acetogenins in the pulp of both *A. atemoya* cultivars. Two compounds (1 and 16) were reported in common between the cultivars, and three compounds (2, 3 and 8) were reported in the KJ cultivar. For the seed, both cultivars indicated some similarities (2, 3, 6, 7, 8, 10, 11 and 16) with more compounds reported in the AP cultivar. The ethyl acetate crude extracts of both the seeds were richer in acetogenins in comparison with the other crude extracts. Finally, compound (16) was reported for the first time in the pulp of AP and in the seed of KJ cultivar.

# CHAPTER 5

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## **Cytotoxic and Protective Activities of *A. atemoya* Crude Extracts**

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## 5.1. Introduction

Primary liver cancer is the sixth-most diagnosed cancer in the world and the third-most common cancer caused mortality [321]. In 2020, more than 906,000 new cases were reported globally, and approximately 830,000 deaths [322]. The highest incidence of liver cancer is found in both Asia and Africa, with Mongolia has recording the overall highest [323]. Primary liver cancer involves hepatocellular carcinoma (HCC) and intrahepatic cholangiocarcinoma [322]. HCC also known as hepatic cancer and is the most common primary liver cancer, accounting for 75 to 85% of all liver cancers [324, 325]. According to the World Health Organization (WHO), HCC is the fifth most common tumour worldwide and the second leading cause of cancer-related death [326-328]. In 2018, more than 841,000 cases were reported of HCC with approximately 263,000 deaths [329]. The main risk factors associated with the development of HCC are chronic infection with hepatitis B or C, excess body weight, heavy alcohol intake, smoking, aflatoxin-contaminated foods and type 2 diabetes [322, 323, 330, 331]. The risk factors vary from area to area, for instance in both China and Republic of Korea the main causes of HCC are hepatitis B and aflatoxin-contaminated foods, while, in Japan, Italy and Egypt hepatitis C is the predominant cause [322]. Additionally, co-infection of hepatitis B or C and alcohol consumption are the main risk factors of HCC in Mongolia [322]. In Australia, the incidence of HCC has increased more than fourfold from 1.8 to 7.7 per 100000 population between 1982 and 2016 [332]. The main aetiological drivers of liver cancer in Australia have traditionally been hepatitis B or C infection, alcohol consumption and obesity [332].

At an early stage HCC can be treated curatively with liver transplantation or surgical resection [330, 331, 333]. Several other cures involving microwave ablation, percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), transarterial chemoembolization (TACE), cryoablation, radiation therapy, molecularly targeted and systemic chemotherapy therapies exist [330, 331, 333].



A systemic therapy is the best choice for advanced HCC cases or if the other therapies have failed or are not suitable. Sorafenib is the first line treatment of advanced stage HCC and has been shown to prolong survival by nearly three months in patients with advanced HCC [334]. Additionally, cabozantinib and ramucirumab have been shown to provide clear survival benefits from 8 to 11 months as second line therapies [321, 334, 335]. Sorafenib is a multitargeted kinase inhibitor and was approved by US food and drug administration (FDA) as a first line systemic treatment for advanced HCC in 2017 [321, 334, 335]. An agent recently approved as a second line alternative for patients who have progressed during treatment with sorafenib is regorafenib [336]. The prognosis for HCC is still poor and the outcomes of chemotherapy agents are not significant since HCC is considered to be highly resistance to therapy [337]. Therefore, there is a strong need to discover new treatments for HCC or to find a combination therapy in order to improve the effectiveness of existing treatments. Natural products for instance, herbal extracts or pure compound have attracted the attention of many scientists as complementary anti-cancer agents [329, 338].

Natural product crude extracts are composed of various phytochemicals, several of which are bioactive compounds such as secondary metabolites with anti-cancer activity [339, 340]. For example, piperine, an alkaloid isolated from black peppers has reported for anti-tumour and ant-proliferative activities by increasing both caspase-3 and caspase-9 activity and reducing catalase, resulting in the stimulation of peroxide-driven and mitochondria-mediated apoptosis [341]. It also showed selectivity for and was toxic to liver cancer cells and nontoxic to normal hepatocytes cells [342]. Another natural product found in a commonly spice that has abundant biological effects in different diseases such as liver cancer is curcumin. In an animal study conducted on rats it enhanced the effect of piperine on inhibiting diethylnitrosamine-induced HCC [343]. Oleocanthal is a phenolic component of extra-virgin olive oil demonstrated to be toxic to Hep3b, HepG2 and Huh7 cells. Various concentrations of oleocanthal induced both

caspase cleavage and PARP cleavage with G<sub>0</sub>/G<sub>1</sub> cell cycle arrest [344]. *Cnidium officinale* Makino an herb used in traditional medicine, and its crude extract demonstrated a decrease in cell viability and an enhancement in the formation of apoptotic bodies in HepG2 cells [345].

The anti-cancer activity of pure compounds and crude extracts of *A. atemoya* have been discussed in chapter 2 section 2.3.3.1. However, despite an unmet need of improved systemic therapies, and reports of anticancer activities of Annona species across a variety of cancer cell lines, little work has been done to understand the actions of annonaceous acetogenins on HCC

Apoptosis is also known as programmed cell death and can be described as a significant mechanism by which cells die if DNA damage is not repaired [346]. It plays an important role in controlling cell number, proliferation, elimination of self-reactive lymphocytes and an essential for the for eliminating lymphocytes with non-productive relocations of the antigen receptor gene [347]. The mechanism of apoptosis occurs predominantly through two main pathways involved in inducing apoptosis: the extrinsic and intrinsic pathways [347]. The extrinsic pathway refers to the death receptor-mediated or cytoplasmic pathway, whereas the intrinsic pathway is a mitochondrial-mediated pathway with the subsequent release of cytochrome *c* from the mitochondria into the cytoplasm that helps to the formation of the caspase 3 activation [348]. The release of cytochrome *c* from the mitochondria can be promoted by pro-apoptotic members of the BCL-2 family such as Bax and Bim, and can be inhibited through anti-apoptotic members of the same family for instance BCL-X<sub>L</sub> and MCL1. Both of extrinsic and intrinsic pathways might lead to the same terminal or execution pathway [349, 350].

The liver is the central and major organ of lipid and glucose metabolism and plays an important role in the production and metabolism of lipid and lipoprotein [351]. An excess accumulation of energy, particularly of fats, are associated with adverse effects on the liver and other organs

[352]. Fatty liver is described in the setting of both alcoholic liver disease (ALD) as well as non-alcoholic fatty liver disease (NAFLD) [353, 354]. ALD is the most prevalent type of chronic liver disease caused by chronic consumption of alcohol and it can be progressed from alcoholic fatty liver (AFL) to alcoholic steatohepatitis (ASH), which can progress to cirrhosis and HCC [354, 355]. The development of NAFLD results from an increased inflow of free fatty acids (FFA) due to metabolic disorders such as insulin resistance, hepatocyte triglyceride accumulation, altered hepatic dietary lipid processing, increased hepatic de novo lipid biosynthesis, and impaired lipid export out of the hepatocytes [351]. NAFLD progresses to non-alcoholic steatohepatitis (NASH), which can eventually lead to liver fibrosis and cirrhosis, and in some cases HCC [354]. Hepatic cirrhosis, which often occurs after the initial stage of liver cancer, substantially damages liver function and influences lipid metabolism in the liver [355]. Additionally, lipotoxicity can result in abnormal activation of intracellular signalling pathways and organelle dysfunction which is a hallmark of NASH [356, 357].

Extreme accumulation of fatty acids might increase lipotoxicity by increasing free fatty acids in the body and lead to diseases like type 2 diabetes, liver disease, coronary heart disease, atherosclerosis and insulin resistance [358]. The mechanisms of lipotoxicity include cellular processes such as endoplasmic reticulum (ER) stress, lysosomal permeabilization and mitochondrial dysfunction, which can lead to cell death [356, 357].

Increases in the amount of palmitic acid (PA) in cells from nonadipose tissues is also known to cause lipotoxicity, resulting in cellular disorder and death [359]. PA is the most abundant saturated free fatty acid in the bloodstream that induces cellular disorder and cell death. The lipotoxicity of PA leads to lipid overload, which is specifically related to the cause of both type 2 diabetes and obesity [359]. A recent study has shown that food containing saturated fatty acids such as PA can promote the growth of various cancers and apoptotic cell death by

triggering ER stress, and also induce lipotoxicity in hepatocytes and cardiomyocytes [360].

According to the most common model of NAFLD, the "two-hit hypothesis"; the "first hit" is lipid accumulation in the hepatocytes and insulin resistance. The "second hit" is caused by oxidative stress, lipid peroxidation, proinflammatory mediators and mitochondrial dysfunction and promotes hepatic injury, inflammation and fibrosis.[361, 362]. Many studies have also indicated that increased levels of circulating free fatty acids can cause obesity and metabolic syndrome by promoting fat deposition in nonadipose tissues. For instance, PA has been reported to induced lipid accumulation in HepG2 cells accompanied by an increase in intracellular triglyceride levels by raising levels of biologically active neutrophil chemoattractant, IL-8, from steatotic hepatocytes [363].

Severe liver disease can disorder the metabolism of lipoproteins and also change the levels of lipids and lipoproteins in the bloodstream [355]. The relationship between cancer development and alterations in intracellular lipid levels is important due to the vital function lipids play in cell proliferation and growth [355]. Thus, there is solid evidence between liver diseases especially HCC, and dysregulation of lipid metabolism.

In this chapter, the cytotoxic activities of *A. atemoya* crude extracts of different plant parts including leaves, fruit's pulp and seeds were assessed by investigating their effects on the Huh7 cells using MTS assay. Fractions that were not cytotoxic, were further investigated for their effects on PA-induced lipotoxic stress using immunoblotting.

## 5.2. Materials and Methods

### 5.2.1. Reagents and crude extracts preparation

All chemicals and solvents were supplied by Sigma-Aldrich (Sydney, Australia). 20 mg of leaf

hexane extract (LH), leaf ethyl acetate extract (LEY), leaf ethanol extract (LE), fruit hexane extract (FH), fruit ethyl acetate extract (FEY), fruit ethanol extract (FE), seed hexane extract (SH), seed ethyl acetate extract (SEY) and seed ethanol extract (SE) were dissolved in 1 ml of dimethyl sulfoxide (DMSO) and then filtered through a 0.22  $\mu\text{m}$  Millex-GP syringe filter.

### **5.2.2. Cell culture**

The Huh7 liver cancer cell lines were obtained from Cell Bank Australia, and the necessary reagents and materials such as TrypLE Express, high glucose Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine, Dulbecco's phosphate buffer saline (DPBS), and penicillin-streptomycin were acquired from Gibco™ Thermo Fisher Scientific (Life Technologies Australia Pty Ltd, Mulgrave, Victoria, Australia). Fetal bovine serum (FBS) was purchased from Cytiva (North Ryde, New South Wales, Australia). The Huh7 cells were cultured in high glucose DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 U/mL penicillin and 100  $\mu\text{g/mL}$  streptomycin, and maintained in a humidified atmosphere with 5%  $\text{CO}_2$  at 37 °C.

### **5.2.3. Evaluation of cytotoxic activity using (MTS) assay**

The cell viability was determined by using cell proliferation assay (MTS) purchased from Promega (Alexandria, New South Wales, Australia). The Huh7 cells were seeded in a 96 well plate with a cell density of 8000 cells/well. Cells were incubated overnight before treatment with various concentrations (10  $\mu\text{g/mL}$ , 100  $\mu\text{g/mL}$  and 1000  $\mu\text{g/mL}$ ) plant part extracts (leaves, fruit and seeds). The extracts were prepared by extraction into hexane, ethyl acetate or ethanol and evaporated to dryness (as described in chapters 3 and 4). The crude extract was then resuspended in DMSO to give a stock solution of (20 mg/mL), with DMSO used as the vehicle control. After 24 hours of treatment, the medium was discarded and 20  $\mu\text{L}$  of MTS solution mixed with 100  $\mu\text{L}$  of new culture medium was added to each well and incubated at

37 °C for 1 hour. After 1 hour of incubation, the absorbance of samples was measured with Multimode Plate spectrophotometer, Victor X4, PerkinElmer at a wavelength of 490 nm. The proportion of cell viability was calculated via using the following formula:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treatment well} - \text{average absorbance of blank}}{\text{Absorbance of DMSO} - \text{Absorbance of blank}} \times 100\%$$

All experiments were run in triplicate and the IC<sub>50</sub> values were calculated by using GraphPad Prism 9 (GraphPad Software, San Diego, CA).

#### **5.2.4. Western blot analysis**

The Huh7 liver cancer cell line (1.5 x 10<sup>6</sup> cells/T25 flask) was incubated overnight until it reached 85% of confluence and was then treated with one concentration (100 µg/mL) of plant extract for 24 hours. The cells were then washed with 1 ml of PBS and harvested into 80 µl of TX buffer. The cells were sonicated for 20 minutes using QSonica-Q800R2, and the cell lysates were centrifuge for 3 – 5 minutes at 15000 x g and the supernatant was collected in new tubes and stored at -80°C. The protein concentration was determined via Bicinchoninic acid Protein Assay (BCA). Total protein was then separated via sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) (12%) for 85 minutes and transferred to 0.45 µm polyvinylidene fluoride (PVDF) membrane for 90 minutes. The membrane was blocked with 5% non-fat milk in phosphate-buffered saline with tween 20 (PBST) buffer for 45 minutes at room temperature. Afterward, the membrane was washed three times with (PBST) buffer before incubation with the primary antibody (1:1000 dilution) at 4°C overnight. The membrane was washed three times with (PBST) buffer and incubated with the peroxidase conjugated secondary antibody for 1.5 hour at room temperature. The membrane was covered with 1 ml of enhanced chemiluminescence (ECL) substrate and was visualized using a ChemiDoc™ Touch Imaging System (Bio-Rad laboratories) and processed using ImageLab™ Software

(Bio-Rad laboratories).

### 5.2.5. Statistical analysis

The statistical analyses were performed using GraphPad Prism 9, and all data were expressed as mean  $\pm$  standard deviation (SD) and presented in tables or graphs. The IC<sub>50</sub> was estimated by non-linear regression with fixed a maximum and variable slope and the 95% confidence interval (95% CI) of IC<sub>50</sub> was presented to allow comparison between groups.

## 5.3. Results

### 5.3.1. Cytotoxic activity

To screen the crude extracts of *A. atemoya* for cytotoxic activity, HCC Huh-7 cells were treated firstly with 10  $\mu$ g/mL of each crude extract (LH, LEY, LE, FH, FEY, FE, SH, SEY and SE) overnight and cell viability was assessed via MTS assay. Following 24 hours of treatment, all the crude extracts did not demonstrate any activity at 10  $\mu$ g/mL. The proportion of cell viability of Huh7 cells treated with 10  $\mu$ g/mL of crude extracts for 24 hours is summarised in figure 5.1 and table 5.1.

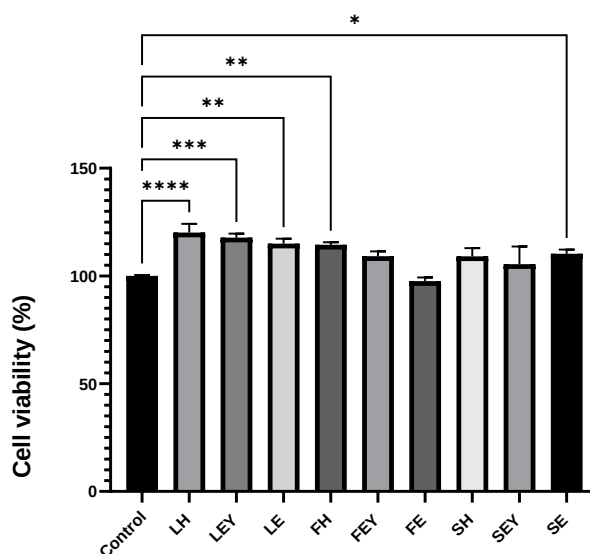


Figure 5.1 The effect of *Annona atemoya* crude extracts of leaves, fruits and seeds, on Huh7 cell viability using MTS assay. For 24 hours, the Huh7 cells were treated with DMSO as vehicle and all nine crude extracts (10  $\mu$ g) of different parts of *Annona*

*atemoya*. The results are described as mean  $\pm$  SD from three independent experiments, each repeated in triplicate. (\*\*\*\*  $p < 0.0001$ , \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  relative to control by one-way ANOVA.)

Table 5.1 Proportion of cell viability of Huh7 cells following treatment with 10  $\mu$ g of selected crude extracts for 24 hours.

| Crude extracts | Cell viability (%) |
|----------------|--------------------|
| LH             | 120.1 $\pm$ 4.098  |
| LEY            | 117.8 $\pm$ 1.910  |
| LE             | 115.0 $\pm$ 2.308  |
| FH             | 114.4 $\pm$ 1.271  |
| FEY            | 109.1 $\pm$ 2.271  |
| FE             | 97.53 $\pm$ 1.790  |
| SH             | 109.9 $\pm$ 3.920  |
| SEY            | 105.4 $\pm$ 8.292  |
| SE             | 110.3 $\pm$ 2.002  |

Data are described as mean  $\pm$  SD from at least three independent experiments carried out in triplicate.

Huh-7 were then incubated with all nine crude extracts at an increased concentration of 100  $\mu$ g/ml for 24 hours. Of the nine crude extracts, only two extracts LH and SEY inhibited Huh7 cell viability at 100  $\mu$ g. SEY crude extract exhibited the strongest cell viability inhibition with 2.90% compared to LH crude extract that reported at 40.56%. The data for cell viability of Huh7 cells treated with 100  $\mu$ g of selected crude extracts for 24 hours were summarised in figure 5.2 and table 5.2.



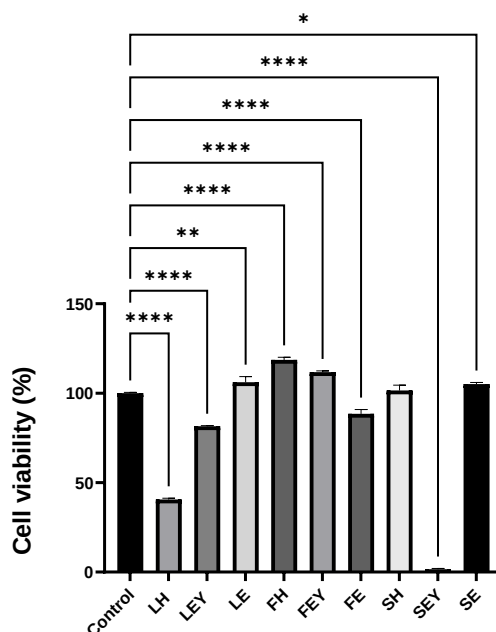


Figure 5.2 The effect of *Annona atemoya* crude extracts of leaves, fruits and seeds, on Huh7 cell viability using MTS assay. For 24 hours, the Huh7 cells were treated with DMSO as vehicle and all nine crude extracts (100 µg) of different parts of *Annona atemoya*. The results are described as mean ± SD from three independent experiments, each repeated in triplicate. (\*\*\*\*  $p < 0.0001$ , \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  relative to control by one-way ANOVA.)

Table 5.2 Proportion of cell viability of Huh7 cells following treatment with 100 µg of selected crude extracts for 24 hours. Data are described as mean ± SD from at least three independent experiments carried out in triplicate.

| Crude extracts | Cell viability (%) |
|----------------|--------------------|
| LH             | 40.56 ± 0.692      |
| LEY            | 81.45 ± 0.362      |
| LE             | 106.1 ± 3.120      |
| FH             | 118.6 ± 1.471      |
| FEY            | 111.7 ± 0.814      |
| FE             | 88.40 ± 2.481      |
| SH             | 101.5 ± 2.941      |
| SEY            | 2.907 ± 0.472      |
| SE             | 105.0 ± 0.939      |

Since some of the crude extracts did not exhibit any cytotoxic activity at 100 µg/mL, Huh-7 cells were then incubated with all nine crude extracts at an increased concentration of 1000 µg/ml for 24 hours. Unfortunately, most of the crude extracts exhibited strong inhibition of

Huh7 cell viability with proportion ranging from 1.52% to 75.70%. The data for cell viability of Huh7 cells treated with 1000 µg of *A. atemoya* extracts for 24 hours were summarised in figure 5.3 and table 5.3.

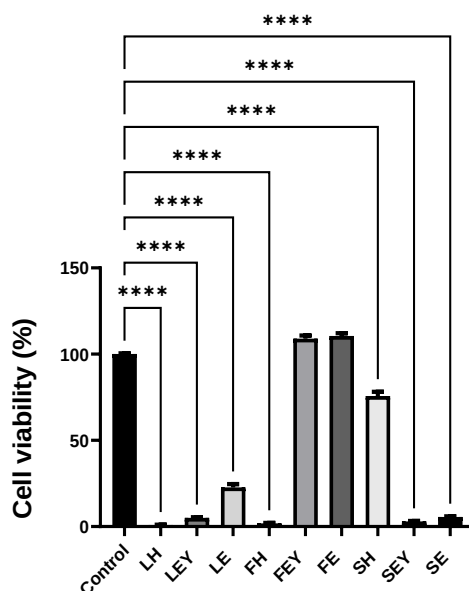


Figure 5.3 The effect of *Annona atemoya* crude extracts of leaves, fruits and seeds, on Huh7 cell viability using MTS assay. For 24 hours, the Huh7 cells were treated with DMSO as vehicle and all nine crude extracts (1000 µg) of different parts of *Annona atemoya*. The results are described as mean ± SD from three independent experiments, each repeated in triplicate. (\*\*\*\*  $p < 0.0001$ , relative to control by one-way ANOVA.)

Table 5.3 Proportion of cell viability of Huh7 cells following treatment with 1000 µg of selected crude extracts for 24 hours. Data are described as mean ± SD from at least three independent experiments carried out in triplicate.

| Crude extracts | Cell viability (%) |
|----------------|--------------------|
| LH             | 3.916 ± 0.177      |
| LEY            | 4.993 ± 0.428      |
| LE             | 22.70 ± 1.986      |
| FH             | 4.803 ± 0.325      |
| FEY            | 110.8 ± 0.00       |
| FE             | 112.3 ± 0.00       |
| SH             | 75.70 ± 2.452      |
| SEY            | 1.520 ± 0.337      |
| SE             | 5.537 ± 0.439      |

As the LH crude extract displayed potent inhibitory effect against Huh-7 cell line we focused on investigating the anti-cancer activities of this crude extract. Concentrations between 10 to

200  $\mu\text{g/mL}$  were applied to the Huh-7 cell line for 24 hours. Across all concentrations, a significant inhibitory effect was observed starting at concentration of 50  $\mu\text{g/mL}$  which steadily increased with increasing concentrations as shown in figure 5.4.

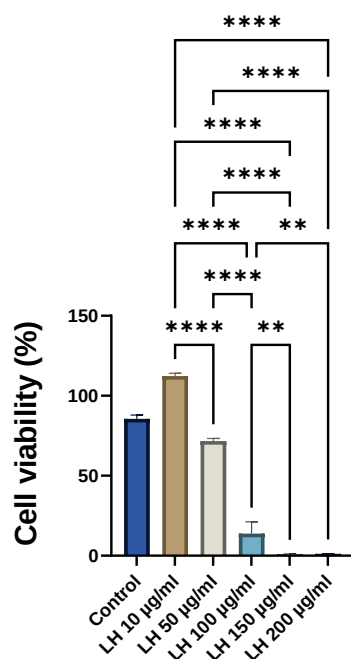


Figure 5.4 The effect of hexane extract of leaves (LH) on Huh7 cell viability using MTS assay. For 24 hours, the Huh7 cells were treated with DMSO as vehicle and various concentrations of LH (10  $\mu\text{g}$ , 50  $\mu\text{g}$ , 100  $\mu\text{g}$ , 150  $\mu\text{g}$  and 200  $\mu\text{g}$ ). The results are described as mean  $\pm$  SD from three independent experiments, each repeated in triplicate. (\*\*\*\*  $p < 0.0001$ , \*\* $p < 0.01$ , relative to control by one-way ANOVA.)

The SEY exhibited the strongest inhibitory effect against Huh-7 cell viability and concentrations from 10 to 100  $\mu\text{g/mL}$  were applied to the Huh-7 cell line overnight. No inhibitory effect was reported at a concentration of 10  $\mu\text{g/mL}$ ; however, a marked inhibition was noted starting at concentration of 25  $\mu\text{g/mL}$  which progressively increased with increasing concentrations as shown in figure 5.5.

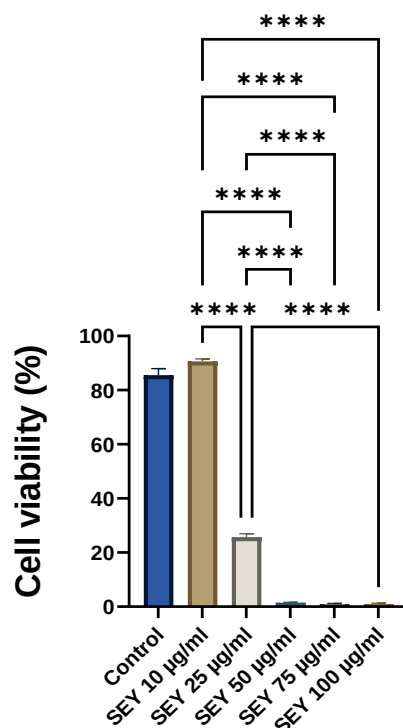


Figure 5.5 The effect of ethyl acetate extract of seeds (SEY) on Huh7 cell viability using MTS assay. For 24 hours, the Huh7 cells were treated with DMSO as vehicle and various concentrations of SEY (10 µg, 25 µg, 50 µg, 75 µg, and 100 µg). The results are described as mean  $\pm$  SD from three independent experiments, each repeated in triplicate. (\*\*\*\*  $p < 0.0001$ , relative to control by one-way ANOVA.)

Further experiments were undertaken for both LH and SEY crude extracts as shown in figures 5.6, and 5.7 respectively. For LH, the concentration was narrowed from 20 to 100 µg/mL, and 10 to 50 µg/mL for SEY. The Huh-7 cells were incubated with these concentrations for 24 hours and the inhibitory effect was assessed using MTS assay. A significant inhibitory effect was noted starting at a concentration of 60 µg/mL which was gradually increased with increasing concentrations as indicated in figure 5.6, and table 5.4. The SEY crude extract, also demonstrated a substantial inhibitory action starting at a concentration of 20 µg/mL which increased at higher concentrations as shown in figure 5.7, and table 5.5.

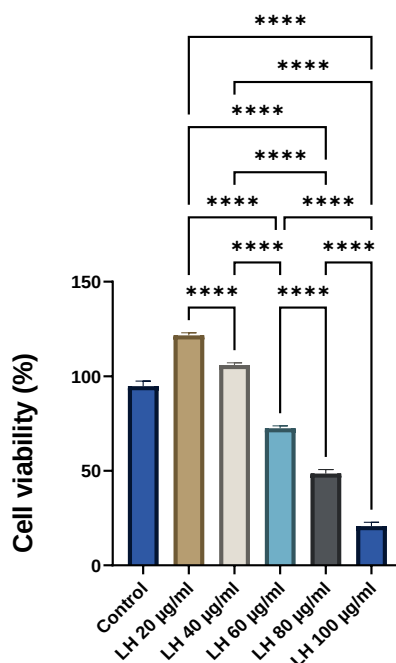


Figure 5.6 The percentages of cell viability vs concentrations of hexane extract of leaves (LH) on Huh7 cell viability using MTS assay. For 24 hours, the Huh7 cells were treated with DMSO as vehicle and various concentrations of LH (20 µg, 40 µg, 60 µg, 80 µg and 100 µg). The results are described as mean  $\pm$  SD from three independent experiments, each repeated in triplicate. (\*\*\*\*  $p < 0.0001$ , relative to control by one-way ANOVA.)

Table 5.4 Proportion of cell viability of Huh7 cells following treatment with various concentrations (20 µg, 40 µg, 60 µg, 80 µg and 100 µg) of LH for 24 hours. Data are described as mean  $\pm$  SD from at least three independent experiments carried out in triplicate.

| LH        | Cell viability (%) |
|-----------|--------------------|
| 20 µg/ml  | 121.6 $\pm$ 1.352  |
| 40 µg/ml  | 105.9 $\pm$ 1.198  |
| 60 µg/ml  | 72.51 $\pm$ 1.227  |
| 80 µg/ml  | 48.56 $\pm$ 2.141  |
| 100 µg/ml | 20.68 $\pm$ 2.069  |

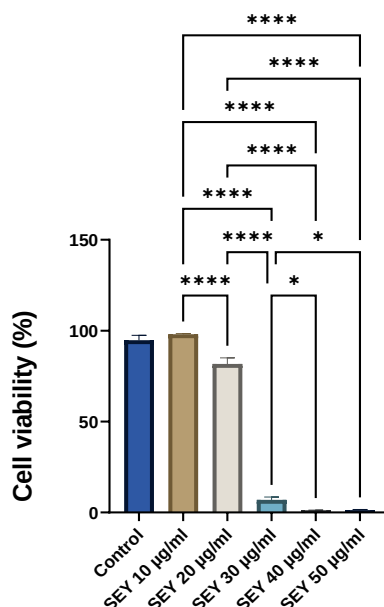


Figure 5.7 The percentages of cell viability vs concentrations of ethyl acetate extract of seeds (SEY) on Huh7 cell viability using MTS assay. For 24 hours, the Huh7 cells were treated with DMSO as vehicle and various concentrations of SEY (10 µg, 20 µg, 30 µg, 40 µg and 50 µg). The results are described as mean  $\pm$  SD from three independent experiments, each repeated in triplicate. (\*\*\*\*  $p < 0.0001$ , \*\*\* $p < 0.001$ , \*\* $p < 0.01$ , \* $p < 0.05$  relative to control by one-way ANOVA.)

Table 5.5 Proportion of cell viability of Huh7 cells following treatment with various concentrations (10 µg, 20 µg, 30 µg, 40 µg and 50 µg) of SEY for 24 hours.

| SEY      | Cell viability (%) |
|----------|--------------------|
| 10 µg/ml | 97.98 $\pm$ 0.378  |
| 20 µg/ml | 81.71 $\pm$ 3.361  |
| 30 µg/ml | 6.883 $\pm$ 1.651  |
| 40 µg/ml | 1.210 $\pm$ 0.062  |
| 50 µg/ml | 1.290 $\pm$ 0.308  |

Data are described as mean  $\pm$  SD from at least three independent experiments carried out in triplicate.

The dose-response curves ( $IC_{50}$ ) were subsequently determined for these crude extracts. The Huh-7 cells were treated with different concentrations of LH (20 – 200 µg), and SEY (10 – 50 µg) for 24 hours and cell viability assessed using the MTS assay. Dose-response curves are shown in figures 5.8 and 5.9, and  $IC_{50}$  values were calculated and reported in Table 5.6.

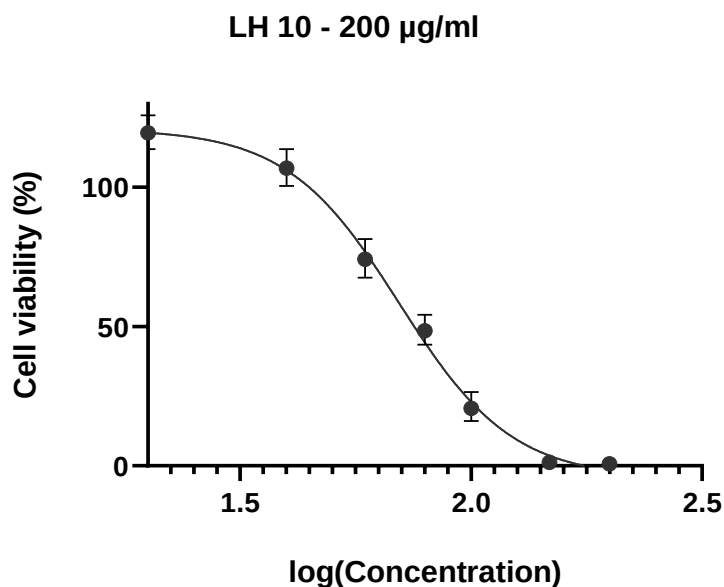


Figure 5.8 Dose-response curve of percentages of cell viability vs concentrations of hexane extract of leaves (LH) on Huh7 cell viability using MTS assay. For 24 hours, the Huh7 cells were treated with DMSO as vehicle and various concentrations of LH (20 µg, 40 µg, 60 µg, 80 µg, 100 µg, 150 µg and 200 µg). The results are described as mean  $\pm$  SD from three independent experiments, each repeated in triplicate.

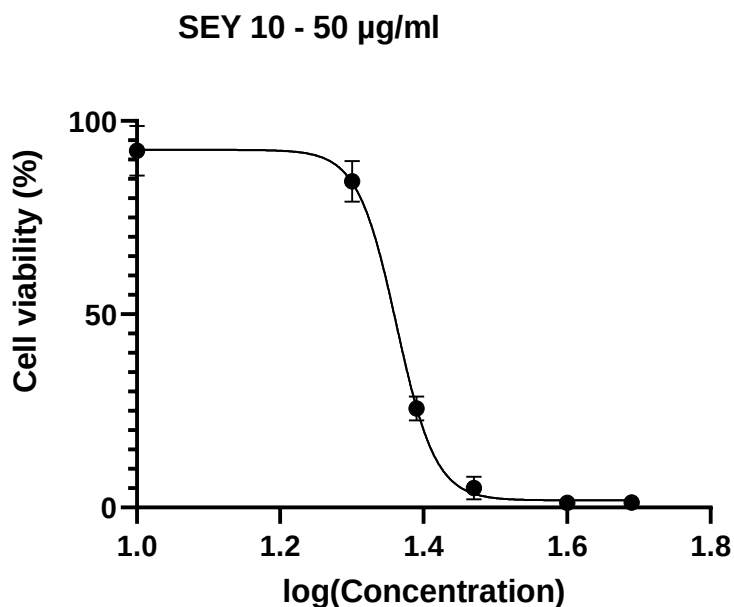


Figure 5.9 Dose-response curve of percentages of cell viability vs concentrations of ethyl acetate extract of seeds (SEY) on Huh7 cell viability using MTS assay. For 24 hours, the Huh7 cells were treated with DMSO as vehicle and various concentrations of SEY (10 µg, 20 µg, 25 µg, 30 µg, 40 µg and 50 µg). The results are described as mean  $\pm$  SD from three independent experiments, each repeated in triplicate.

Table 5.6 IC<sub>50</sub> values of LH and SEY in Huh-7 cell lines for 24 hours.

| Crude extract | IC <sub>50</sub> |
|---------------|------------------|
| LH            | 67.21 ± 72.76    |
| SEY           | 22.55 ± 23.52    |

### 5.3.2. Protective activities

For the crude extracts that did not show significant cytotoxicity, their protective activity against PA-induced damage was examined. Huh-7 cells were incubated with PA (500 µM) alone or PA (500 µM) in the presence of 100 µg/mL of the extracts (LEY, LE, FH, FEY, FE, SH and SE) for 24 hours. PA alone (500 µM) was toxic to the Huh-7 and inhibited the cell growth by 49.2%. In the presence of LEY + PA Huh-7 cells and inhibited the cell growth by 71.9%. The other crude extracts (FH, FEY, and FE) showed protective activity against PA-induced toxicity and as shown in figure 5.10 and table 5.7. At the lower concentration of 10 µg, all the crude extracts still demonstrated protective activity against PA-induced toxicity as illustrated in figure 5.11.

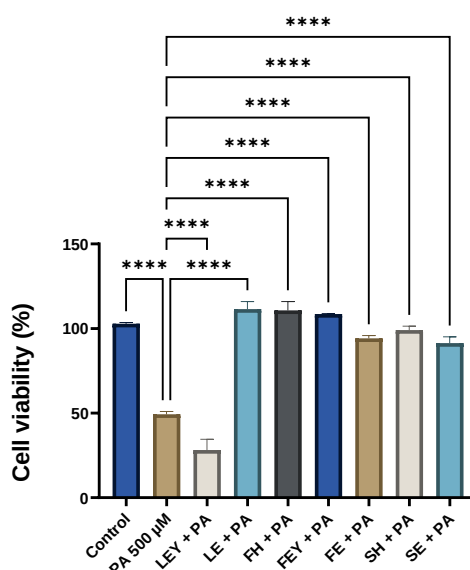


Figure 5.10 The effect of *Annona atemoya* crude extracts of leaves, fruits and seeds, on Huh7 cell viability using MTS assay. For 24 hours, the Huh7 cells were treated with DMSO as vehicle, palmitic acid (PA), and combination of both PA and seven crude extracts (100 µg) of different parts of *Annona atemoya*. The results are described as mean ± SD from three independent experiments, each repeated in triplicate. (\*\*\*\* p<0.0001, relative to control by one-way ANOVA.)

Table 5.7 Proportion of cell viability of Huh7 cells following treatment with a combination of PA and 100 µg of selected crude extracts for 24 hours.



| Treatment | Cell viability (%) |
|-----------|--------------------|
| PA        | 49.2 ± 0.017       |
| LEY + PA  | 28.1 ± 0.063       |
| LE + PA   | 115.3 ± 0.045      |
| FH + PA   | 107.1 ± 0.052      |
| FEY + PA  | 108.5 ± 0.003      |
| FE + PA   | 94.1 ± 0.016       |
| SH + PA   | 99.0 ± 0.023       |
| SE + PA   | 91.3 ± 0.038       |

Data are described as mean ± SD from at least three independent experiments carried out in triplicate.

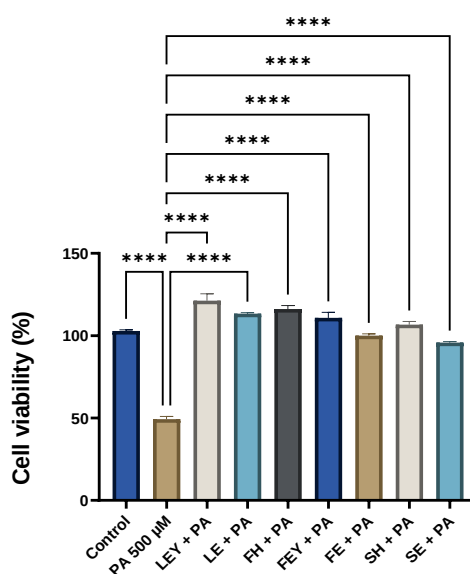


Figure 5.11 The effect of *Annona atemoya* crude extracts of leaves, fruits and seeds, on Huh7 cell viability using MTS assay. For 24 hours, the Huh7 cells were treated with DMSO as vehicle, palmitic acid (PA), and combination of both PA and seven crude extracts (10 µg) of different parts of *Annona atemoya*. The results are described as mean ± SD from three independent experiments, each repeated in triplicate. (\*\*\*\*  $p < 0.0001$ , relative to control by one-way ANOVA.)

### 5.3.3. Mechanism of action

The effect of *A. atemoya* crude extracts on apoptosis of Huh-7 cell was studied by determining the activity of downstream apoptotic proteins such as caspase-3, Bcl-2, Bcl-X<sub>L</sub>, Mcl-1, Bax and Bim using western blot technique. In figure 5.12, PA clearly induced activation of cleaved caspase-3 whereas, all crude extracts at concentrations of 100 µg decreased the expression of cleaved caspase-3. For Bim and Bax, the FH, FEY and FE crude extracts obviously induced the expression of Bcl-2 pro-apoptosis proteins at concentrations of 100 µg/mL for 24 hours.

This protective activity may be due to the various phenolic compounds e.g. flavonoids that the fruit of *A. atemoya* contains, as reported in many studies summarised in our previous review [364]. Simultaneously, most of the crude extracts decreased the expression of the Bcl-2 anti-apoptosis proteins, Bcl-X<sub>L</sub> and Mcl-1. Finally, the effect of the FH, FEY and FE crude extracts on these Bcl-2 proteins indicates that they induce cell apoptosis by the intrinsic pathway.

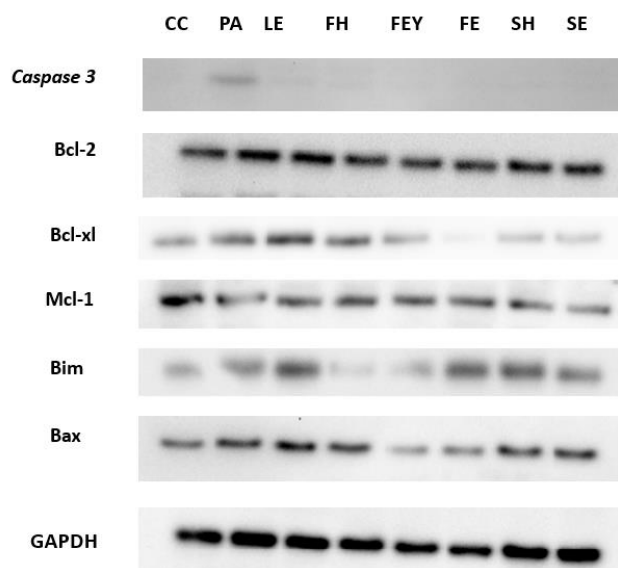


Figure 5.12 The expression of apoptosis markers and Bcl-2 family proteins analysed by western blot following treatment of Huh-7 cells with 100 µg of *Annona atemoya* crude extracts for 24 hours.

Furthermore, figure 5.13 shows that FH induced phosphorylated *p*-AKT, *p*-Erk, *p*-Gsk, and *p*-S6K at a concentration of 100 µg. It is also clearly seen from figure 5.13 that the levels of *p*-AKT, *p*-Erk and *p*-Gsk were decreased in cells treated with all crude extracts except of FH. This result indicates that a constituent of FH induced the expression of these ER stress markers in Huh-7 cell line.

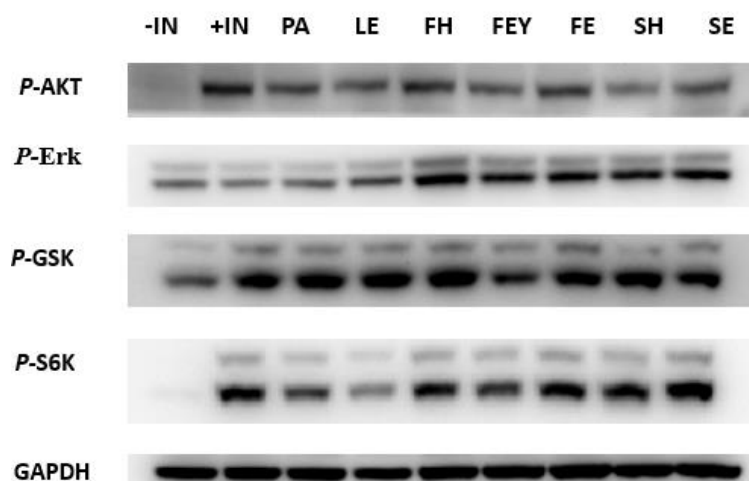


Figure 5.13 The expression of protein-related ER stress markers in Huh-7 cells analysed by western blot following treatment of 100  $\mu$ g of *Annona atemoya* crud extracts for 24 hours.

## 5.4. Discussion

The activity of *A. atemoya* and its chemical constituents specifically annonaceous acetogenins have attracted considerable attention due to their cytotoxic activities [290]. Among *A. atemoya* parts including the leaves, fruit and seeds, only the aqueous-ethanolic extract of the leaves of *A. atemoya* was shown to be cytotoxic to both HeLa and HepG2 cancer cells [60]. Bullatacin which was isolated from the seed of *A. atemoya* elicited a potent cytotoxicity, inhibiting the proliferation of Hep G2/2.2.15 cells by inducing apoptosis [276], via a reduction of intracellular cAMP and cGMP levels. The cytotoxicity of other pure compounds identified in *A. atemoya* were discussed in chapter 2 section 2.3.3.1. It has also been reported that annonaceous acetogenins induce cytotoxicity via inhibiting mitochondrial complex I, which is involved in oxidative phosphorylation and ATP synthesis [365].

In this chapter, three parts of *A. atemoya* (nine crude extracts) were screened at 10 to 1000  $\mu$ g/ml using Huh-7 cell line. From the selected crude extracts, only two crude extracts LH and SEY shown anti-cancer activity in Huh-7 cells with an  $IC_{50}$  values 67.21 and 22.55

respectively. Both crude extracts demonstrated dose dependent inhibition of cell viability at 24 hours using MTS assay. The cytotoxic effect may be due to the presence of the same annonaceous acetogenins in these crude extracts (discussed in chapter 3 and 4). Acetogenins 2-5 were present in both LH and SEY. Although the other crude extracts have similar phytochemical constituents, they did not demonstrate cytotoxicity which suggests that these constituents are only present at low concentrations, which is consistent with the TLC and NMR results in chapters 3 and 4.

With regards to the other crude extracts that did not show cytotoxic effect, their protective activity was assessed against PA-induced toxicity. After 24 hours exposure, the average viability of cells that were treated only with 500  $\mu$ M of PA was calculated to be 49.2%. At 100  $\mu$ g/ml of the seven crude extracts, all exhibited protective activity against PA-induced toxicity, with the average cell viabilities of 91.3% to 115.3%, except LEY which promoted the PA-induced toxicity and the cell viability was reported at 28.1% as shown in figure 5.10. At a concentration of 10  $\mu$ g/ml, all the crude extracts still showed significant protective effects against PA-induced toxicity with cell viabilities ranging from 95.1% to 115.3%.

To better understand the underlying mechanism of the protective effect of the crude extracts against PA-induced toxicity, the capability of these crude extracts to either induce or inhibit apoptosis in Huh7 cells was also examined. It has been reported that HepG2 cells treated with PA resulted in a down-regulation of Bcl-2 ( $p < 0.05$ ) and dramatic up-regulation of Bax ( $p < 0.01$ ) [366]. Additionally, in HepG2 cells PA at (125  $\mu$ M) induced protein expression of Bax and decreased in Bcl-2 protein expression [367]. In our study, it was observed that PA increased cleaved caspase-3 in Huh-7 cells. This apoptotic effect was triggered by the crude extracts FH, FEY and FE through the induction of the pro-apoptotic proteins Bax and Bim, and at the same time a reduction in the anti-apoptotic protein levels of both Bcl-X<sub>L</sub> and Mcl-1. This suggests

that these crude extracts may be acting through the induction of the mitochondrial pathway of apoptosis.

Protein kinase B (Akt) is a serine/threonine kinase that plays an important role in cellular functions such as cell cycle progression, cell size, neovascularization, genome stability, regulation of glucose metabolism and protein synthesis [368]. It can also promote cell survival by blocking apoptosis via the inactivation of pro-apoptotic proteins and mediating cellular growth factors [368]. Our results demonstrate that treatment of Huh-7 cells with the FH crude extract led to an increase of phosphorylated *p*-Akt, *p*-Gsk and *p*-S6k. This result indicates that FH crude extract induced apoptosis-mediated ER stress by triggering *p*-Akt, *p*-Gsk and *p*-S6k in Huh-7 cells. As has been discussed in chapter 2 the major constituents of *A. atemoya* fruits are the phenolic components and the present findings suggested that it is likely that these components are responsible for the protective effect by reduction of excessive lipid accumulation induced by palmitic acid in Huh-7 cells. The protective activity of phenolics such as flavonoids against PA-induced toxicity has been reported in many studies [369, 370]. The findings of this current study indicate that *A. atemoya* fruit potentially ameliorate PA-induced hepatocyte damage, which might provide a new perspective of *A. atemoya* fruits health-promoting effects on preventing NAFLD.

## 5.5. Conclusion

The major findings of this study showed that both LH and SEY crude extracts demonstrated cytotoxic activity in Huh-7 cells with an IC<sub>50</sub> values 67.21 and 22.55 µg/mL, respectively. The cytotoxic effect may be due to the presence of the same annonaceous acetogenins reported in SEY and LH crude extracts. The rest of the crude extracts lacked cytotoxicity. However, their protective effect was study against PA-induced toxicity and some of the extracts were shown

to block PA-induce toxicity by inducing the anti-apoptotic proteins Bax and Bim, and at the same time reducing the pro-apoptotic proteins Bcl-X<sub>L</sub> and Mcl-1. Finally, FH crude extract induced phosphorylated *p*-Akt by triggering *p*-Akt, *p*-Gsk and *p*-S6k in Huh-7 cell.

# **CHAPTER 6**

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## **Conclusions and Future Directions**

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## Conclusion and future directions

The study in this thesis involved investigating the phytochemical constituents of *A. atemoya*, and evaluating the cytotoxicity cancer cell line Huh-7 and protective activity against PA-induced toxicity of the crude extracts of different plant parts. In chapter 2, all the studies that reported the phytochemical constituents and pharmacological effects of *A. atemoya* were summarized and it was the first systematic review of *A. atemoya* (published in 2020). This study showed out of 32 articles, 18 studies examined the phytochemical constituents of various parts of *A. atemoya*, 14 studies investigated the biological activities *in vivo* (n = 5), *in vitro* (n = 7) and two studies discussed the *in vitro* toxicity. Regarding the phytochemical screening of the *A. atemoya* plant, limited studies have reported a range of constituents isolated from various parts including the leaves, fruits and seeds, and examined as extracts or pure compounds in both *in vivo* and *in vitro* studies. The increasing popularity of the flavoursome custard apple (*A. atemoya*) as a fruit particularly in Australia means that this investigation and screening of the phytochemical constituents of different parts of *A. atemoya* is very timely.

In chapters 3 and 4, a comprehensive study of the chemical constituents of the leaves, pulp and seeds of two cultivars of *A. atemoya* was undertaken. Our data are the first to show that annonaceous acetogenins are present in the leaves and pulp of *A. atemoya*. Twenty compounds were identified: sixteen annonaceous acetogenins (1–11 and 16–20) and four alkaloids (12–15). The seeds of the AP cultivar were the richest in annonaceous acetogenins, with fourteen acetogenins identified (1–7, 10, 11, 17–20) in the hexane, ethyl acetate and ethanol extracts, compared with the pulp, which only contained two annonaceous acetogenins (1 and 16). In addition, the ethyl acetate crude extracts of both the leaves and seeds were richer in annonaceous acetogenins in comparison with the crude extracts using hexane or ethanol. Compounds 6 and 7 were isolated, and their structures and absolute configuration were



determined through the interpretation of the spectroscopic data and comparison with previously reported data. Montanacin A (16) is reported for the first time in the pulp of the AP cultivar and the leaves of the KJ cultivar of *A. atemoya*. Our results demonstrated that *A. atemoya* contains a range of acetogenins and alkaloids, similar to those found in other *Annona* species, including *A. squamosa* and *A. cherimola* the parent species of the *A. atemoya* hybrid. Although the two cultivars contained similar annonaceous acetogenins, the KJ cultivar contained only nine annonaceous acetogenins compared with the sixteen detected in the AP cultivar, indicating that further study of other cultivars should be undertaken. In addition, future research could be undertaken to investigate and compare various phytochemical constituents of different cultivars, including other constituents such as the polyphenolics in the fruit.

In chapter 5, the biological activity of *A. atemoya* crude extracts were examined using Huh-7 cell line. Firstly, the cytotoxicity of each crude extract was investigated, and the major findings of this study showed that both LH and SEY crude extracts demonstrated cytotoxic activity in Huh-7 cells with an  $IC_{50}$  values 67.21 and 22.55 respectively, and this activity is likely due to the abundant presence of annonaceous acetogenins in SEY and LH crude extracts. For the remaining crude extracts their protective effect was studied against PA-induced toxicity. Some of the extracts were found inhibit PA-induced toxicity through the induction of the anti-apoptotic proteins Bax and Bim. Finally, FH crude extract induced phosphorylated *p*-Akt by triggering *p*-Akt, *p*-Gsk and *p*-S6k in Huh-7 cells.

Future studies could further investigate both the cytotoxic and protective activities of *A. atemoya* and it's constituents in different cancer cell lines and normal cell lines. Further studies to explore other underlying mechanisms responsible for the protective activity of these crude extracts as well as identify the phytochemical constituents responsible for the activity is required. Finally, this study was conducted during the pandemic including two periods of

lockdown. Several initially planned experiments such as Seahorse real-time metabolic assays to analyse hepatocytes metabolic reprogramming in the presence of free fatty acid overload in the presence and absence of *A. atemoya* extracts and flow cytometric analyses of cell death to supportive data for the Western Blot experiments that demonstrated decreased expression of cleaved caspase-3, could not be conducted due time constraints resulting from the COVID-19 associated lockdowns.

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## **Appendices:**

### **Publication list, Supplementary data and Publications**

# Appendix A. Publication list

## Publications in support of this thesis

1. **Bassam SM Al Kazman**, Joanna E. Harnett, and Jane R. Hanrahan. "Traditional Uses, Phytochemistry and Pharmacological Activities of *Annonaceae*." *Molecules* 27.11 (2022): 3462. (**Chapter 1**)
2. **Bassam SM Al Kazman**, Joanna E. Harnett, and Jane R. Hanrahan. "The Phytochemical Constituents and Pharmacological Activities of *Annona atemoya*: A Systematic Review." *Pharmaceuticals* (14248247) 13.10 (2020). (**Chapter 2**)
3. **Bassam SM Al Kazman**, Joanna Elizabeth Harnett, and Jane Rouse Hanrahan. "Identification of Annonaceous Acetogenins and Alkaloids from the Leaves, Pulp, and Seeds of *Annona atemoya*." *International Journal of Molecular Sciences* 24.3 (2023): 2294. (**Chapters 3 and 4**)



## Conference proceedings

1. Al Kazman, Bassam SM, Joanna Harnett, and Jane R. Hanrahan. "Annonaceous acetogenins reported for the first time in the leaves and fruit's pulp of *Annona atemoya*." (2022), online. Oral presentation.
2. Al Kazman, Bassam SM, Joanna Harnett, and Jane R. Hanrahan. "Preliminary studies on phytochemical constituents of the leaves and fruits of *Annona atemoya*, in *Proceedings of the 7th International Electronic Conference on Medicinal Chemistry*, 1–30 November 2021, MDPI: Basel, Switzerland." (2021), online. Oral presentation.

## **Appendix B. Supplementary data from Chapter 2**

The phytochemical constituents and pharmacological activities of *Annona atemoya*: A systematic review

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Supporting Information

**Table S1.** Phytochemical constituents found in different parts of the *Annona atemoya* plant.

| Plant part | Chemical class | Phytochemical constituents                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | Reference |
|------------|----------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Leaf       | Alkaloids      | Anonaine<br>Asimilobine<br>Lanuginosine<br>Liriodenine<br>Lysicamine<br>Proaporphine<br>Pronuciferine<br>Stepharine                                                                                                                                                                                                                                                                                                                                                                                                                                                            | [1, 2]    |
|            | Flavonoids     | Quercetin-3-O-rutinoside-7-O-glucoside<br>Quercetin-3-O-rutinoside-7-O-pentoside<br>Quercetin-3-O-rutinoside,<br>Kaempferol-3-galactoside-7-rhamnoside<br>Quercetin-3-O-glucoside<br>Kaempferol-3-O-glucoside<br>Luteolin-3-galactoside-7-rhamnoside<br>Luteolin-3-glucoside-7-rhamnoside<br>Apigenin-8-C-glucoside<br>Catechin<br>Epicatechin                                                                                                                                                                                                                                 | [1]       |
| Fruit      | Terpenes       | $\alpha$ -Pinene<br>$\beta$ -Pinene<br>Camphene<br>Myrcene<br>$\alpha$ -Phellandrene<br>$\alpha$ -Terpinene<br>Limonene<br>$\beta$ -Phellandrene<br>1,8-Cineole<br>(Z)-Ocimene<br>$\beta$ -Elemene<br>Terpinen-4-ol<br>$\beta$ -Caryophyllene<br>Aromadendrene<br>(Z)-p-menth-2-en-1-ol<br>(E)-p-Menth-2-en-1-ol<br>Isopinocarveol<br>$\alpha$ -Humulene<br>$\alpha$ -Terpineol<br>Borneol<br>Viridiflorene<br>(E)-Ocimene<br>p-Cymene<br>Terpinolene<br>$\alpha$ -Dimethylstyrene<br>$\beta$ -Dimethylstyrene<br>$\alpha$ -Cubebene<br>$\beta$ -Cubebene<br>$\alpha$ -Copaene | [3-7]     |

|       |                    |                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  |        |
|-------|--------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------|
|       |                    | Linalool<br>Bornyl acetate<br>Germacrene D<br>Bicyclogermacrene<br>$\delta$ -Cadinene<br>Globulol<br>Viridiflorol<br>Spathulenol<br>epi-Muurolool<br>$\delta$ -Cadinol<br>$\alpha$ -Cadinol<br>epi-Cadinol<br>Sabinene<br>Sabinene hydrate<br>$\alpha$ -Terpinolene<br>Myrtenal<br><i>cis</i> -Verbenol<br><i>trans</i> -Verbenol<br><i>trans</i> -Pinocarveol<br>$\beta$ -Selinene<br>$\gamma$ -Cadinene<br>Myrtenol<br><i>trans</i> -Carveol<br>Calamenene<br>epi-Cubebol<br>Caryophyllene oxide<br>Ledol<br>$\alpha$ -Kaurene |        |
|       | Phenolic compounds | 3,4-Dihydroxybenzoic acid<br><i>p</i> -Coumaric acid.<br>Chlorogenic acid<br>Catechin<br>Epicatechin                                                                                                                                                                                                                                                                                                                                                                                                                             | [4]    |
| Seeds | Acetogenins        | Almunequin<br>Annonacin<br>Annonisin<br>Annotemoyin-1<br>Annotemoyin-2<br>Asimicin<br>Artemoin-A<br>Artemoin-B<br>Artemoin-C<br>Artemoin-D<br>Atemotetrolin<br>Atemoyacin-A<br>Atemoyacin-E<br>Atemoyin<br>Bulladecin<br>Bullatacin<br>Bullatalicin<br>12,15- <i>cis</i> -Bullatalicin                                                                                                                                                                                                                                           | [8-15] |

|       |                            |                                                                                                                                                                                                                                                                                                                                                                                 |      |
|-------|----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------|
|       |                            | Bullatanocin<br>12,15- <i>cis</i> -Bullatanocin<br>Cherimolin-1<br>Cherimolin-2<br>Desacetylvaricin<br>Isodesacetylvaricin<br>Motrilin<br>Molvizarin<br>Neoannonin<br>Parviflorin<br>Reticulatain-1<br>Rolliniastatin-2<br>Squamocin<br>Squamostatin A<br>12,15- <i>cis</i> -Squamostatin A<br>squamostatin D<br>12,15- <i>cis</i> -Squamostatin D<br>Uvariamicin-III           |      |
|       | Phenolic compounds         | Chlorogenic acid<br>Ferulic acid<br>Vanillic acid<br>Myricetin                                                                                                                                                                                                                                                                                                                  | [16] |
|       | Alkaloids                  | Atempoine<br>Cleistopholine.                                                                                                                                                                                                                                                                                                                                                    | [17] |
|       | <i>N</i> -acyl tryptamines | <i>N</i> -Nonadecanoyltryptamine<br><i>N</i> -Behenoyltryptamine<br><i>N</i> -Lignoceroyltryptamine<br><i>N</i> -Cerotoyltryptamine<br><i>N</i> -Octacosanoyltryptamine<br><i>N</i> -Tricosanoyl-4,5-dihydroxytryptamine<br><i>N</i> -Lignoceroyl-4,5-dihydroxytryptamine<br><i>N</i> -Pentacosanoyl-4,5-dihydroxytryptamine<br><i>N</i> -Heptacosanoyl-4,5-dihydroxytryptamine | [17] |
| Roots | Acetogenins                | Atemoyacin C                                                                                                                                                                                                                                                                                                                                                                    | [18] |

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## **Appendix C. Supplementary data for NMR spectra**



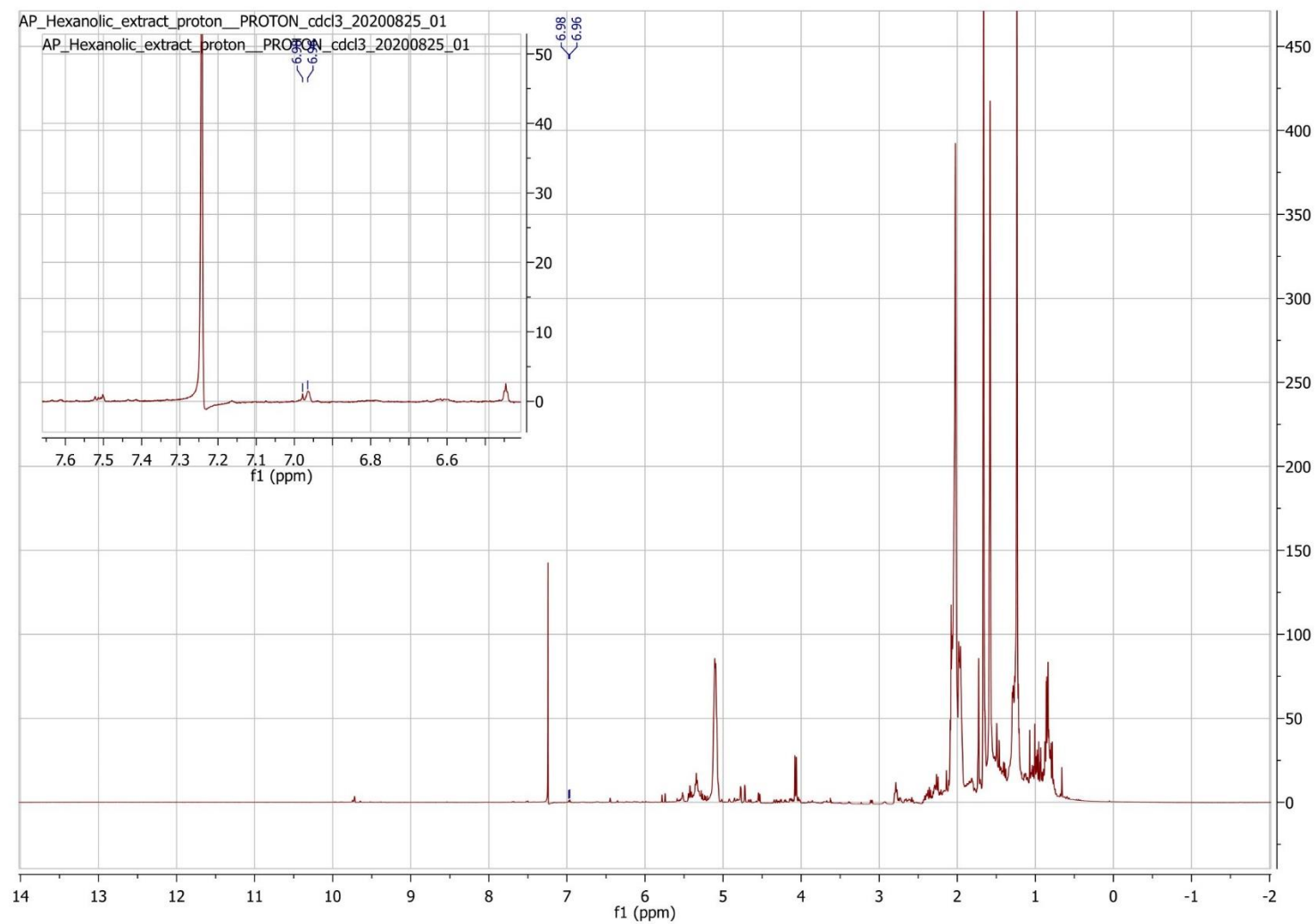


Figure C.1:  $^1\text{H}$  NMR [ $\text{CDCl}_3$ , 400 MHz], chemical shift for AP leaves hexane extract

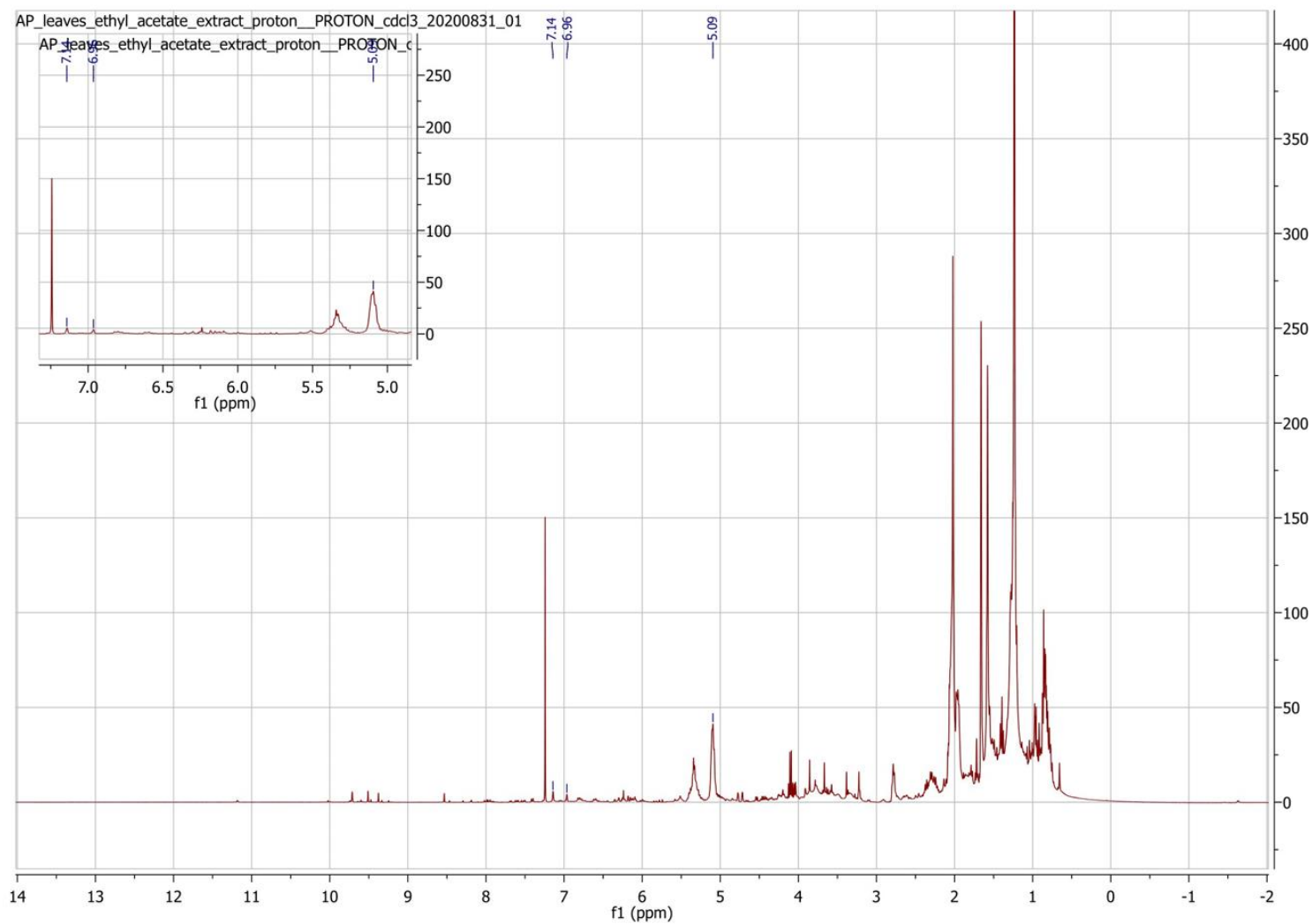
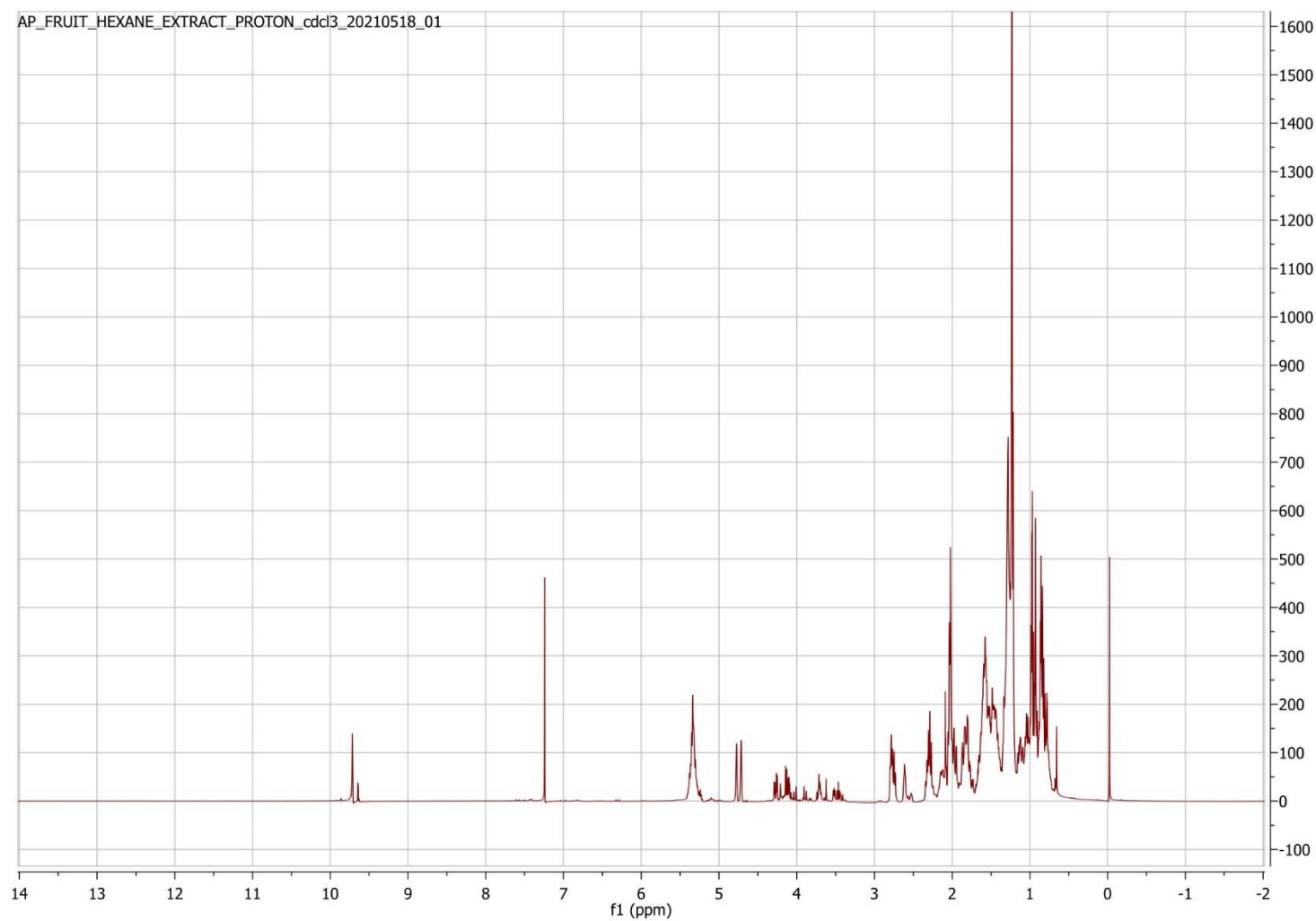
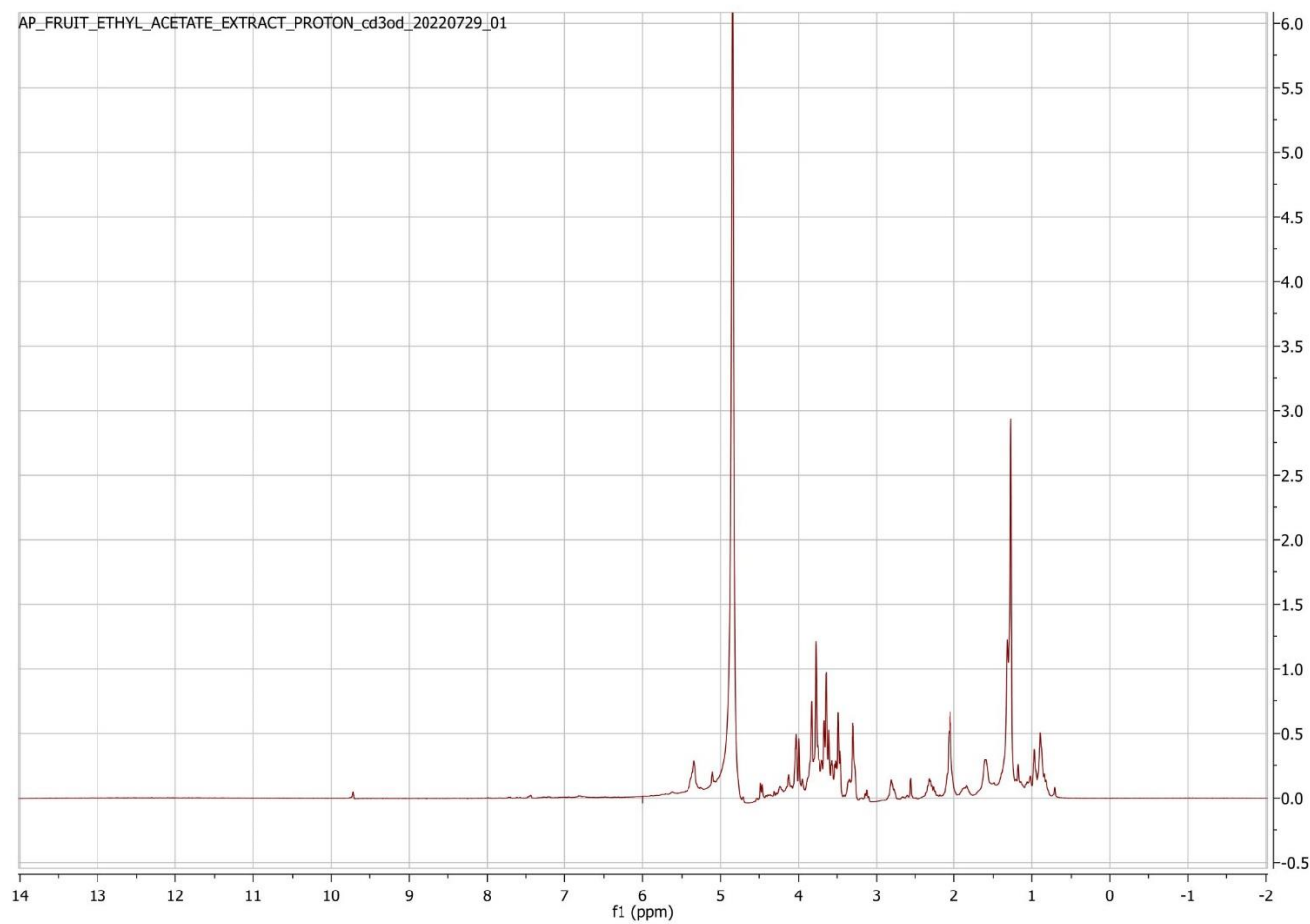


Figure C.2:  $^1\text{H}$  NMR [ $\text{CDCl}_3$ , 400 MHz], chemical shift for AP leaves ethyl acetate extract



**Figure C.3:  $^1\text{H}$  NMR [ $\text{CDCl}_3$ , 400 MHz], chemical shift for AP fruit hexane extract**



**Figure C.4:  $^1\text{H}$  NMR [CD<sub>3</sub>OD, 400 MHz], chemical shift for AP fruit hexane extract**

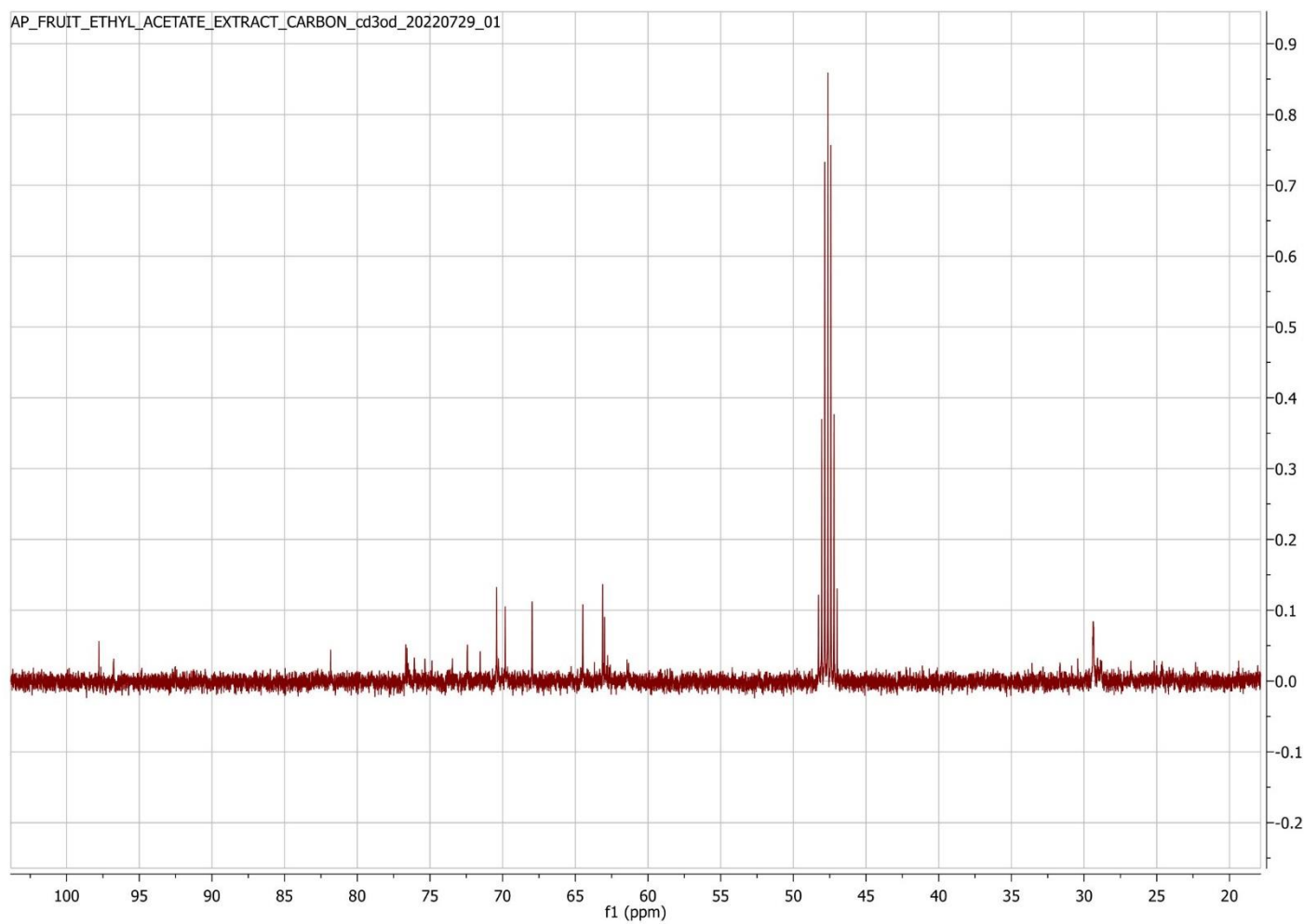


Figure C.5:  $^{13}\text{C}$  NMR [CDCl<sub>3</sub>, 400 MHz], chemical shift for AP fruit hexane extract

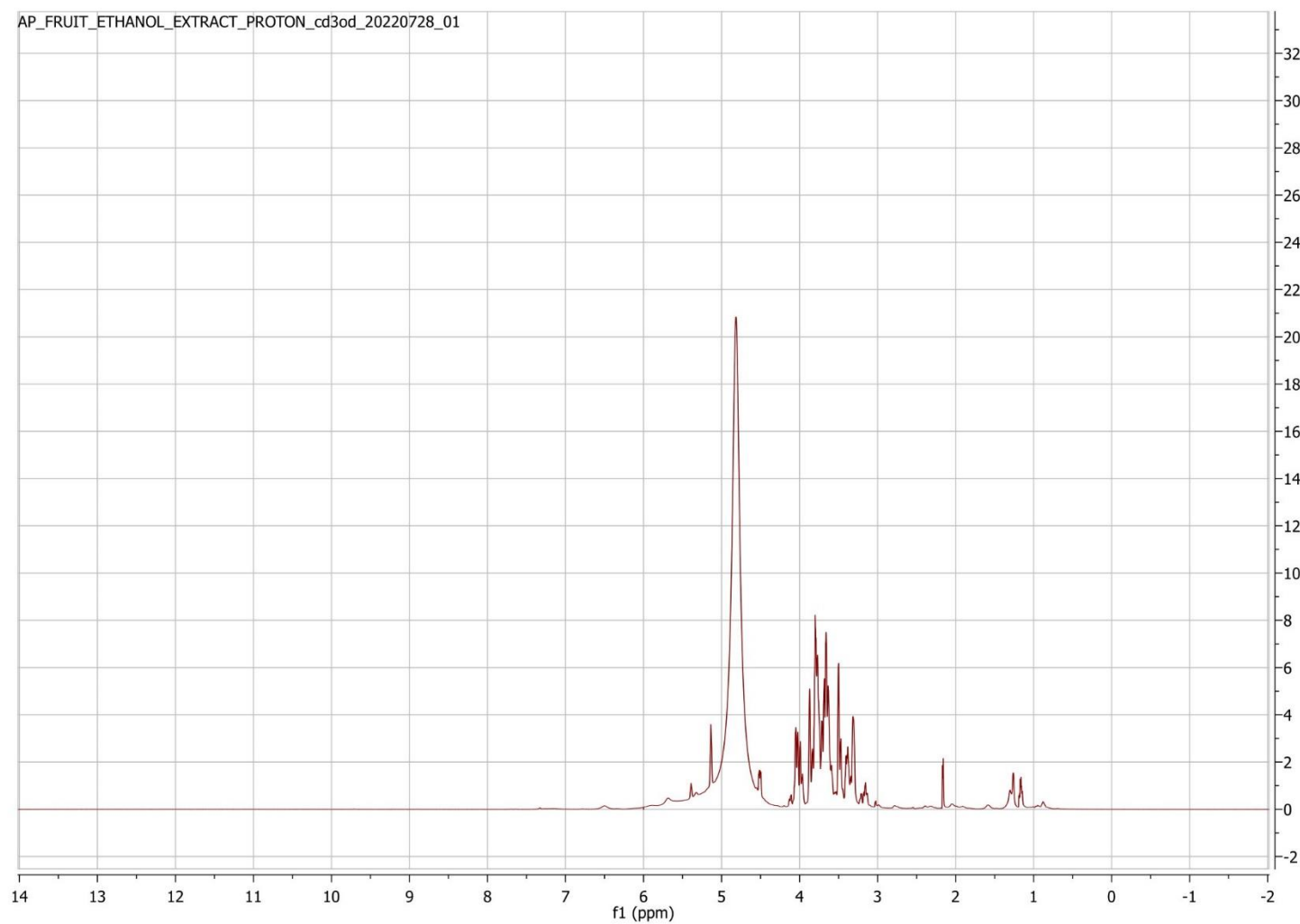


Figure C.6:  $^1\text{H}$  NMR [ $\text{CD}_3\text{OD}$ , 400 MHz], chemical shift for AP fruit ethanol extract

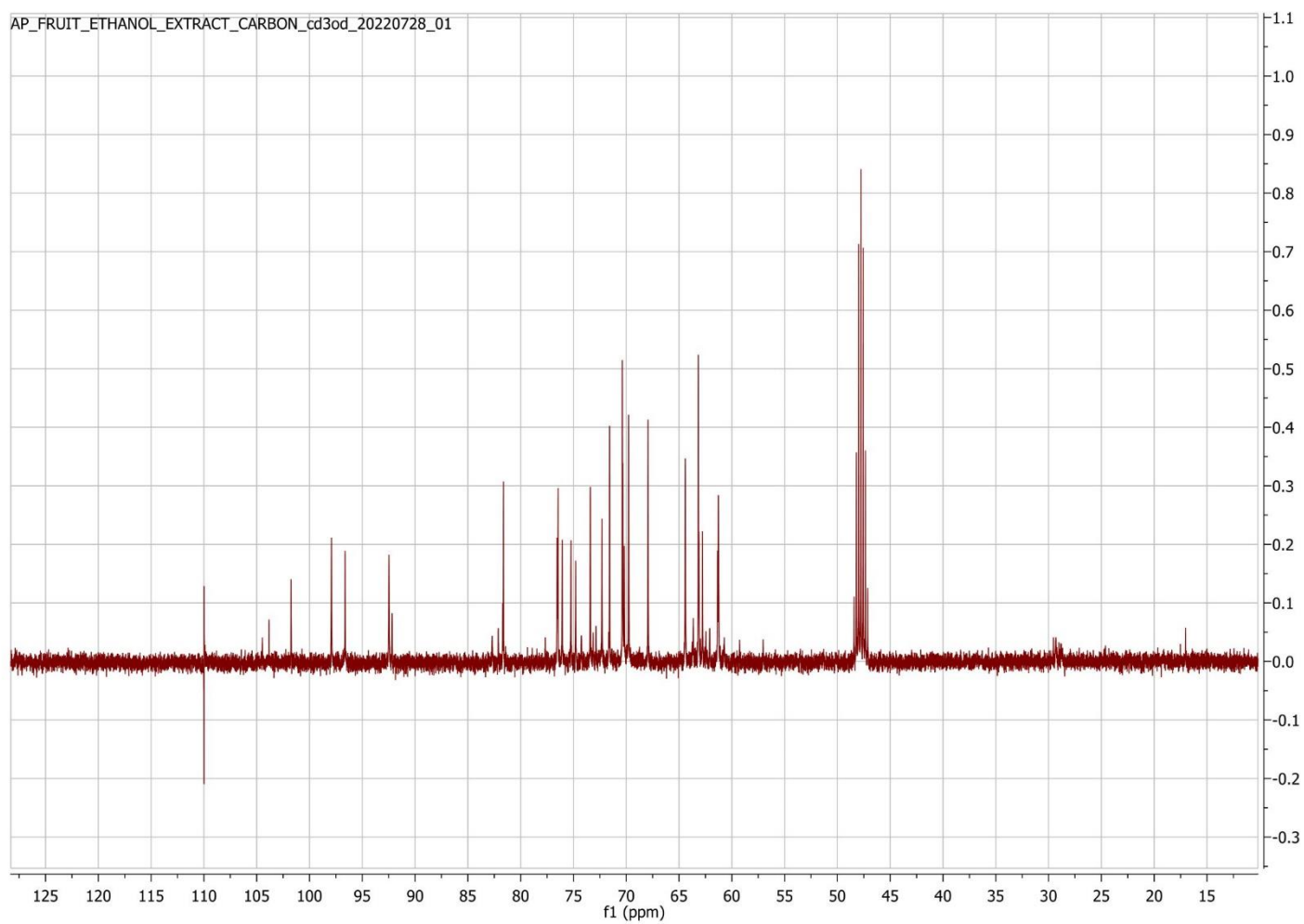


Figure C.7:  $^{13}\text{C}$  NMR [ $\text{CD}_3\text{OD}$ , 400 MHz], chemical shift for AP fruit ethanol extract

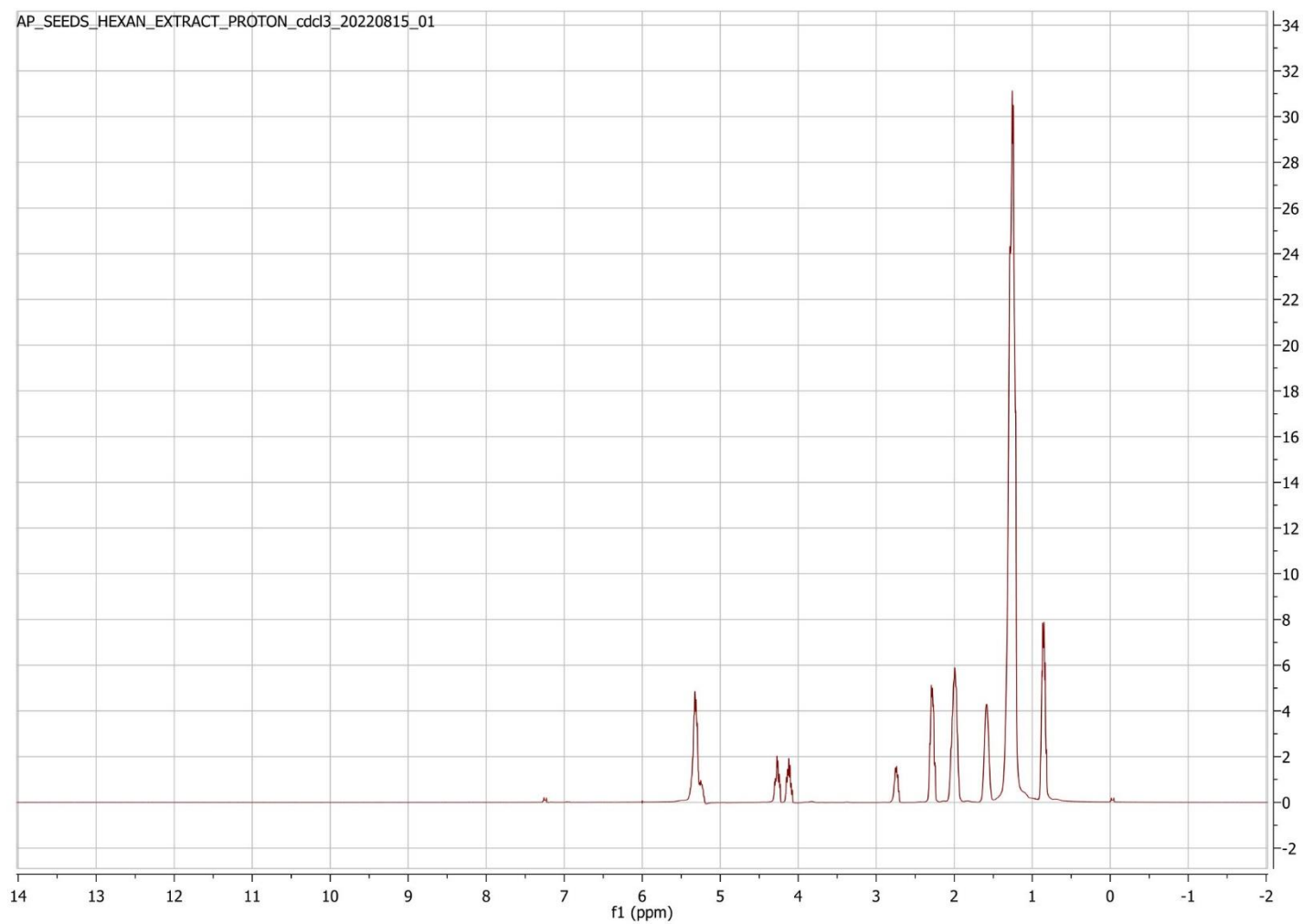
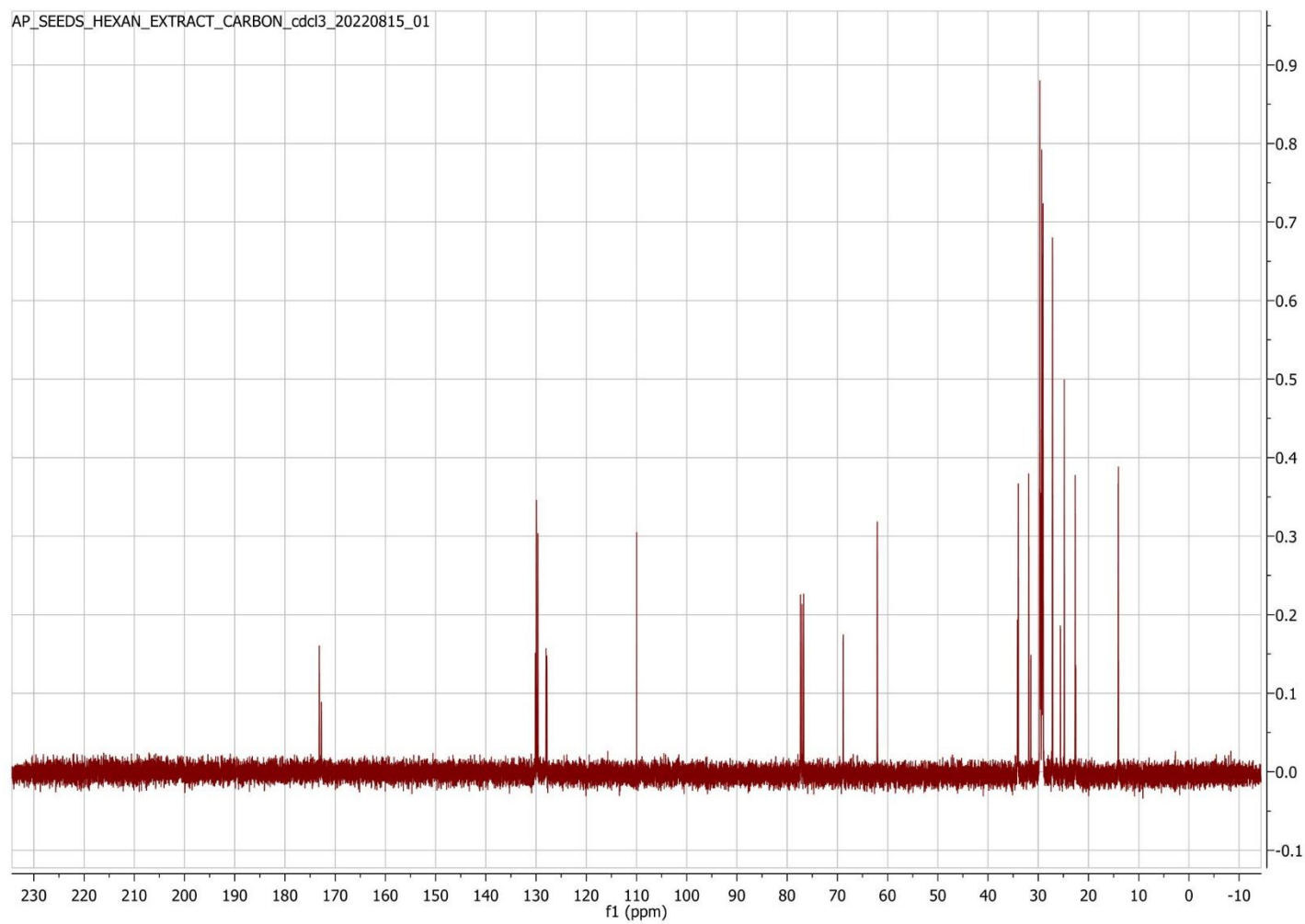


Figure C.8:  $^1\text{H}$  NMR [ $\text{CDCl}_3$ , 400 MHz], chemical shift for AP seed hexane extract





**Figure C.9:  $^{13}\text{C}$  NMR [ $\text{CDCl}_3$ , 400 MHz], chemical shift for AP seed hexane extract**

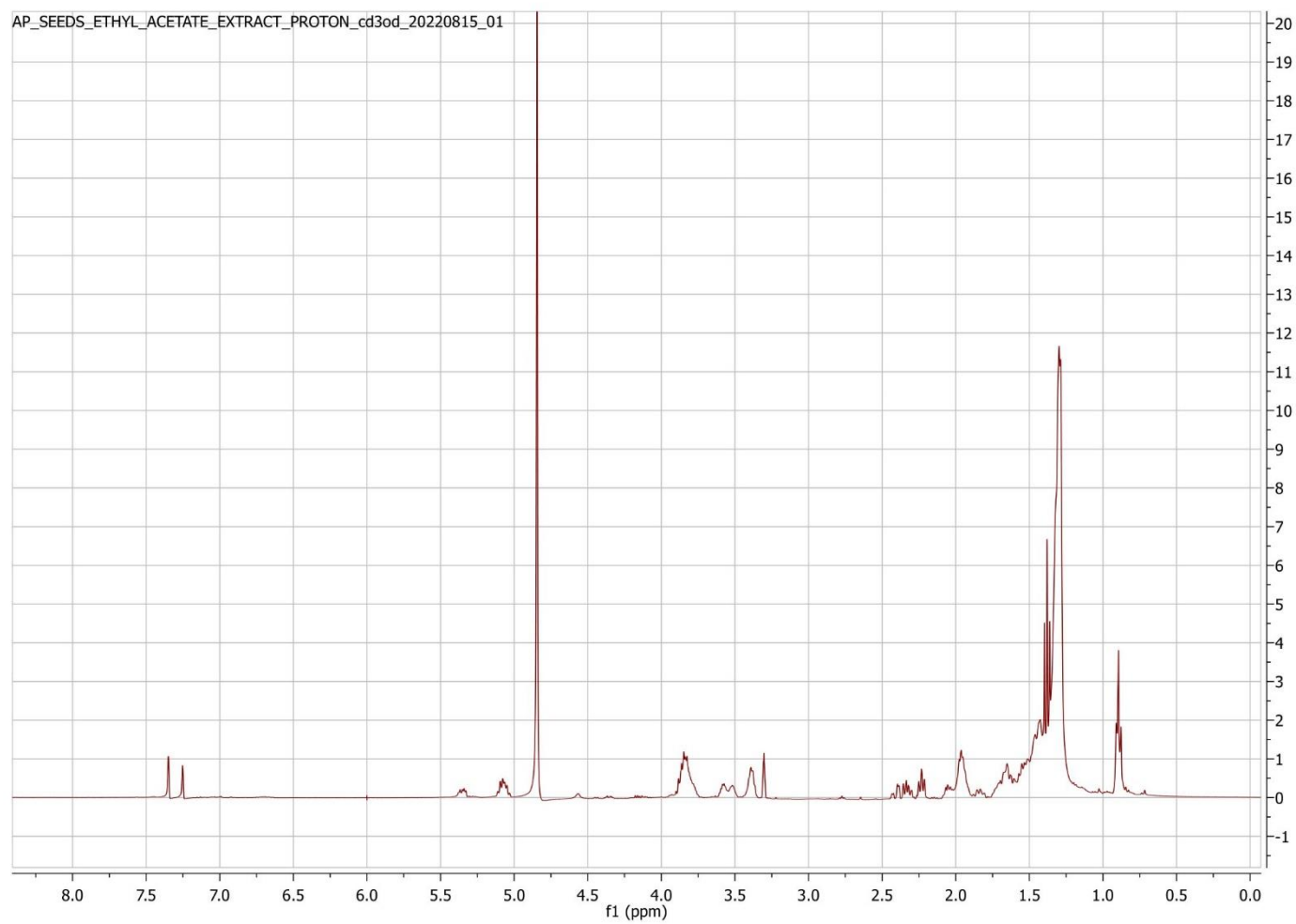


Figure C.10:  $^1\text{H}$  NMR [CD<sub>3</sub>OD, 400 MHz], chemical shift for AP seed ethyl acetate extract

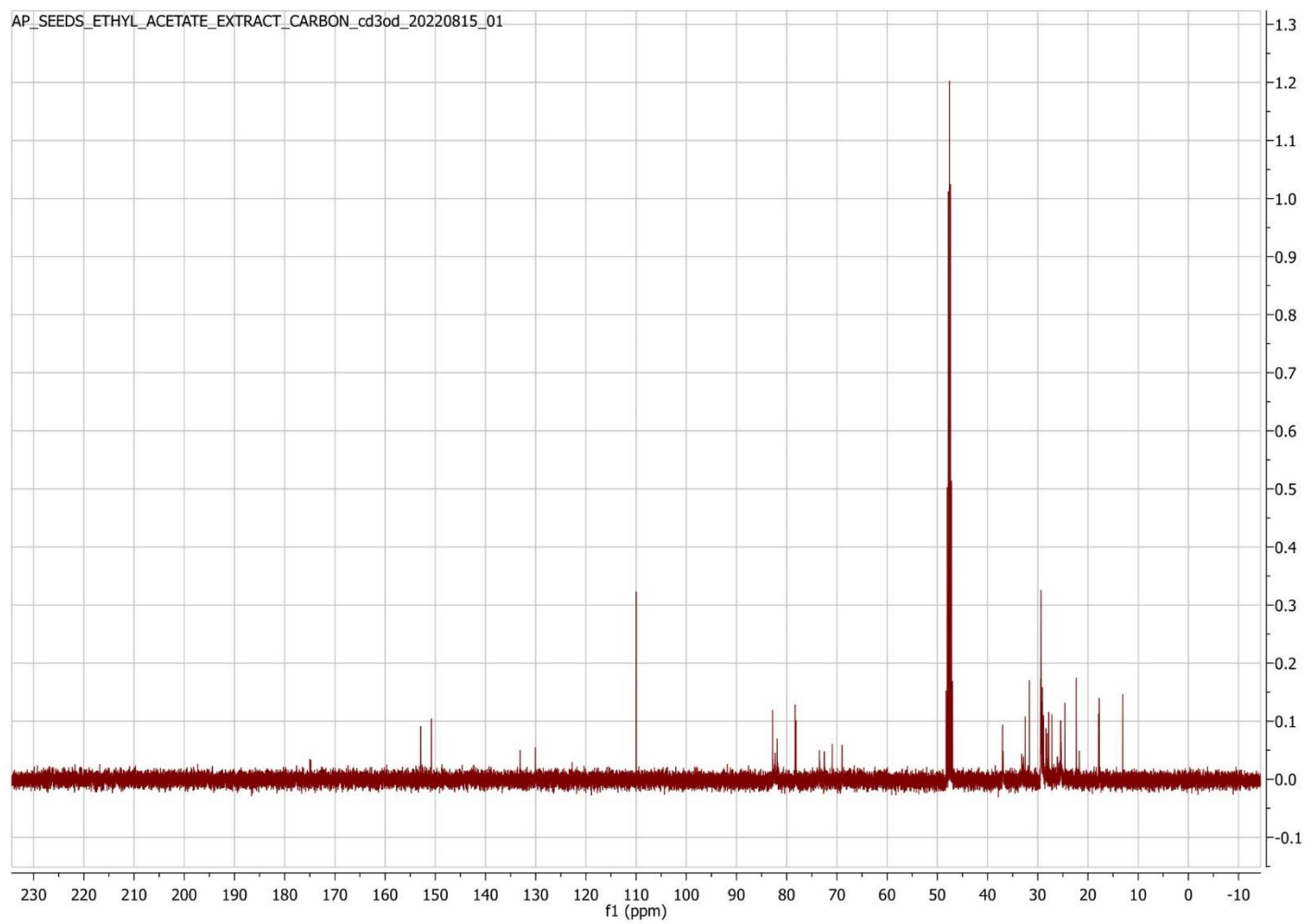


Figure C.11:  $^{13}\text{C}$  NMR [CD<sub>3</sub>OD, 400 MHz], chemical shift for AP seed ethyl acetate extract

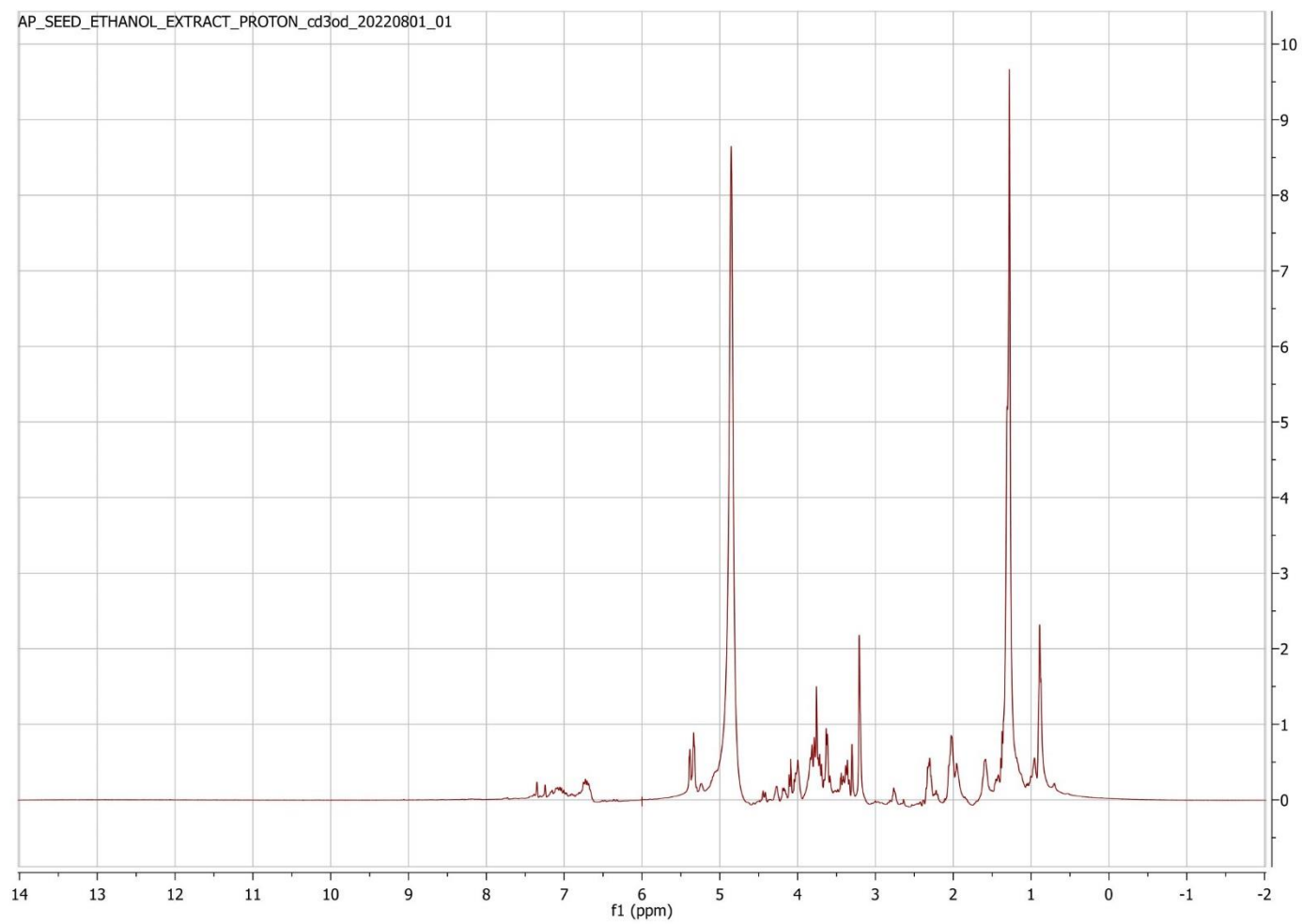


Figure C.12:  $^1\text{H}$  NMR [ $\text{CD}_3\text{OD}$ , 400 MHz], chemical shift for AP seed ethanol extract

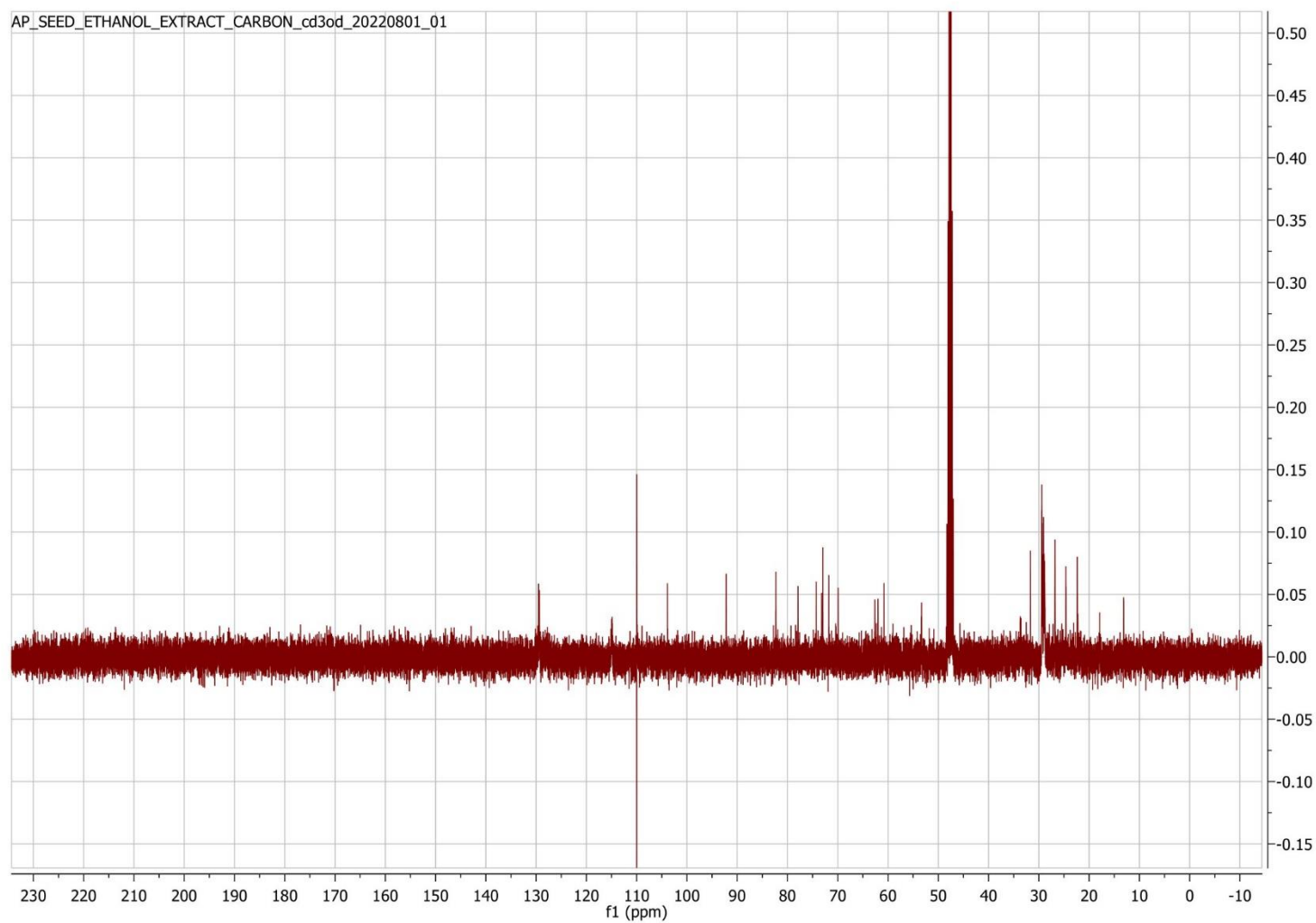


Figure C.13:  $^{13}\text{C}$  NMR [CD<sub>3</sub>OD, 400 MHz], chemical shift for AP seed ethanol extract

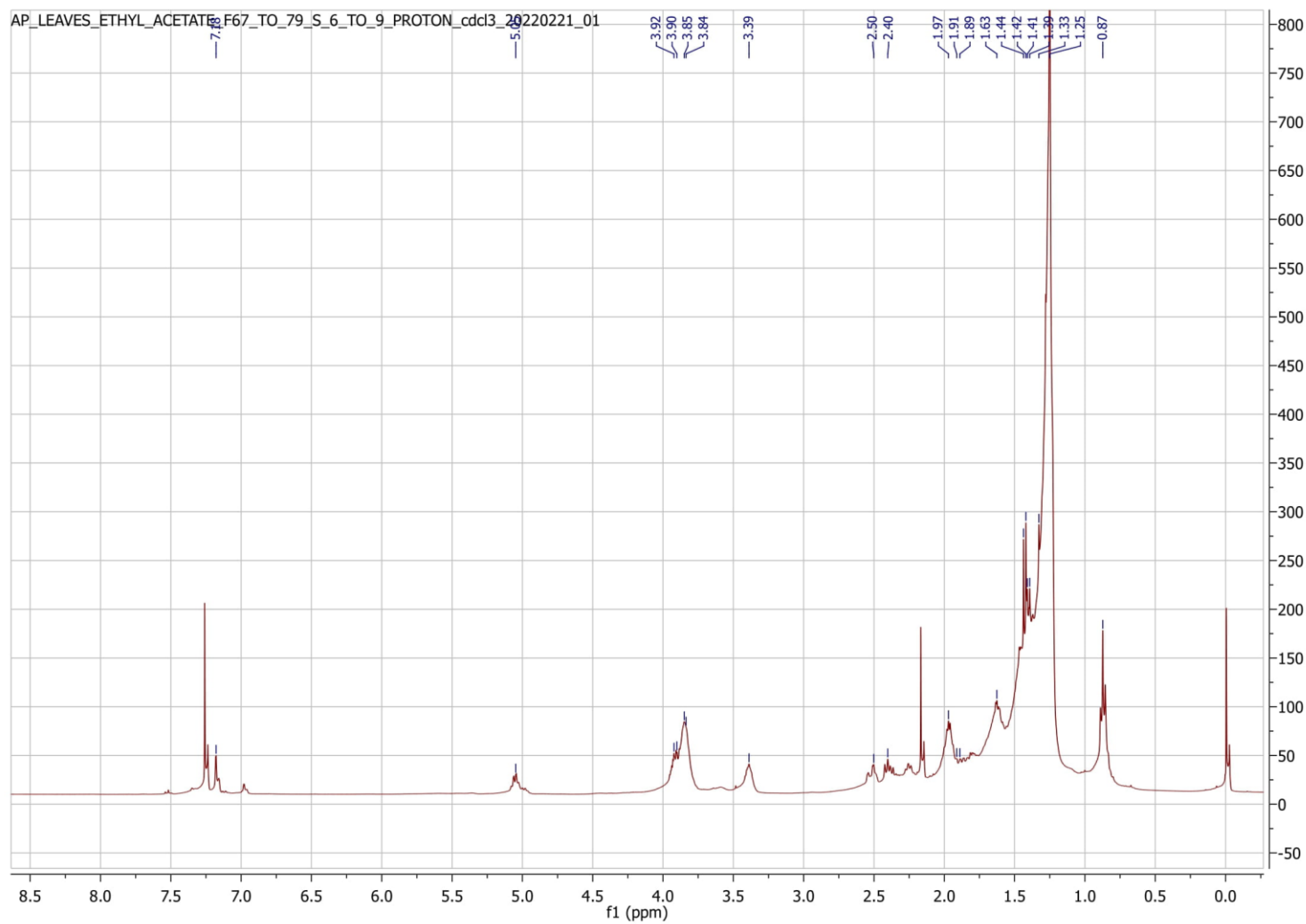


Figure C.14:  $^1\text{H}$  NMR [ $\text{CDCl}_3$ , 400 MHz], chemical shift for Squamocin G

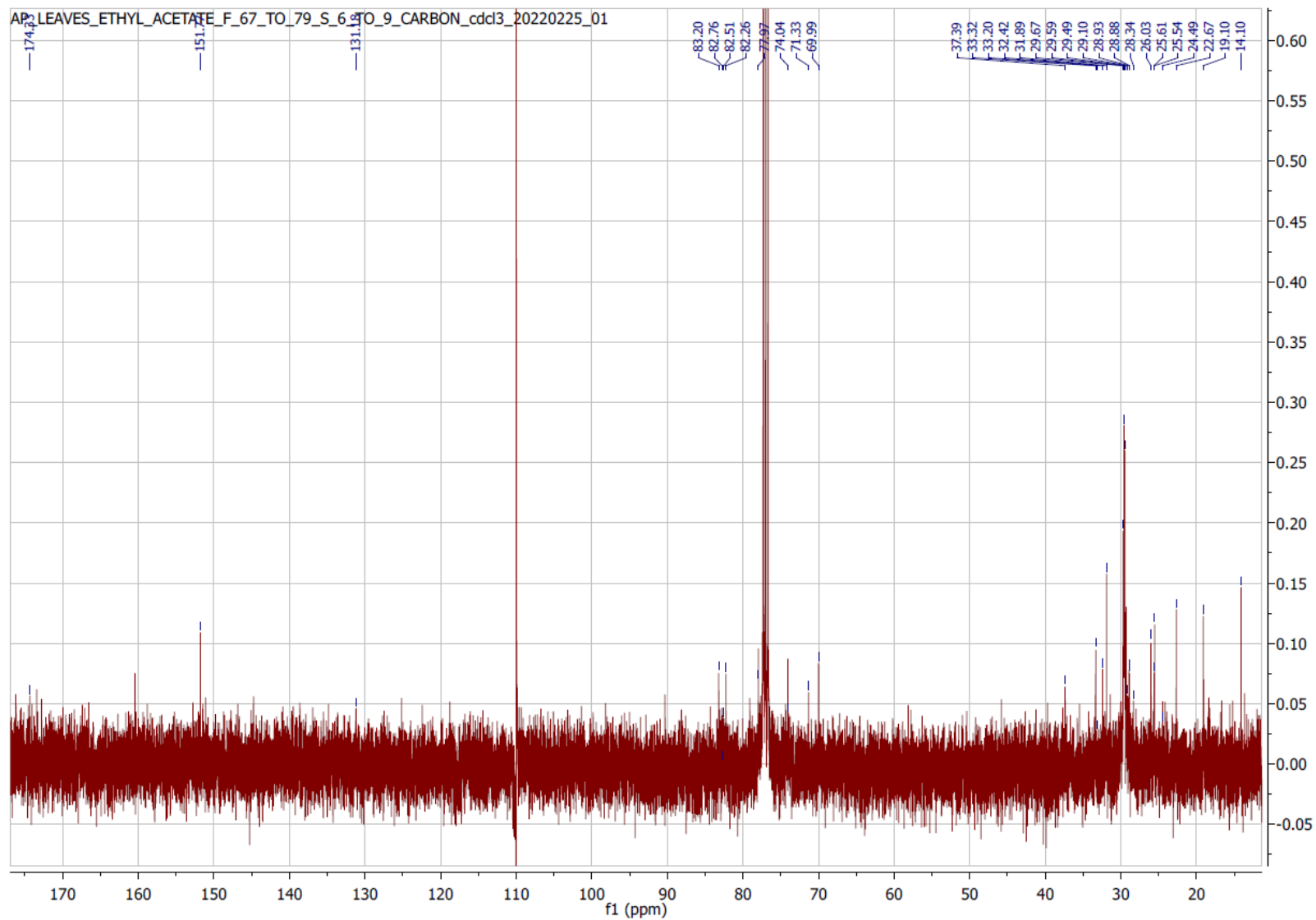


Figure C.15:  $^{13}\text{C}$  NMR [ $\text{CDCl}_3$ , 400 MHz], chemical shift for Squamocin G

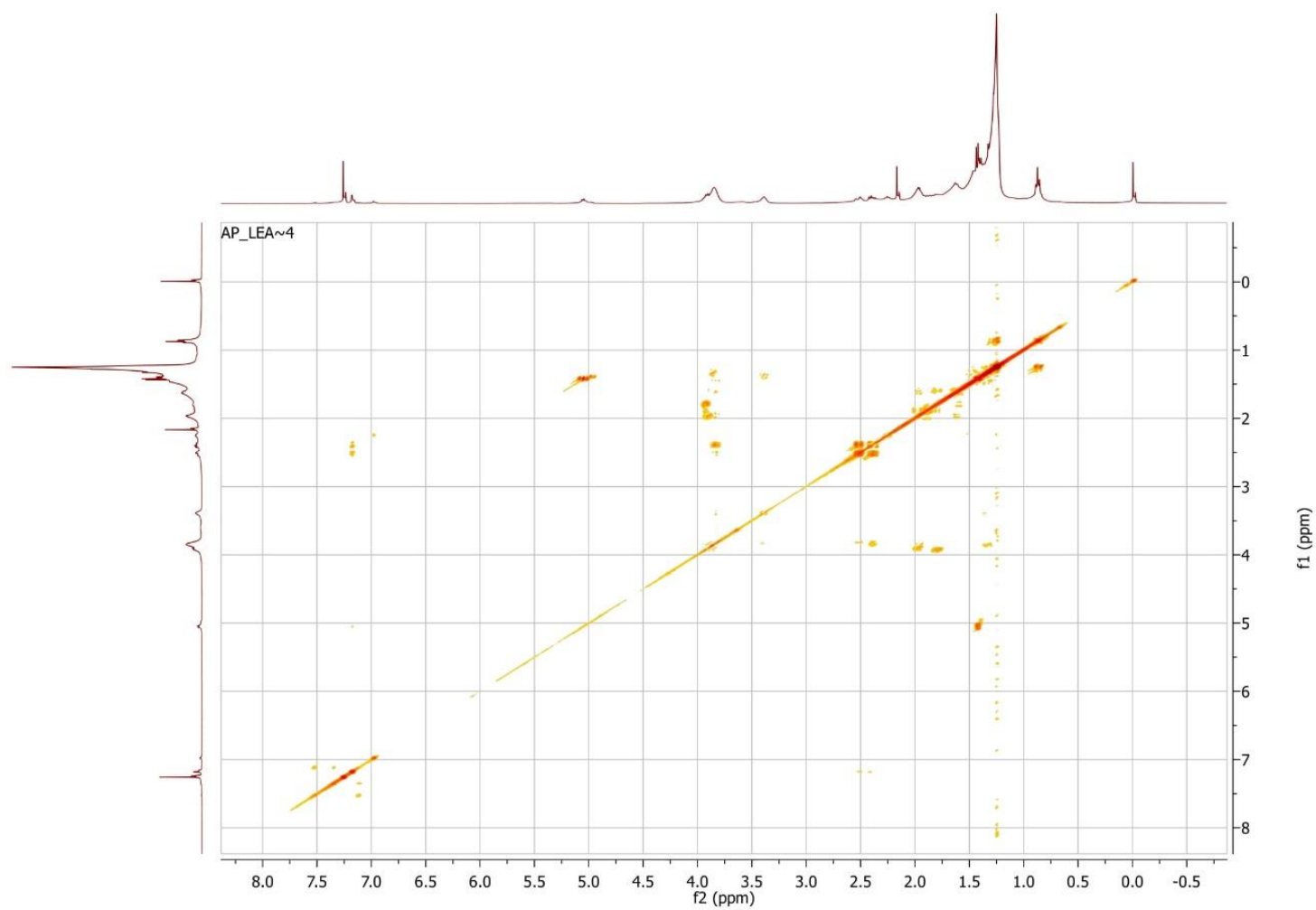


Figure C.16: COSY correlation for Squamocin G



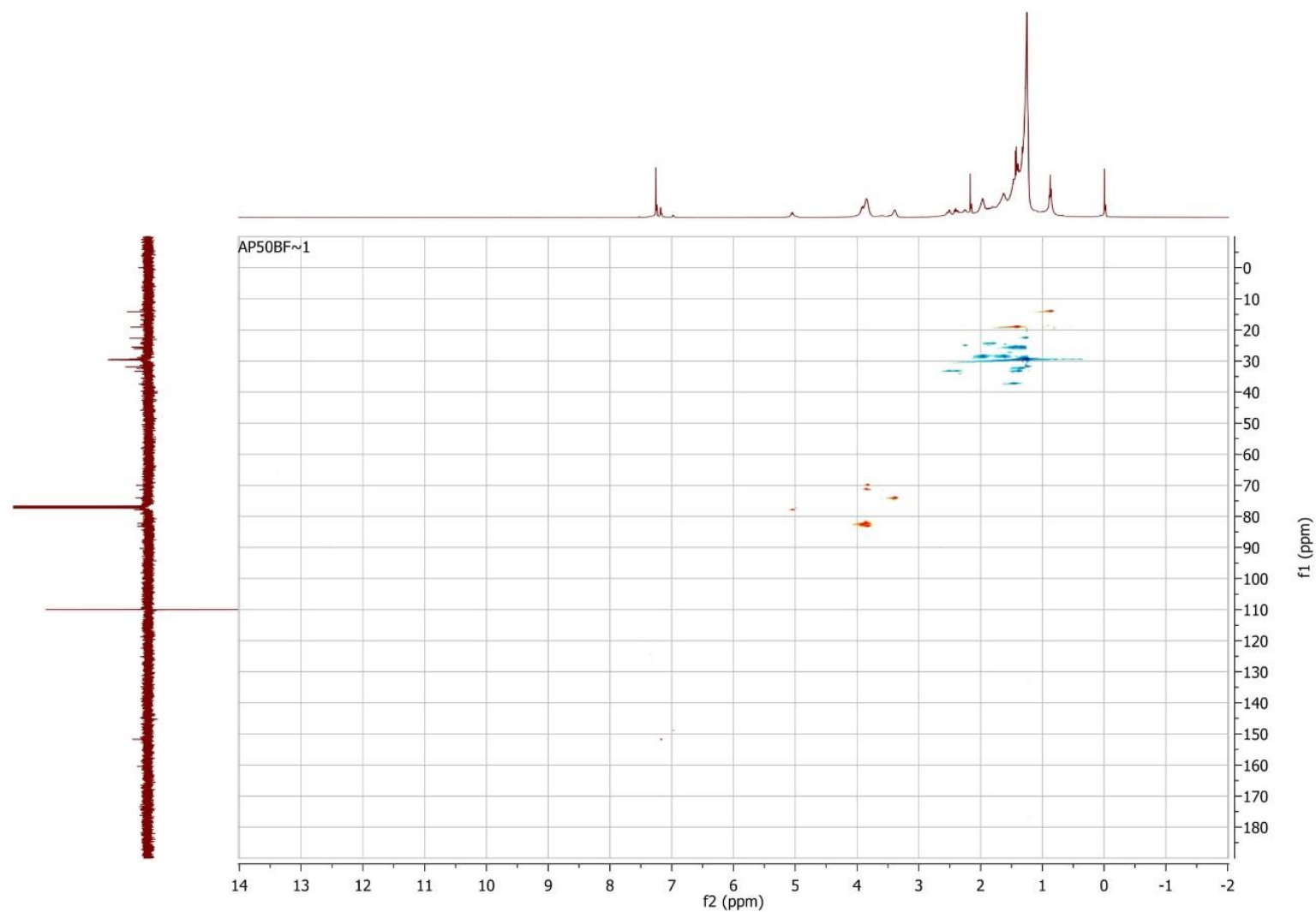


Figure C.17: HSQCAD correlation for Squamocin G

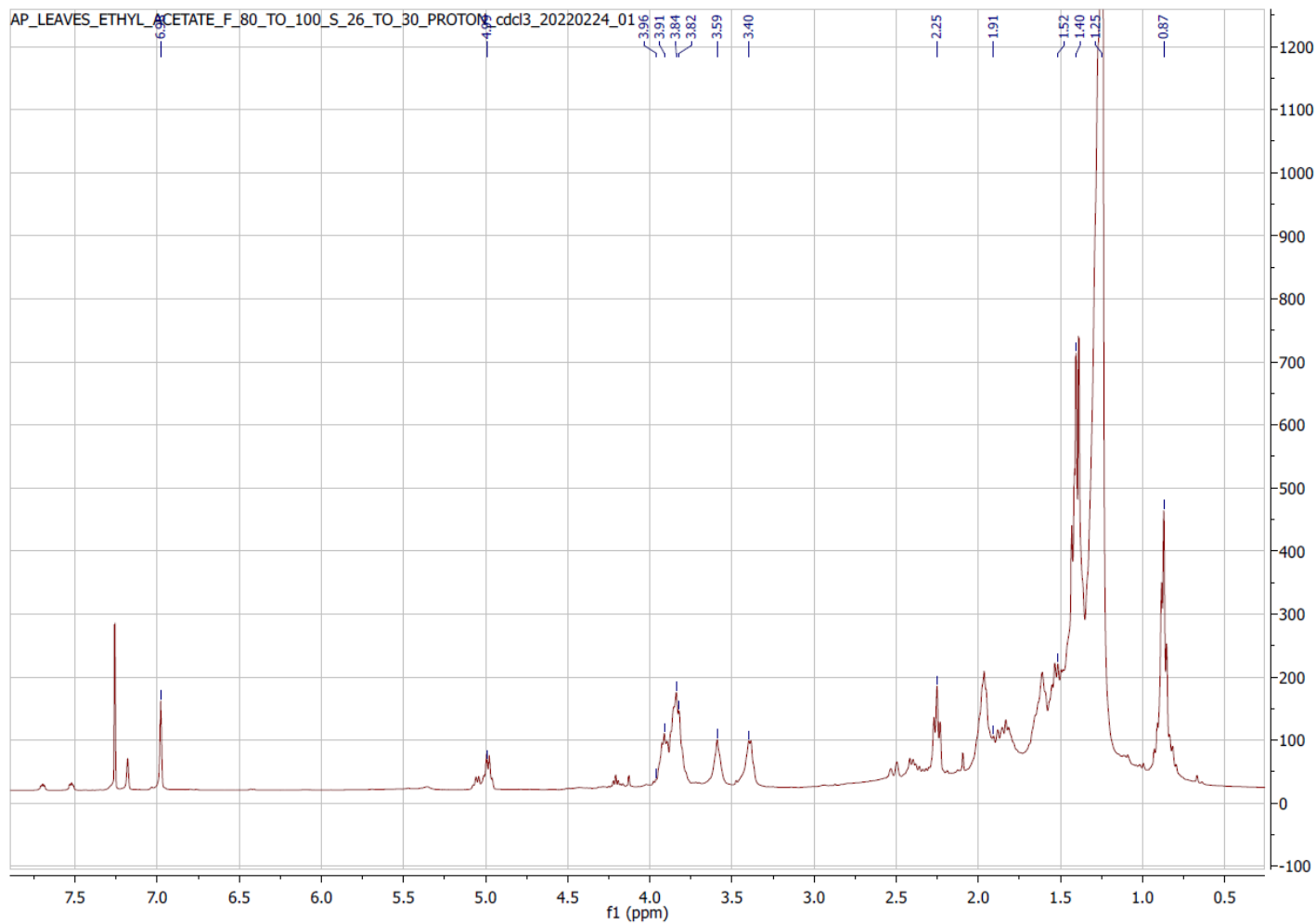


Figure C.18:  $^1\text{H}$  NMR [ $\text{CDCl}_3$ , 400 MHz], chemical shift for Squamocin C

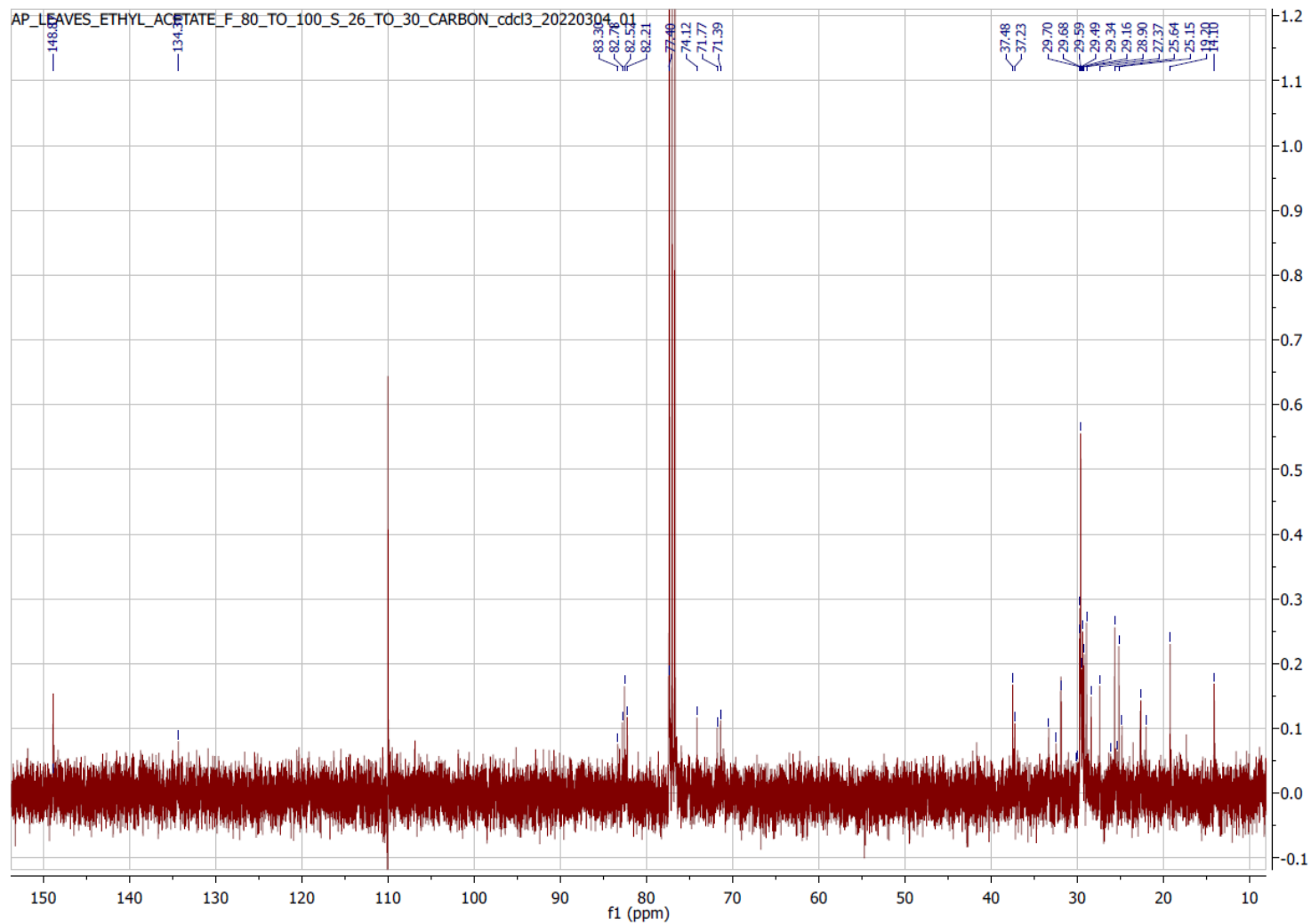


Figure C.19:  $^{13}\text{C}$  NMR [ $\text{CDCl}_3$ , 400 MHz], chemical shift for Squamocin C

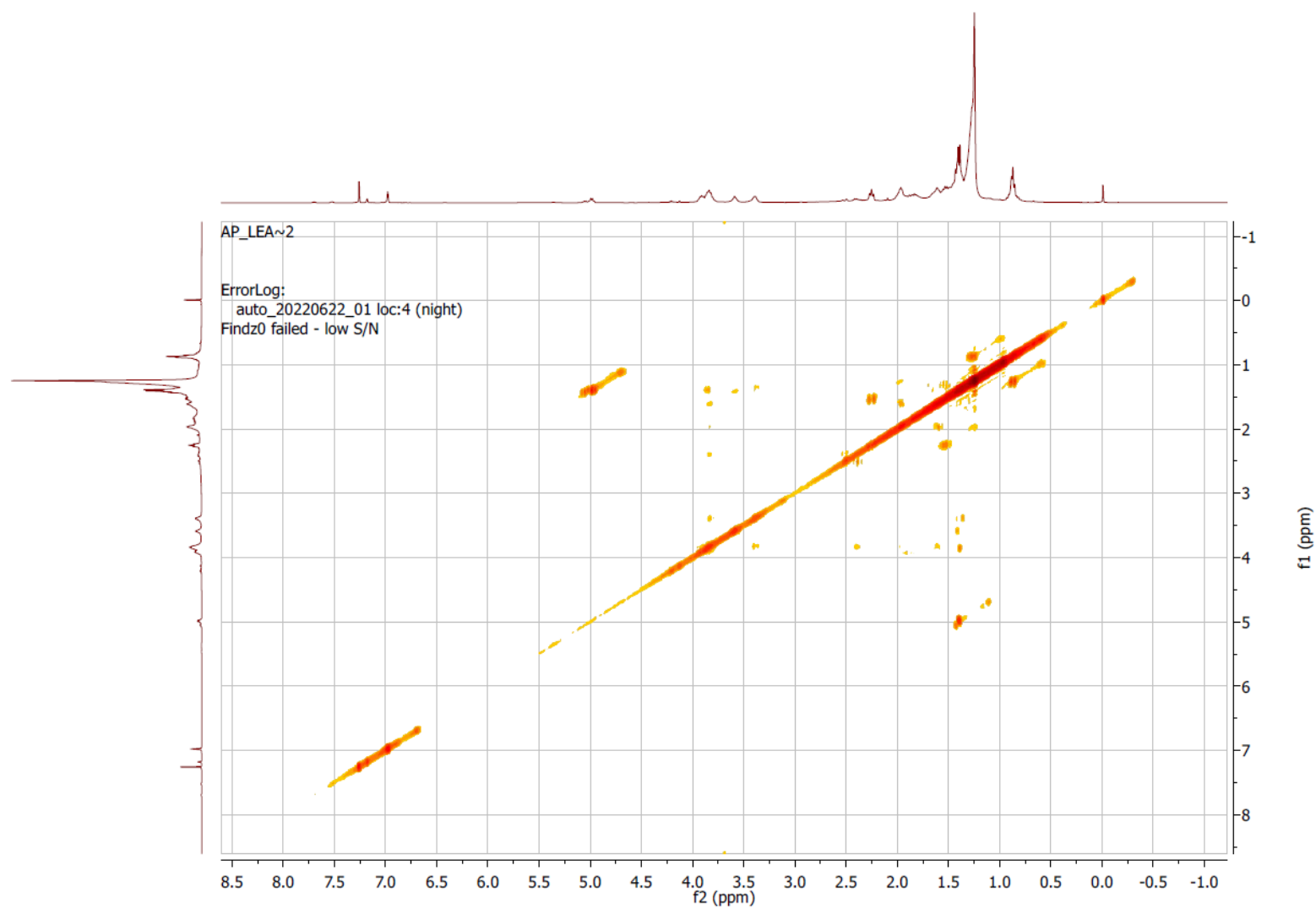


Figure C.20: COSY correlation for Squamocin C

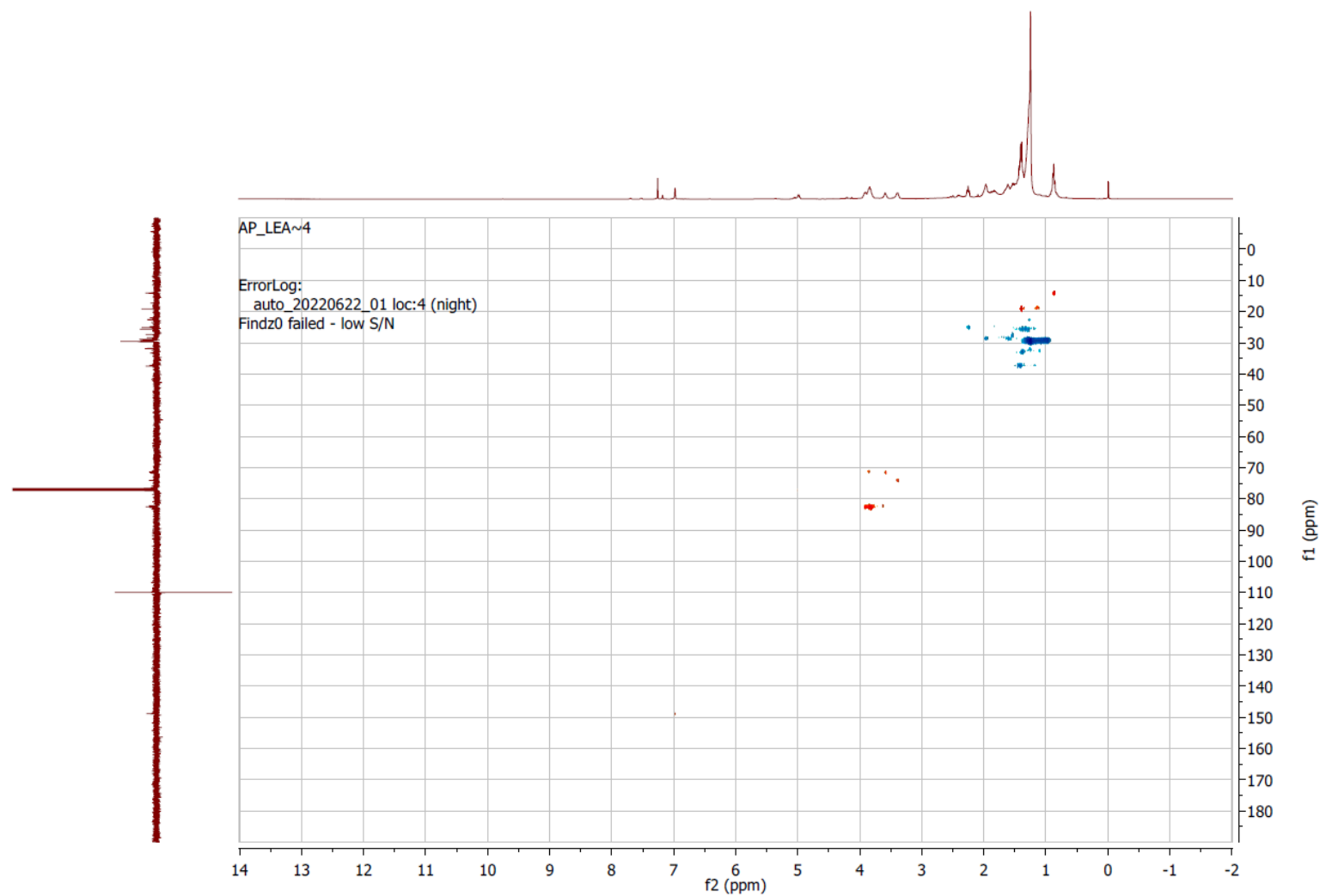


Figure C.21: HSQCAD correlation for Squamocin C

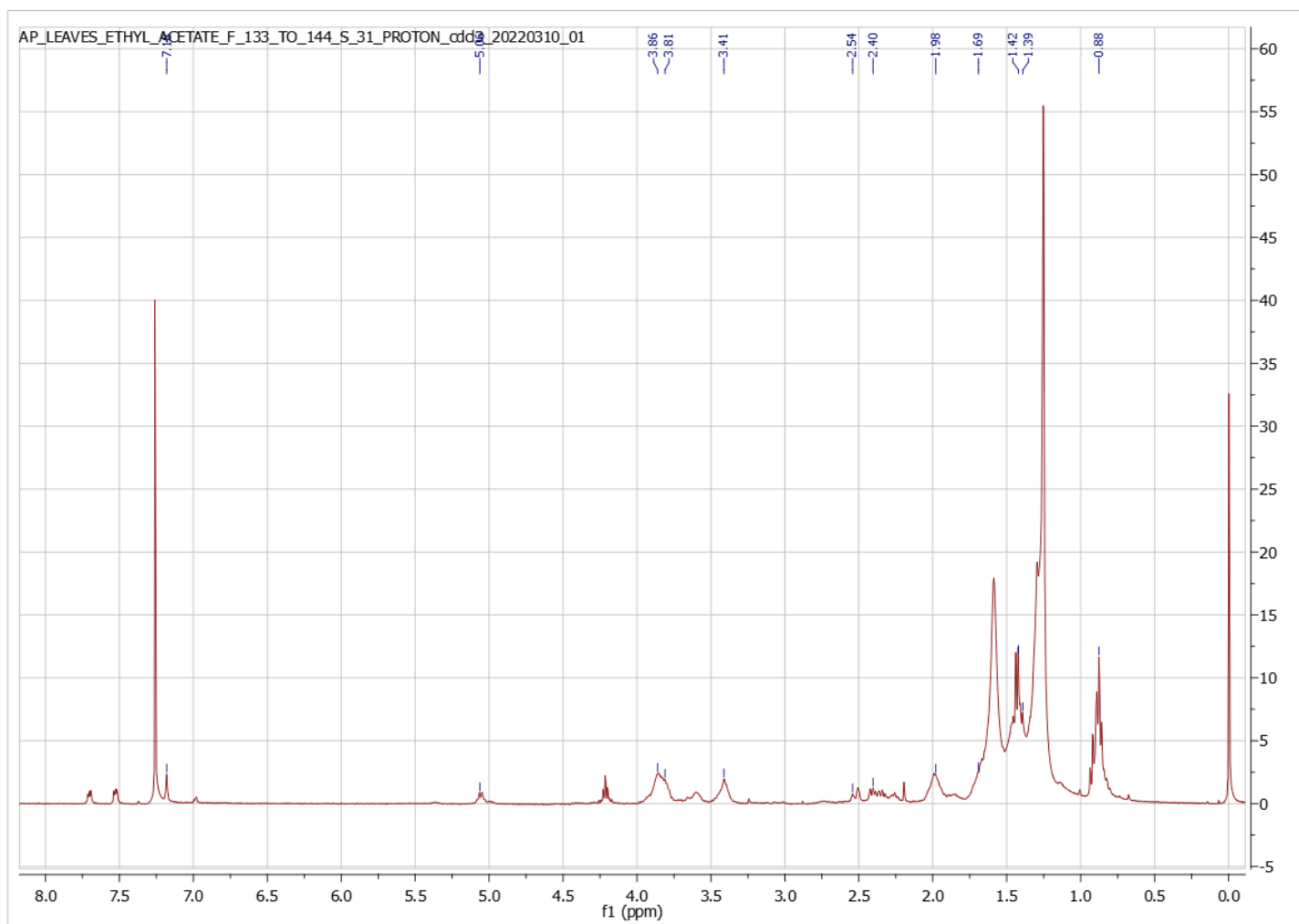


Figure C.22:  $^1\text{H}$  NMR [ $\text{CDCl}_3$ , 400 MHz], chemical shift for Bullatanocin

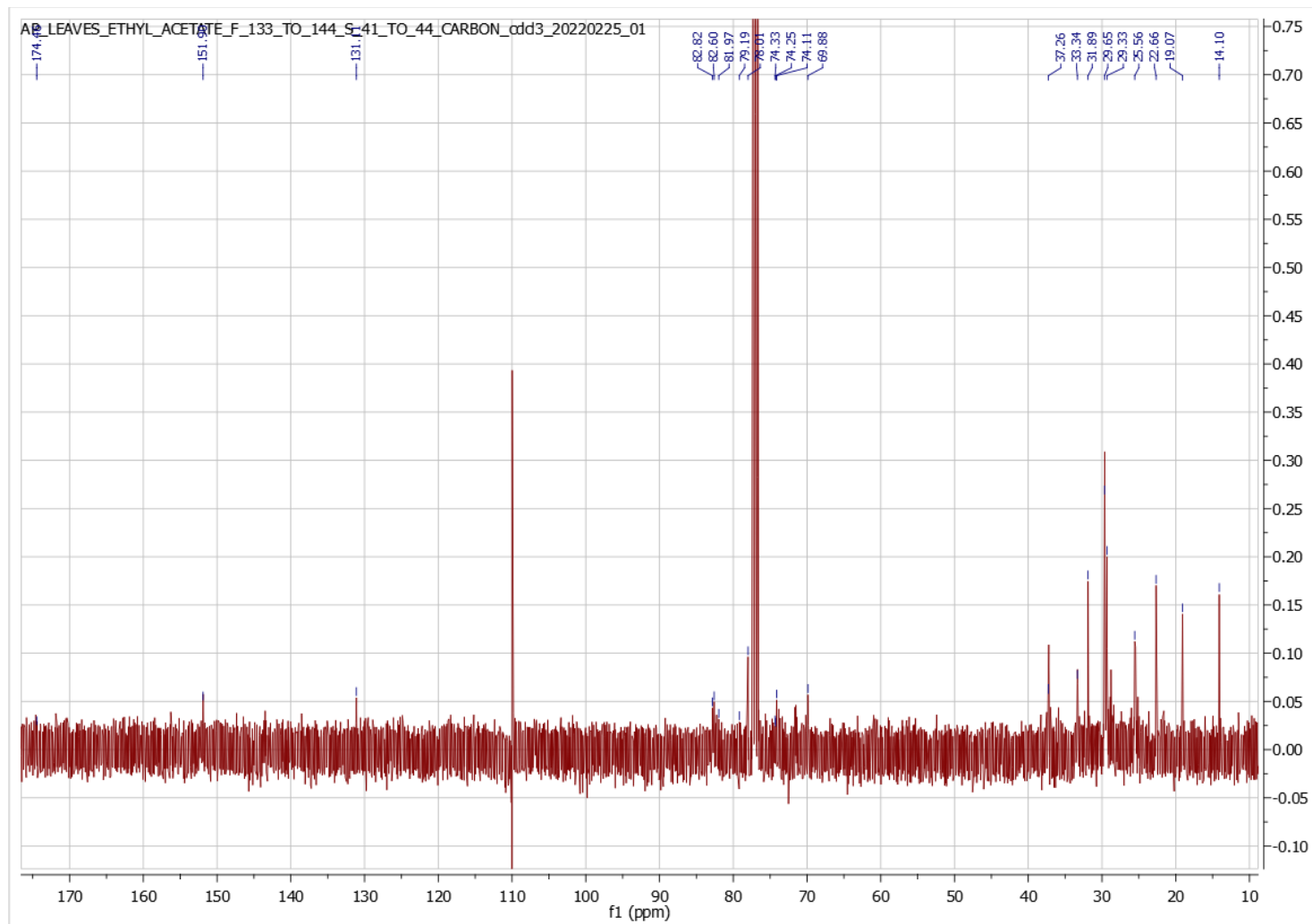


Figure C.23:  $^{13}\text{C}$  NMR [ $\text{CDCl}_3$ , 400 MHz], chemical shift for Bullatanocin

## **Appendix D. Supplementary data for MS spectra**



Paddy\_Bassam\_20220519\_APLHpos

05/21/22 11:11:22

Paddy\_Bassam\_20220519\_APLHpos #5-23 RT: 0.07-0.40 AV: 19 NL: 4.8  
T: +p ESI Q1MS [50.000-1000.000]

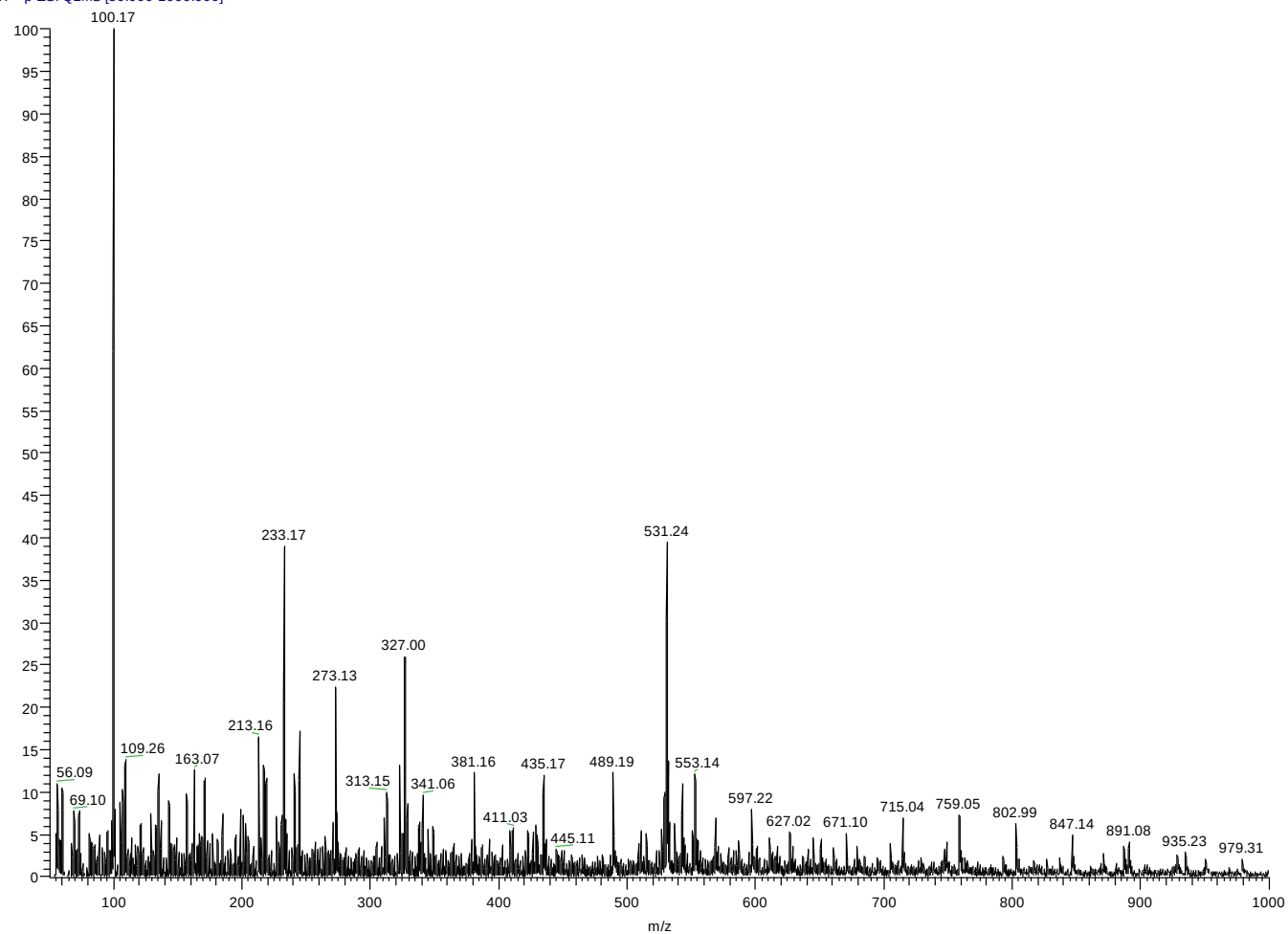


Figure D.1: MS analysis of African Pride leaves hexanolic extract

Paddy\_Bassam\_20220519\_APLEYpos

05/21/22 11:00:00

Paddy\_Bassam\_20220519\_APLEYpos #4-21 RT: 0.06-0.36 AV: 18 NL: 4.  
T: +p ESI Q1MS [50.000-1000.000]

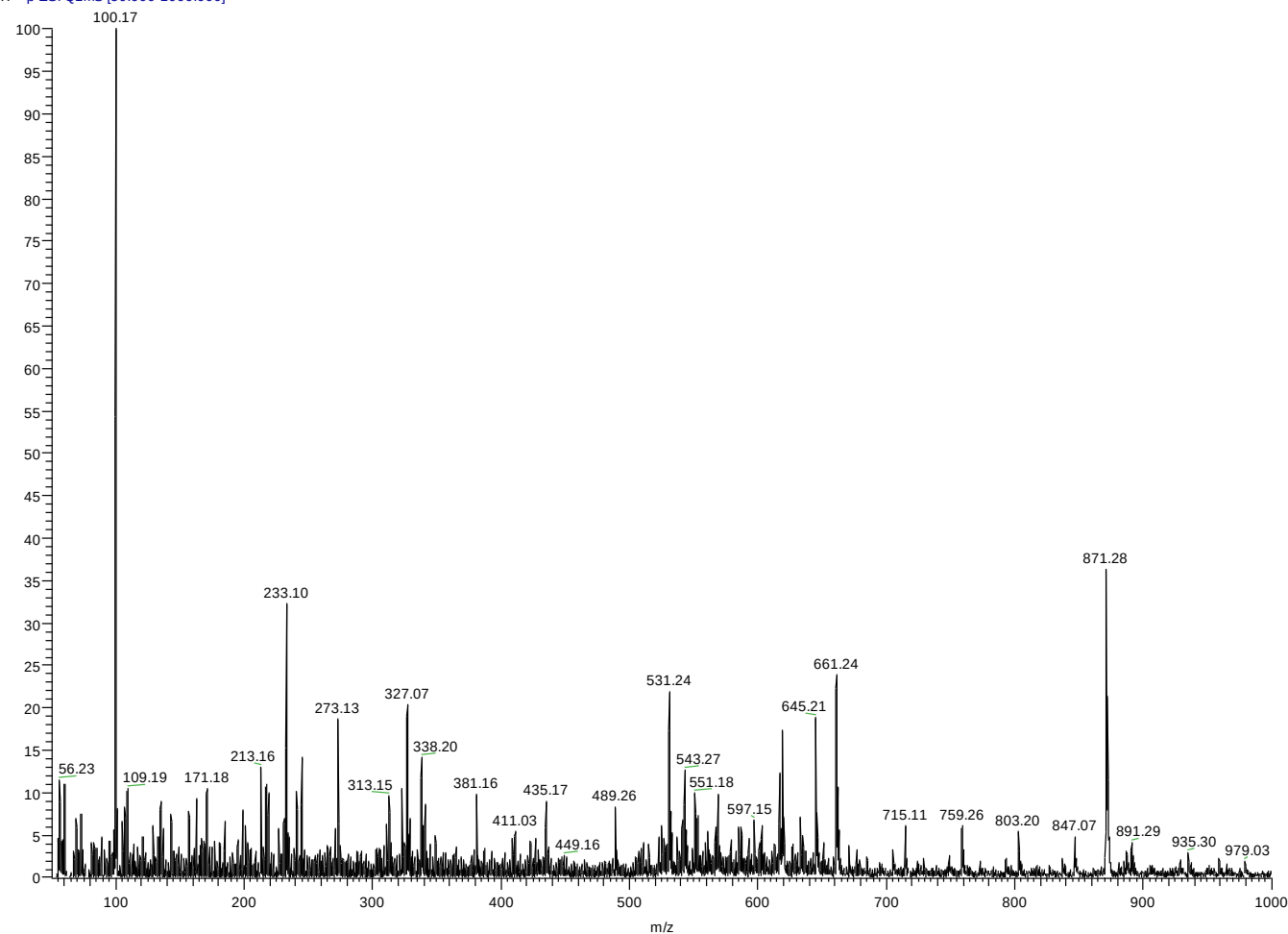


Figure D.2: MS analysis of African Pride leaves ethyl acetate extract

Paddy\_Basaam\_20220519\_APLEpos

05/21/22 11:55:00

Paddy\_Basaam\_20220519\_APLEpos #5-22 RT: 0.07-0.38 AV: 18 NL: 3.6

T: +p ESI Q1MS [50.000-1000.000]

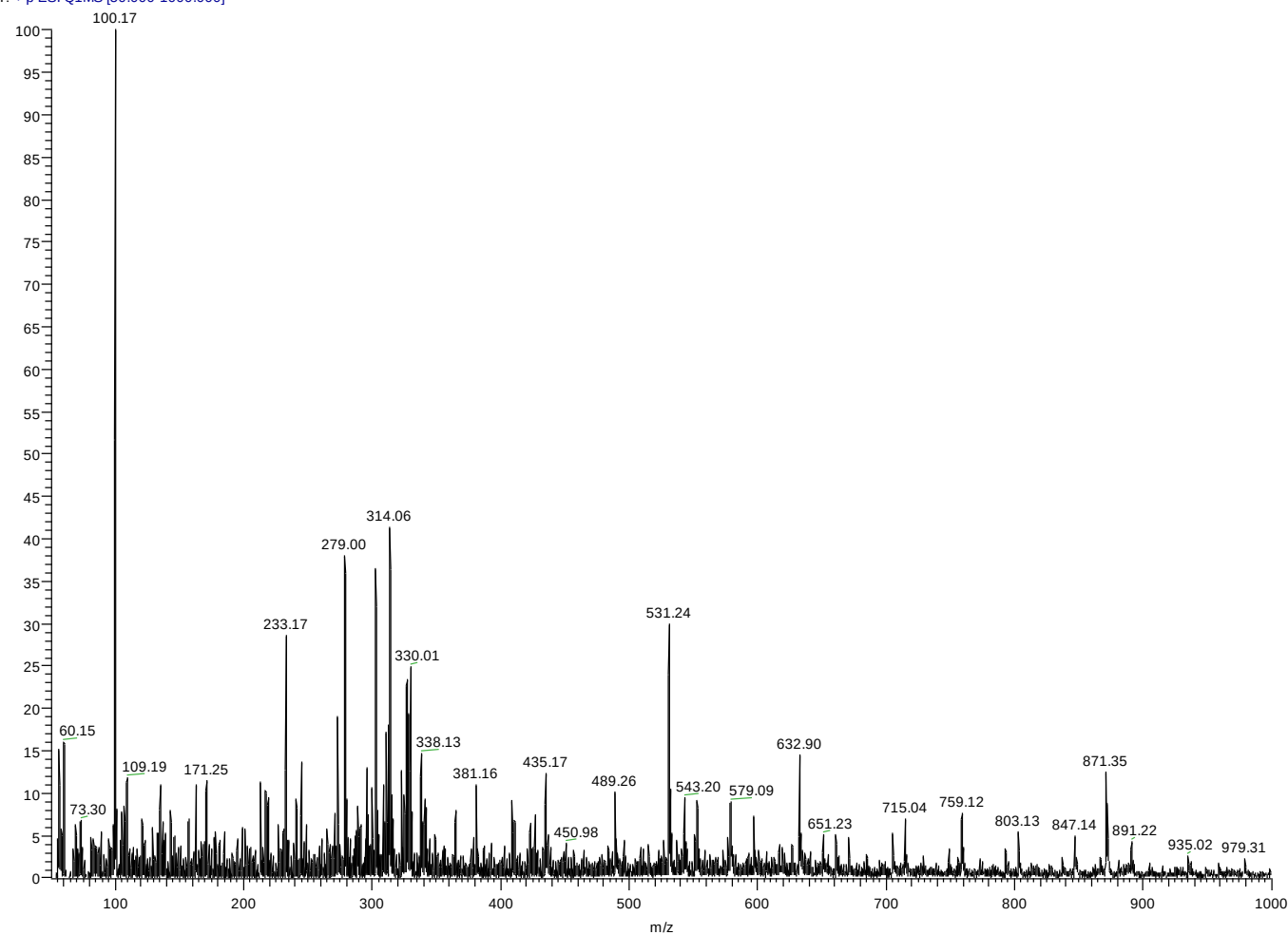


Figure D.3: MS analysis of African Pride leaves ethanolic extract

Paddy\_Bassam\_20220519\_APFHpos

05/21/22 11:00:15

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T: + p ESI Q1MS [50.000-1000.000]

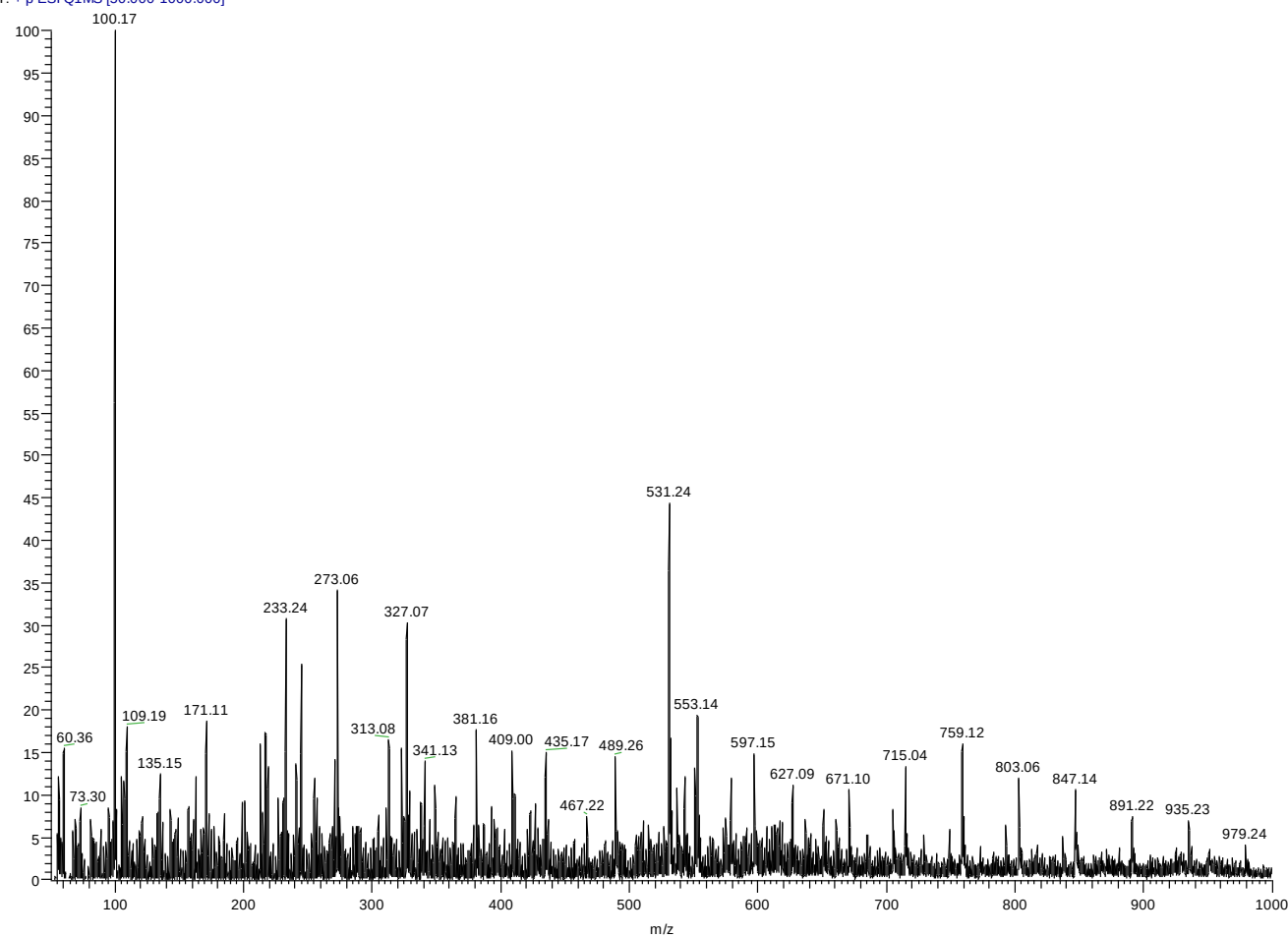


Figure D.4: MS analysis of African Pride fruit hexanolic extract

Paddy\_Bassam\_20220519\_APFYpos

05/21/22 12:00:10

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T: + p ESI Q1MS [50.000-1000.000]

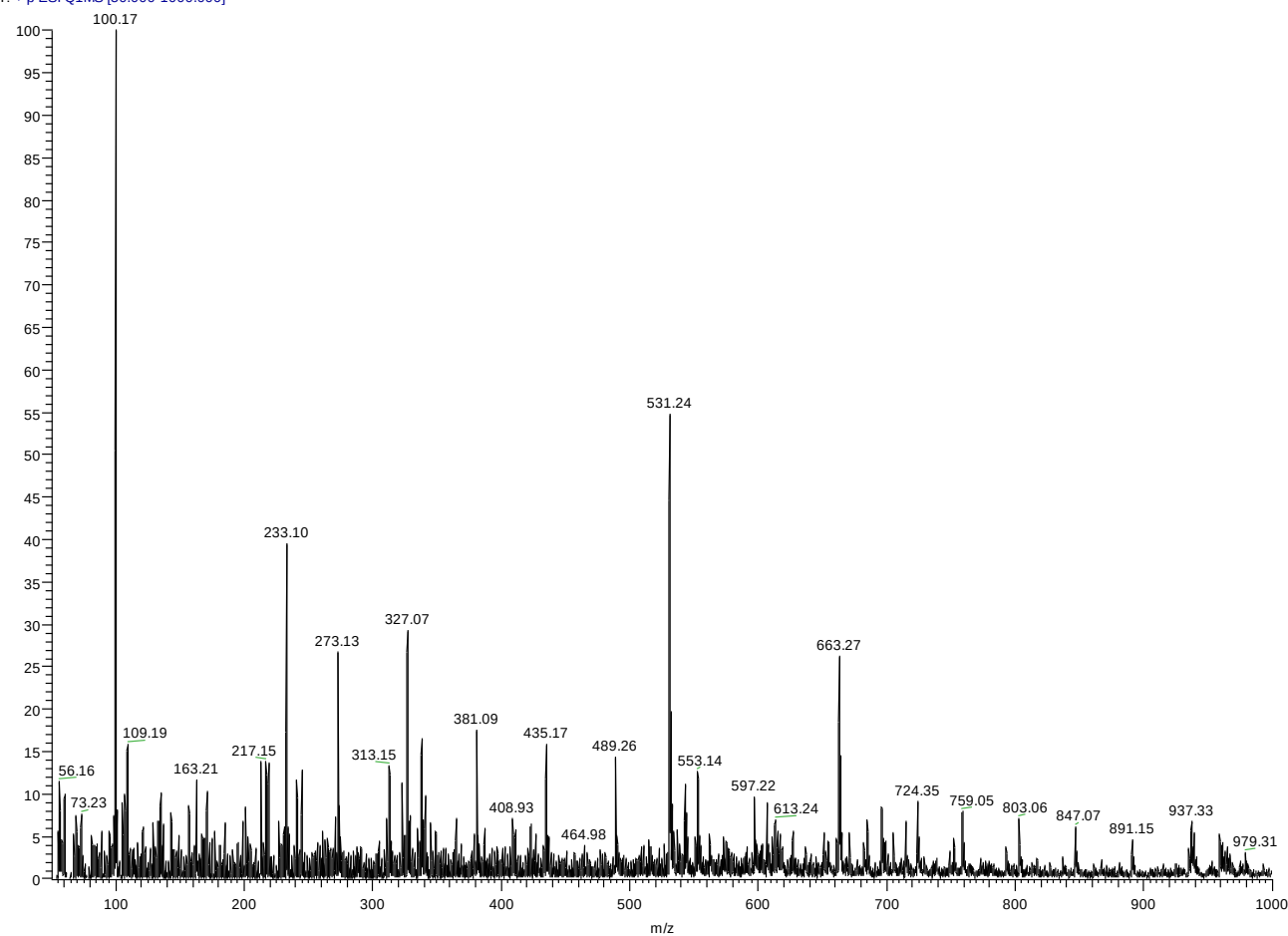


Figure D.5: MS analysis of African Pride fruit ethyl acetate extract

Paddy\_Bassam\_20220519\_APFEPpos

05/21/22 12:00:55

Paddy\_Bassam\_20220519\_APFEPpos #5-21 RT: 0.07-0.36 AV: 17 NL: 3.6  
T: + p ESI Q1MS [50.000-1000.000]

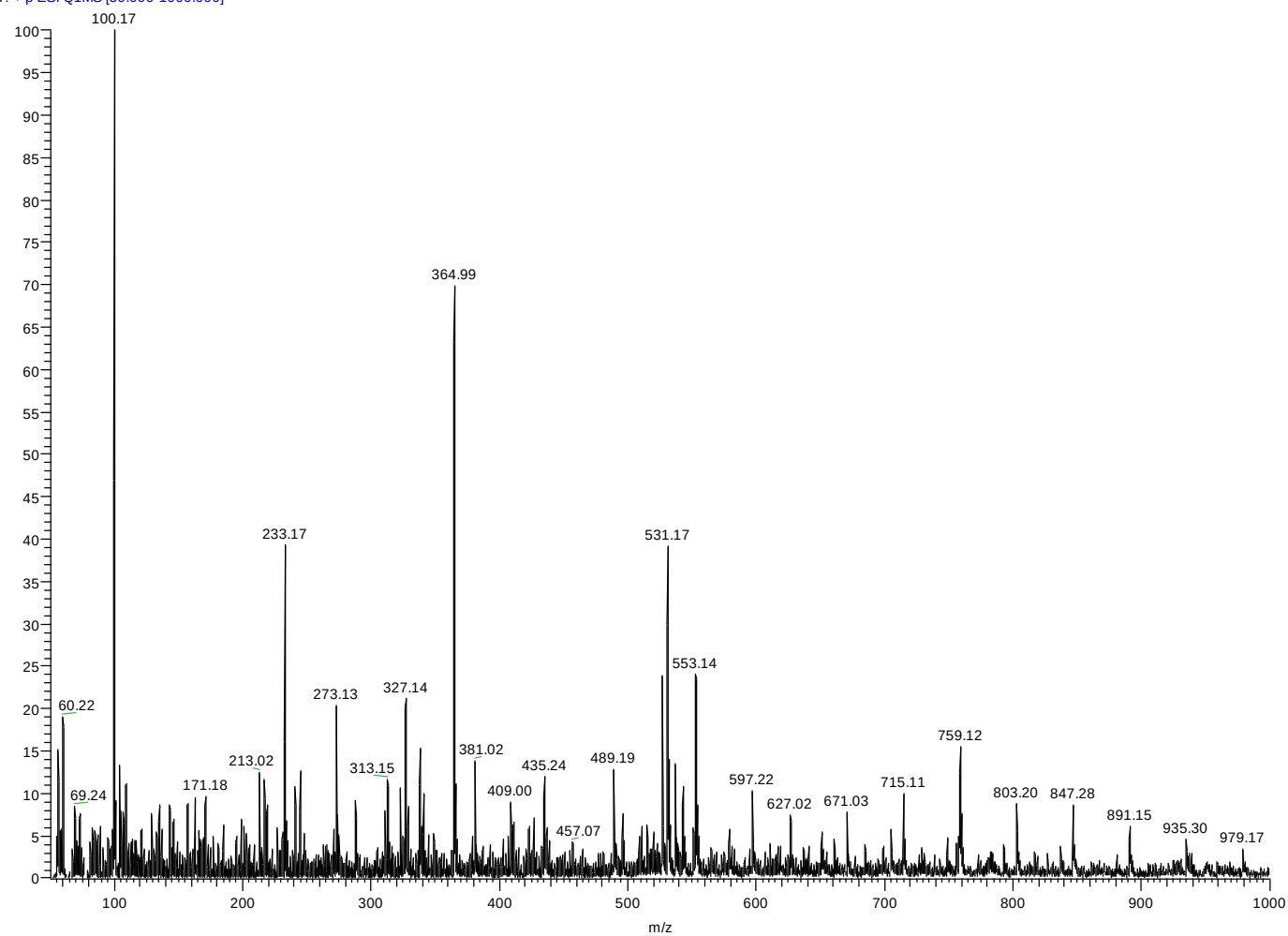


Figure D.6: MS analysis of African Pride fruit ethanolic extract

Paddy\_Bassam\_20220519\_APSHpos

05/21/22 12:00:00

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T: + p ESI Q1MS [50.000-1000.000]

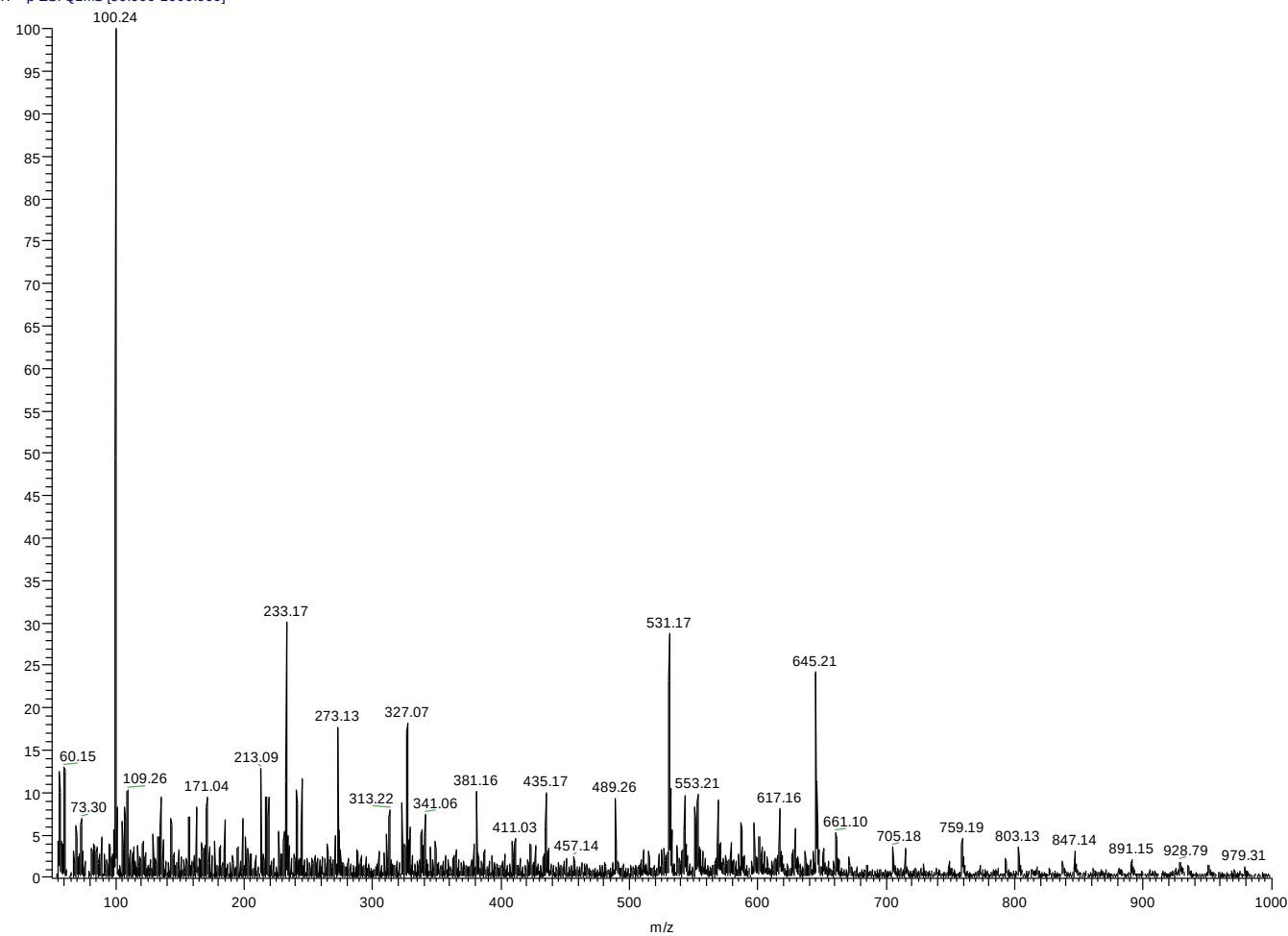


Figure D.7: MS analysis of African Pride seed hexanolic extract

Paddy\_Bassam\_20220519\_APSEYpos

05/21/22 12:00:00

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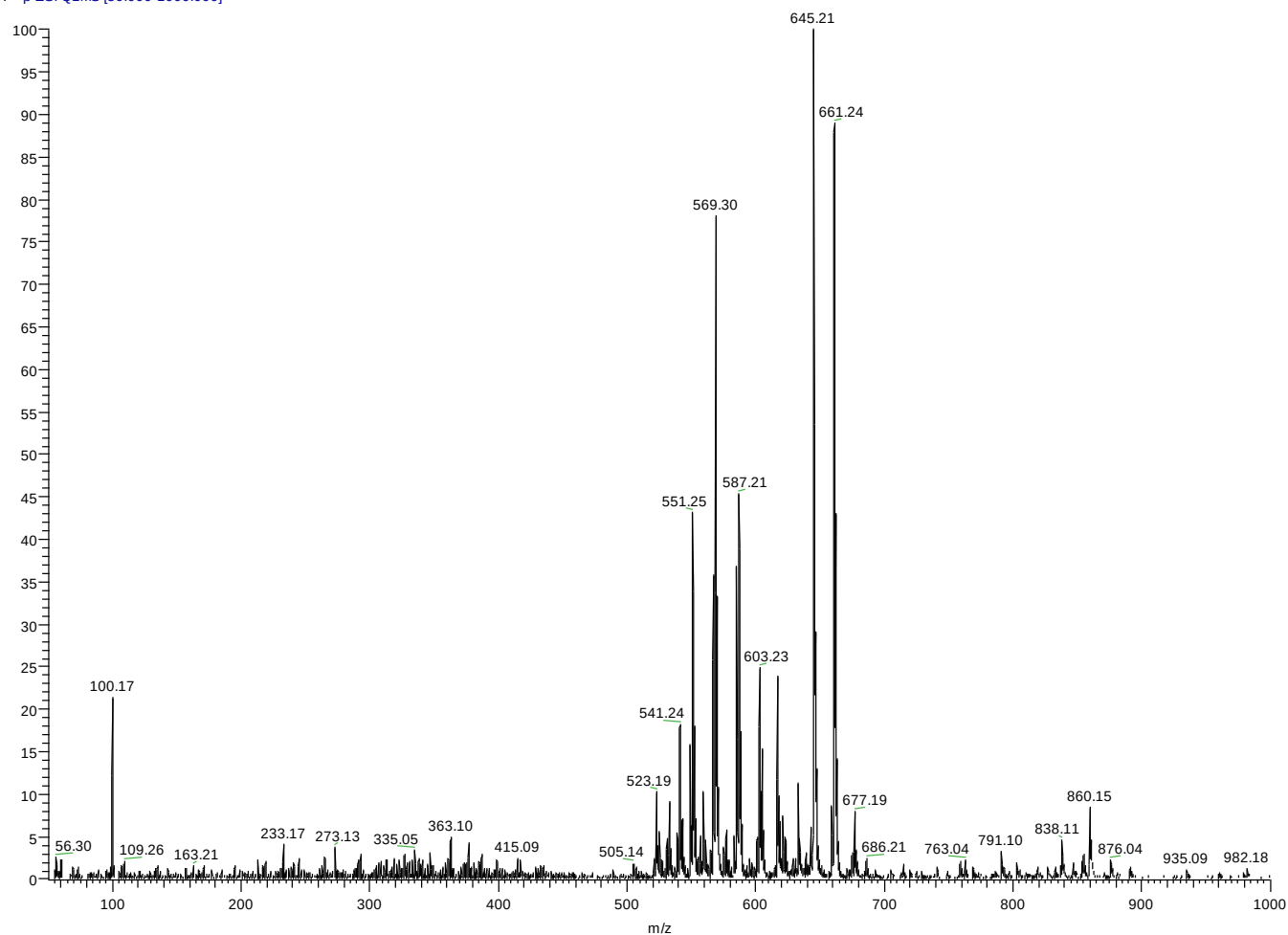


Figure D.8: MS analysis of African Pride seed ethyl acetate extract



Paddy\_Bassam\_20220519\_APSEpos

05/21/22 12:11:56

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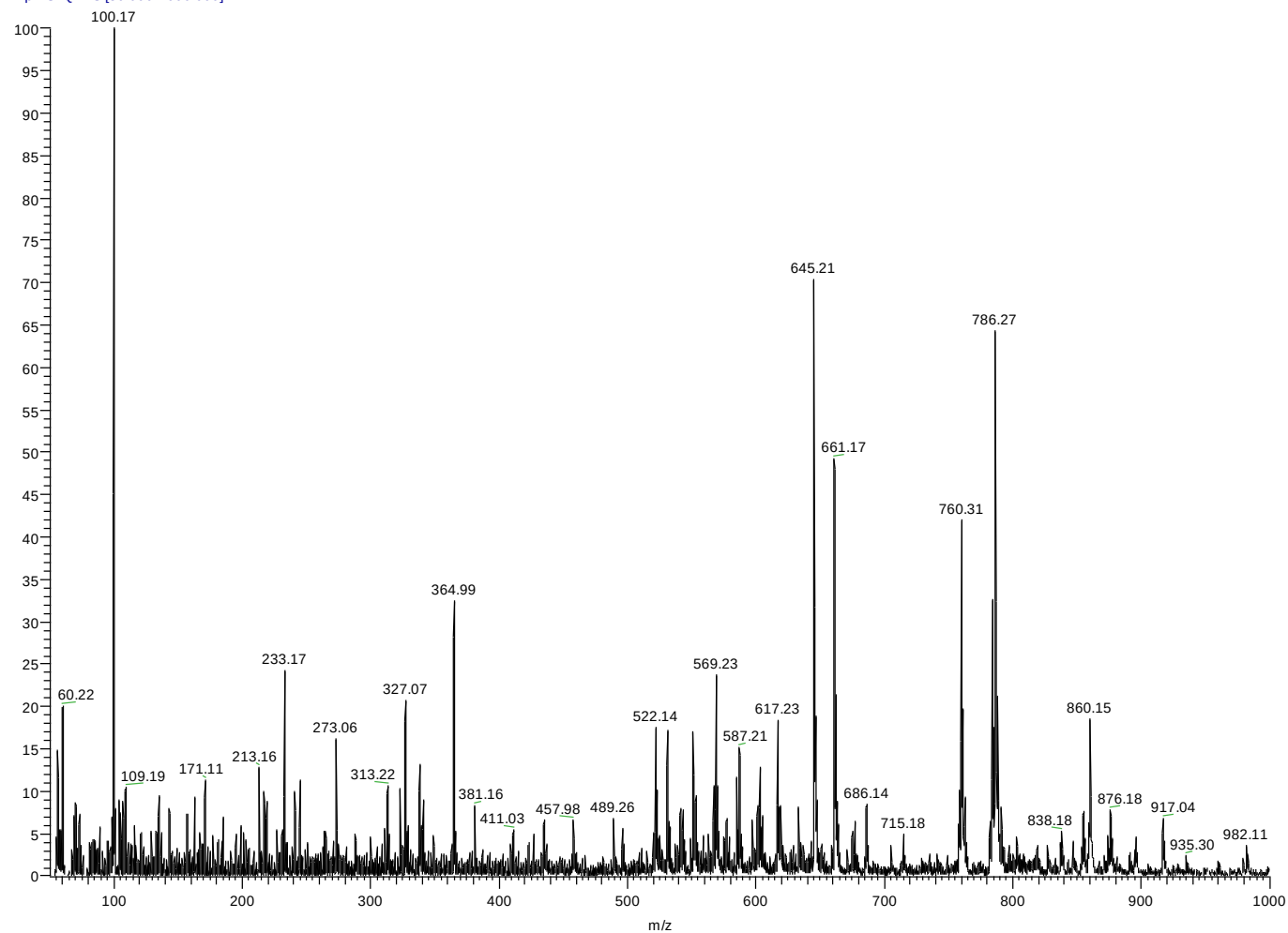


Figure D.9: MS analysis of African Pride seed ethanolic extract

Wislon\_Bassam\_20221021\_KJLH

10/21/22 13:50:00

Wislon\_Bassam\_20221021\_KJLH #1 RT: 0.00 AV: 1 NL: 9.03E6

T: +p ESI Q1MS [50.000-1000.000]

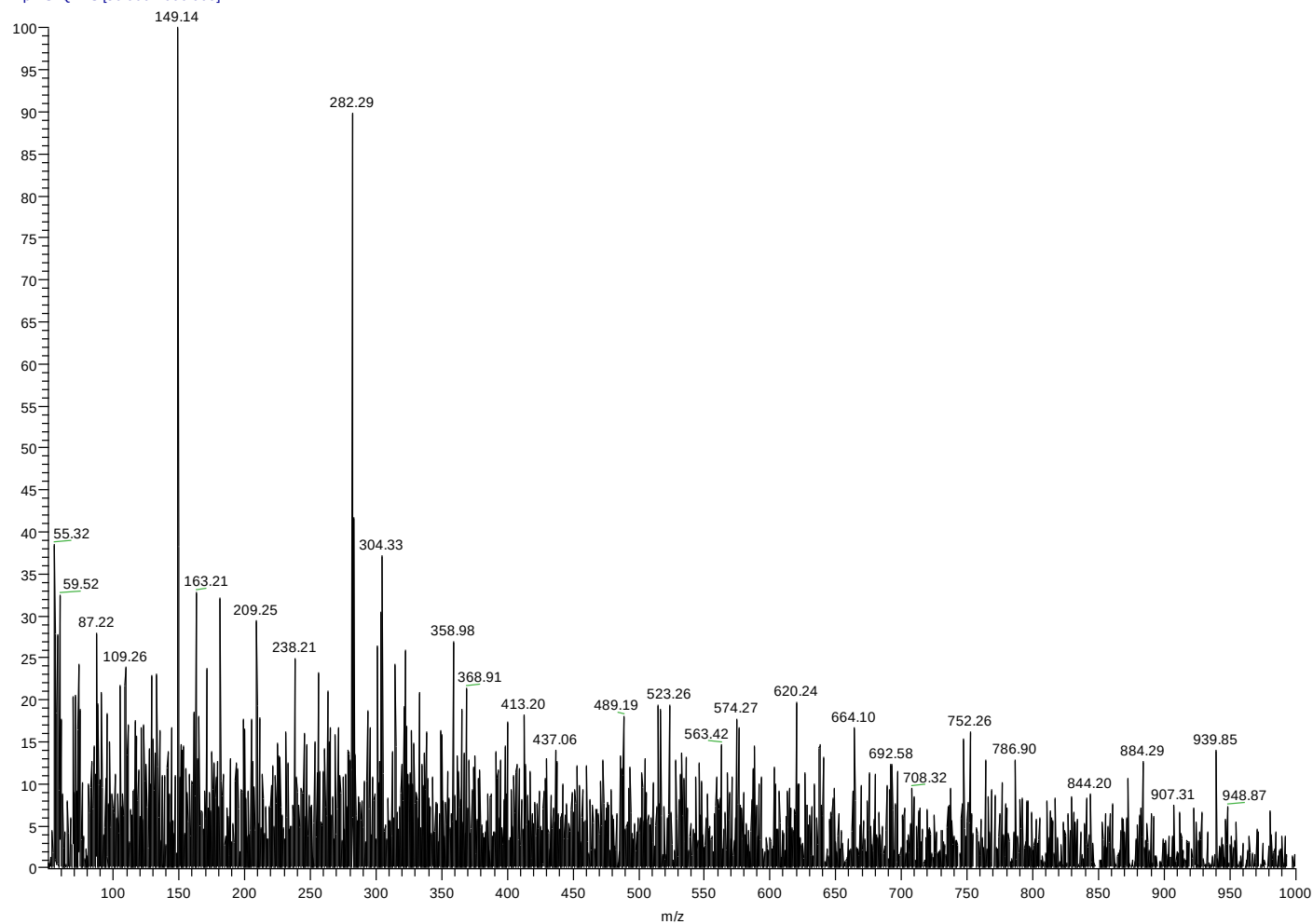


Figure D.10: MS analysis of KJ leaves hexanolic extract

Wislon\_Bassam\_20221021\_KJLEY

10/21/22 14:10:00

Wislon\_Bassam\_20221021\_KJLEY #1 RT: 0.00 AV: 1 NL: 9.47E6  
T: +p ESI Q1MS [50.000-1000.000]

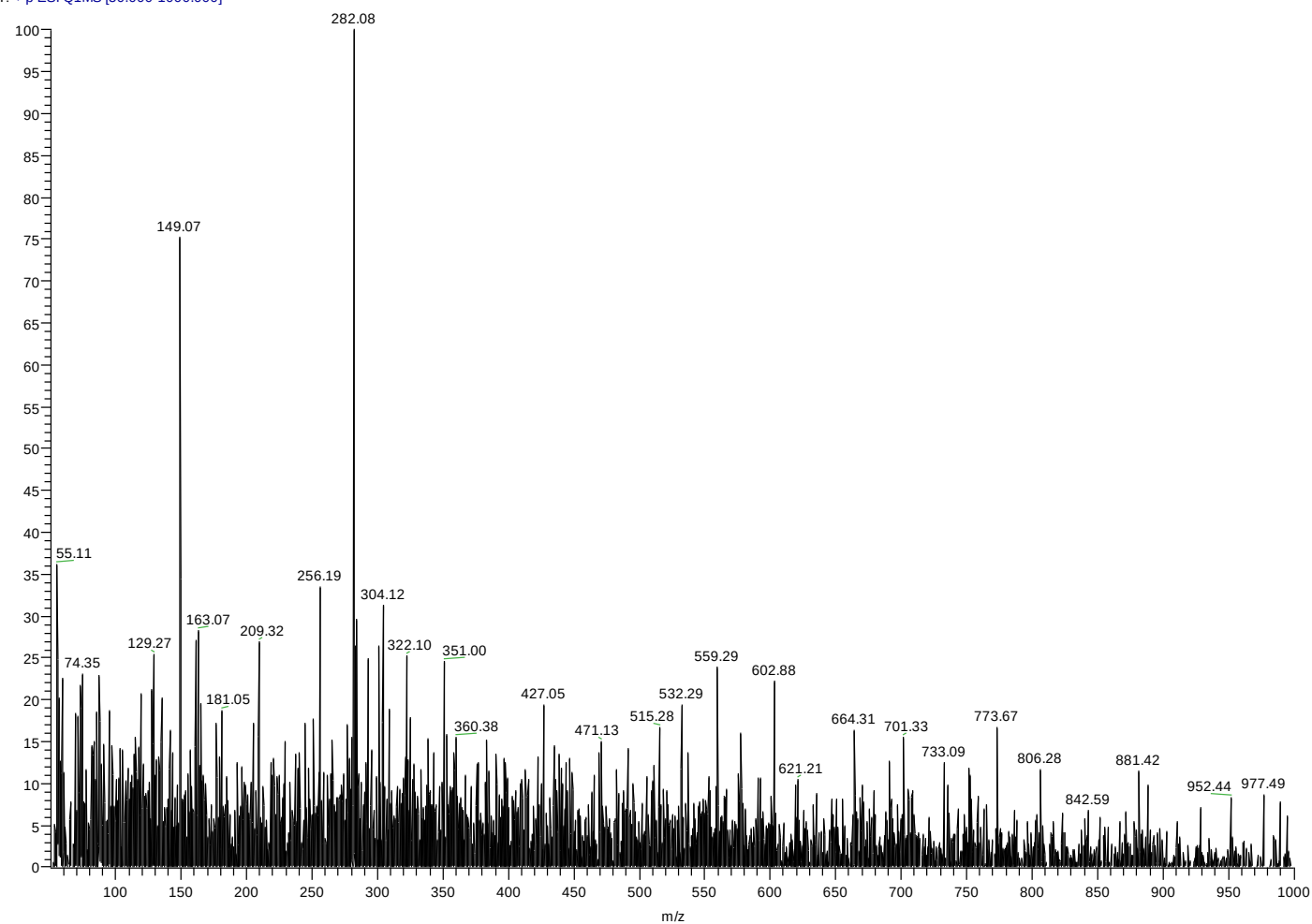


Figure D.11: MS analysis of KJ leaves ethyl acetate extract

Wislon\_Bassam\_20221021\_KJLE

10/21/22 14:11:00

Wislon\_Bassam\_20221021\_KJLE #1 RT: 0.00 AV: 1 NL: 1.07E7  
T: +p ESI Q1MS [50.000-1000.000]

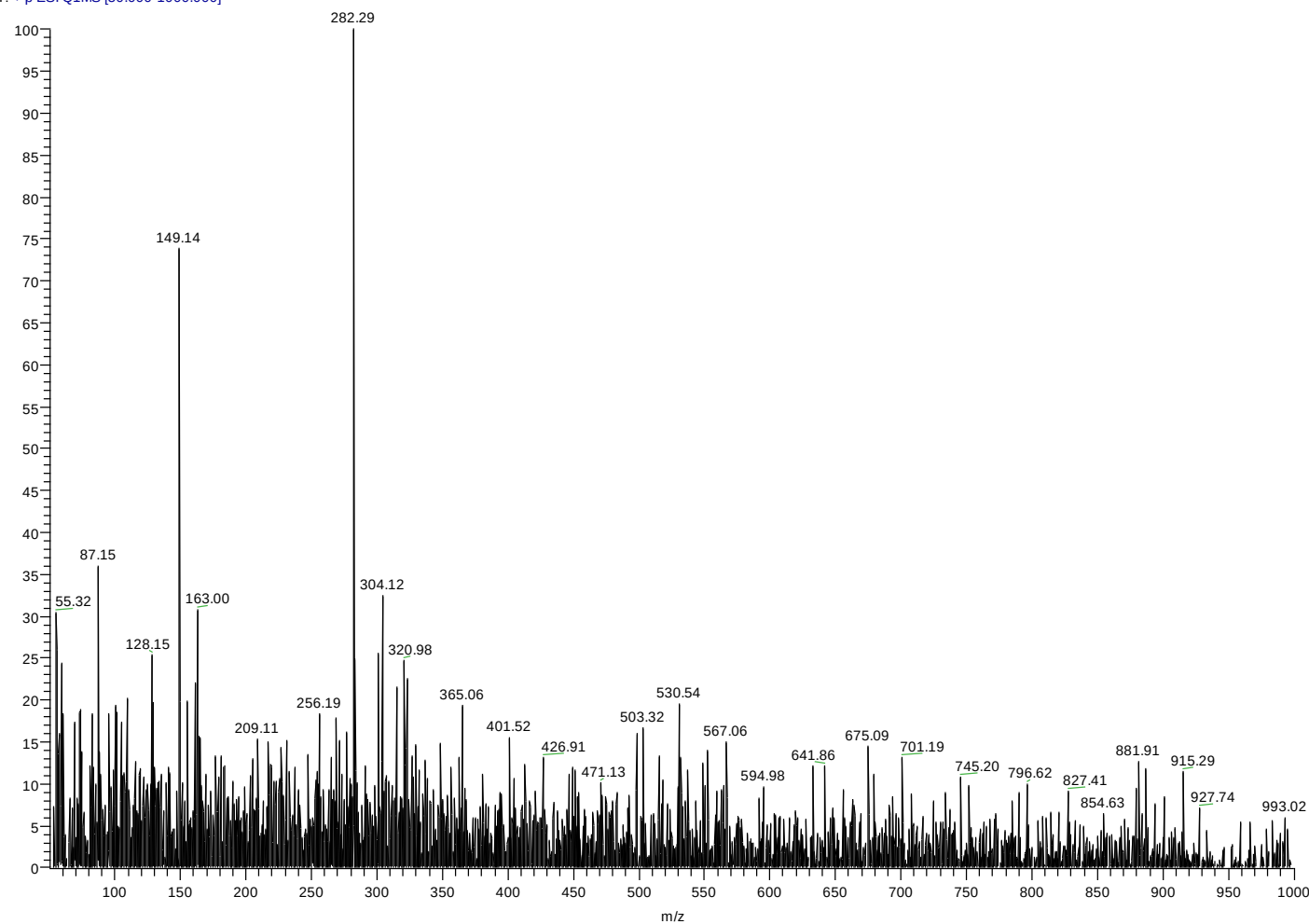


Figure D.12: MS analysis of KJ leaves ethanolic extract

Wislon\_Bassam\_20221021\_KJFH

10/21/22 14:11:11

Wislon\_Bassam\_20221021\_KJFH #1 RT: 0.00 AV: 1 NL: 1.04E7  
T: + p ESI Q1MS [50.000-1000.000]

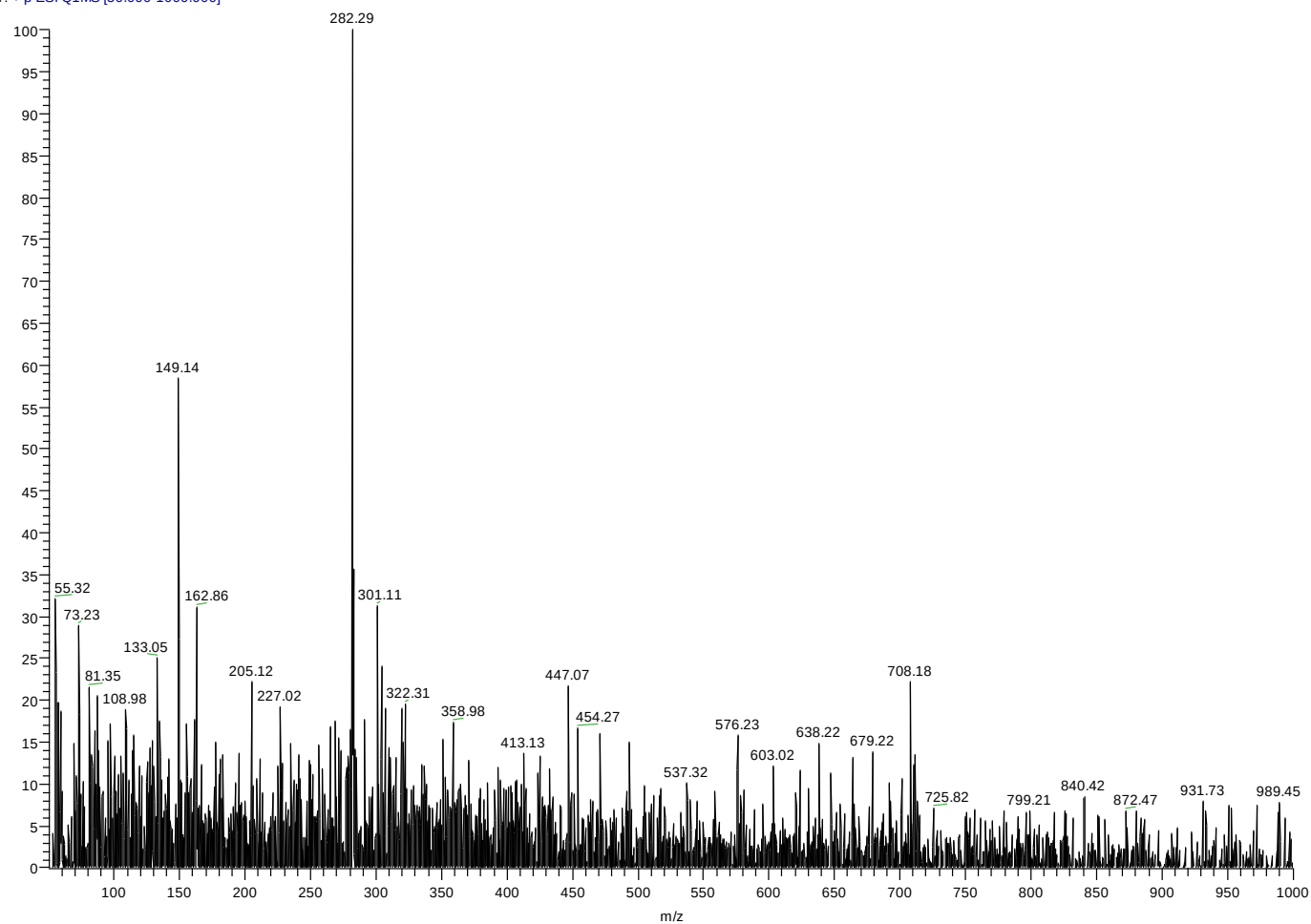


Figure D.13: MS analysis of KJ fruit hexanolic extract

Wislon\_Bassam\_20221021\_KJFEY

10/21/22 14:22:00

Wislon\_Bassam\_20221021\_KJFEY #1 RT: 0.00 AV: 1 NL: 9.06E6

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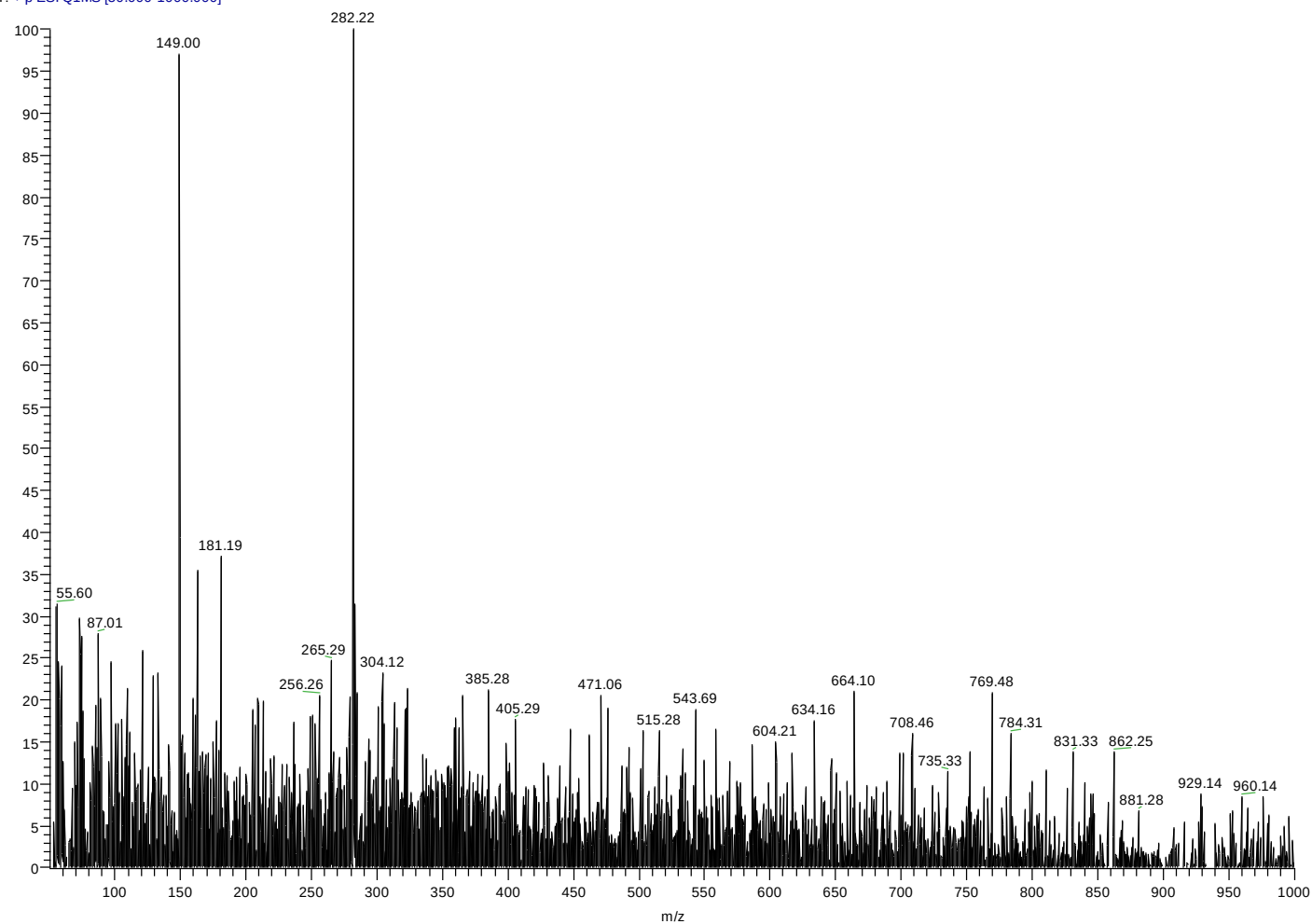


Figure D.14: MS analysis of KJ fruit ethyl acetate extract

Wislon\_Bassam\_20221021\_KJFE

10/21/22 14:25:10

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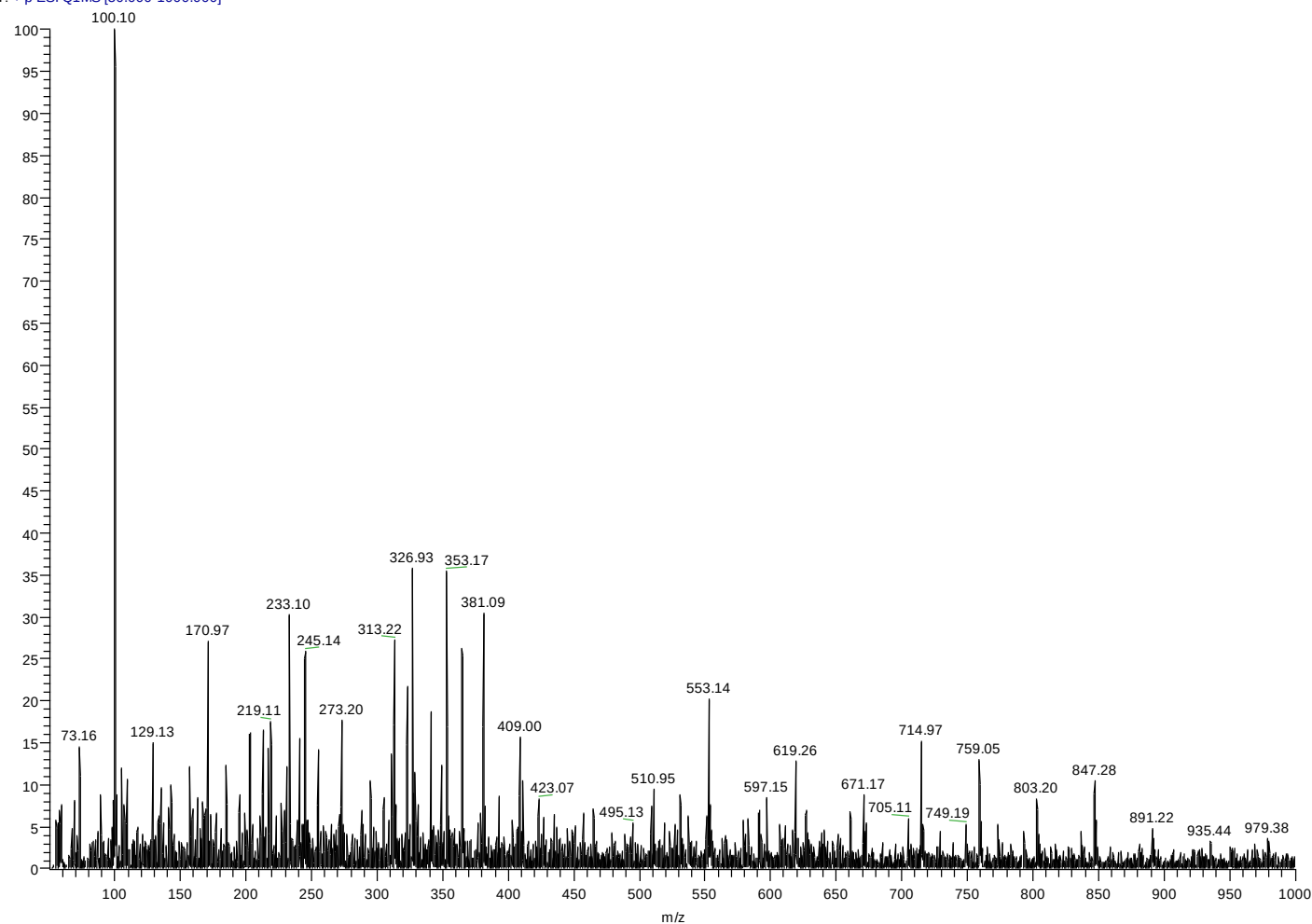


Figure D.15: MS analysis of KJ fruit ethanolic extract

Wislon\_Bassam\_20221021\_KJSH

10/21/22 14:00:50

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T: + p ESI Q1MS [50.000-1000.000]

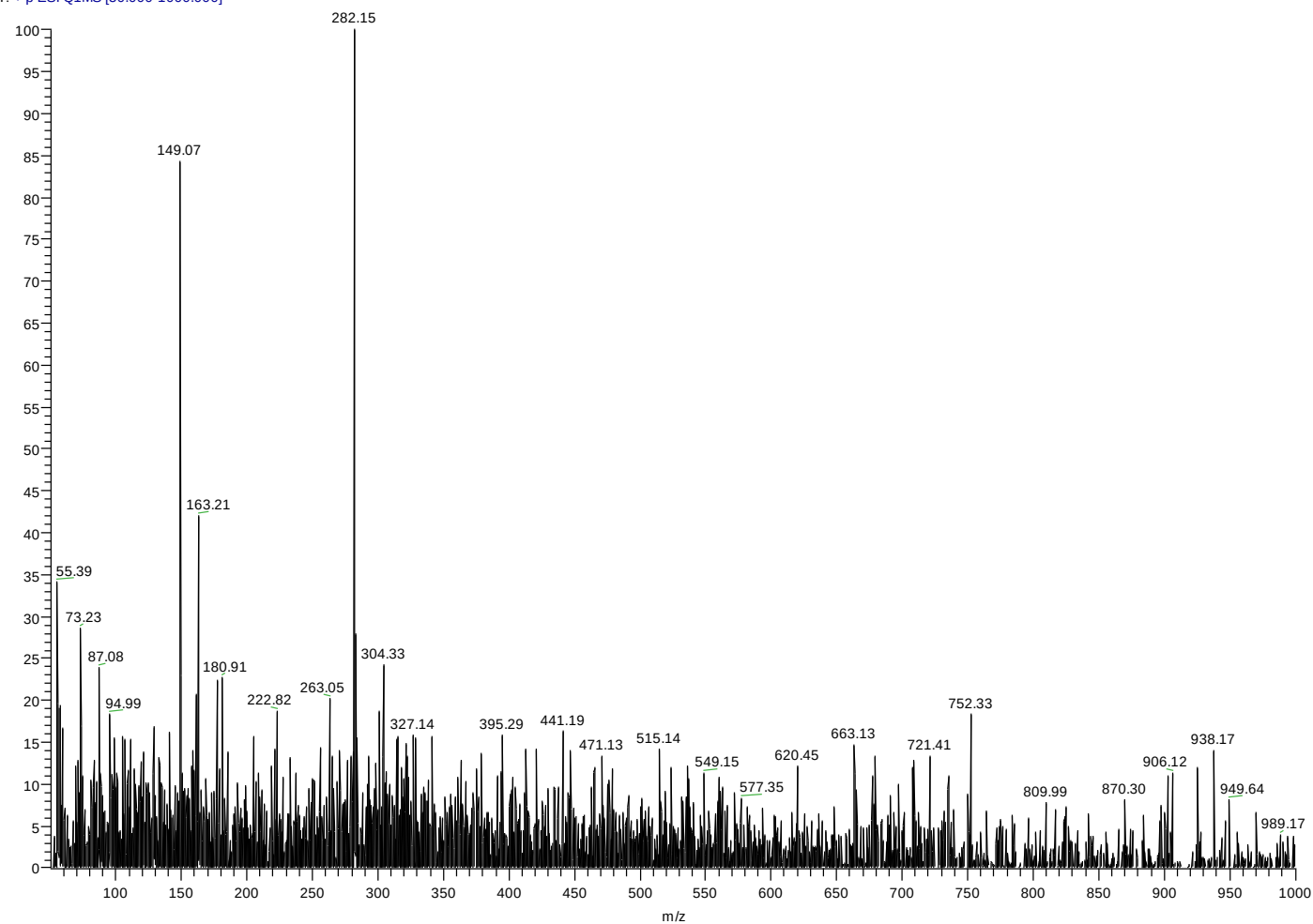


Figure D.16: MS analysis of KJ seed hexanolic extract



Wislon\_Bassam\_20221021\_KJSEY

10/21/22 14:00:00

Wislon\_Bassam\_20221021\_KJSEY #61 RT: 1.07 AV: 1 NL: 9.83E6  
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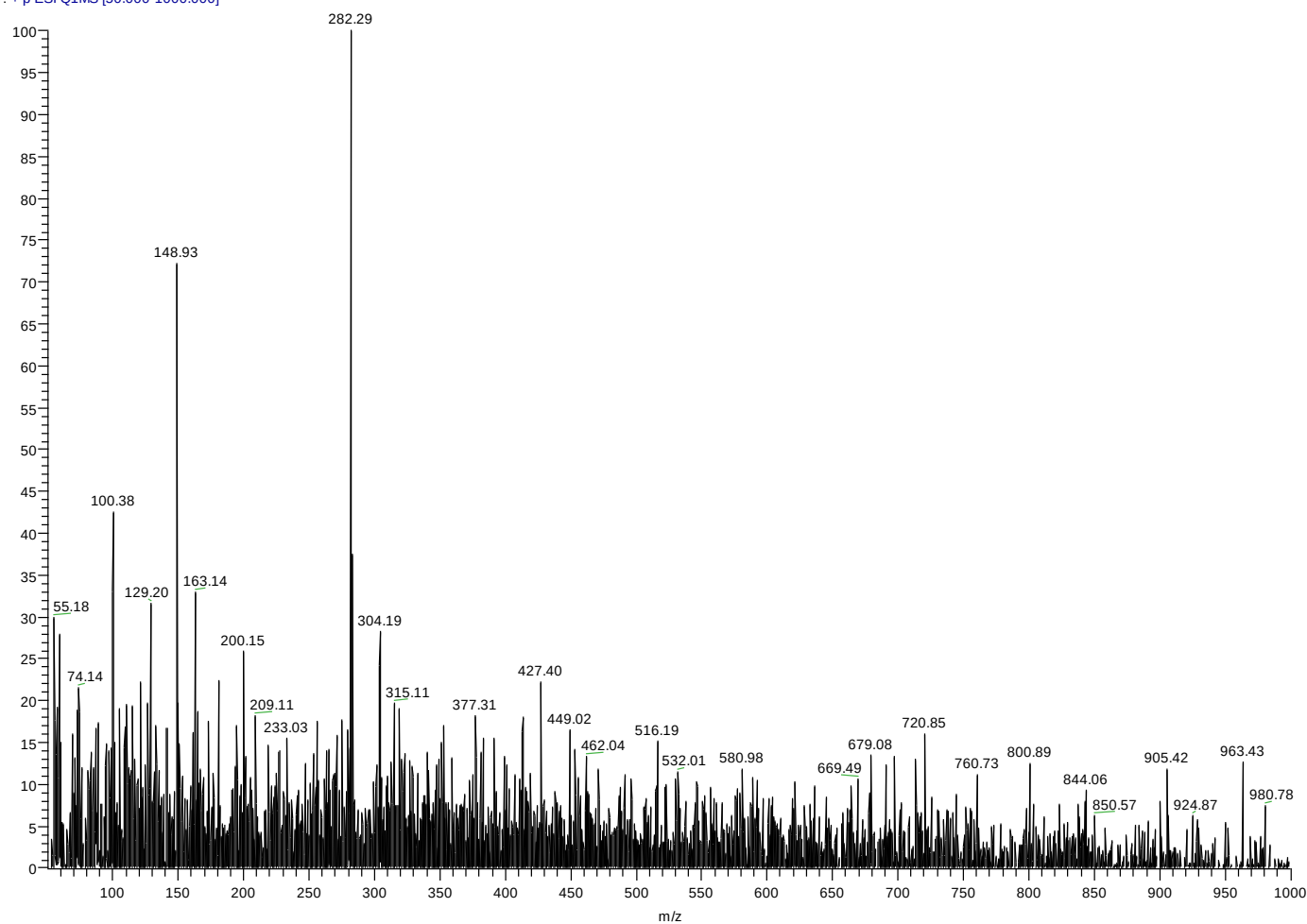


Figure D.17: MS analysis of KJ seed ethyl acetate extract

Wilson\_Bassam\_20221021\_KJSE

10/21/22 14:00:00

Wilson\_Bassam\_20221021\_KJSE #1 RT: 0.00 AV: 1 NL: 8.37E6

T: + p ESI Q1MS [50.000-1000.000]

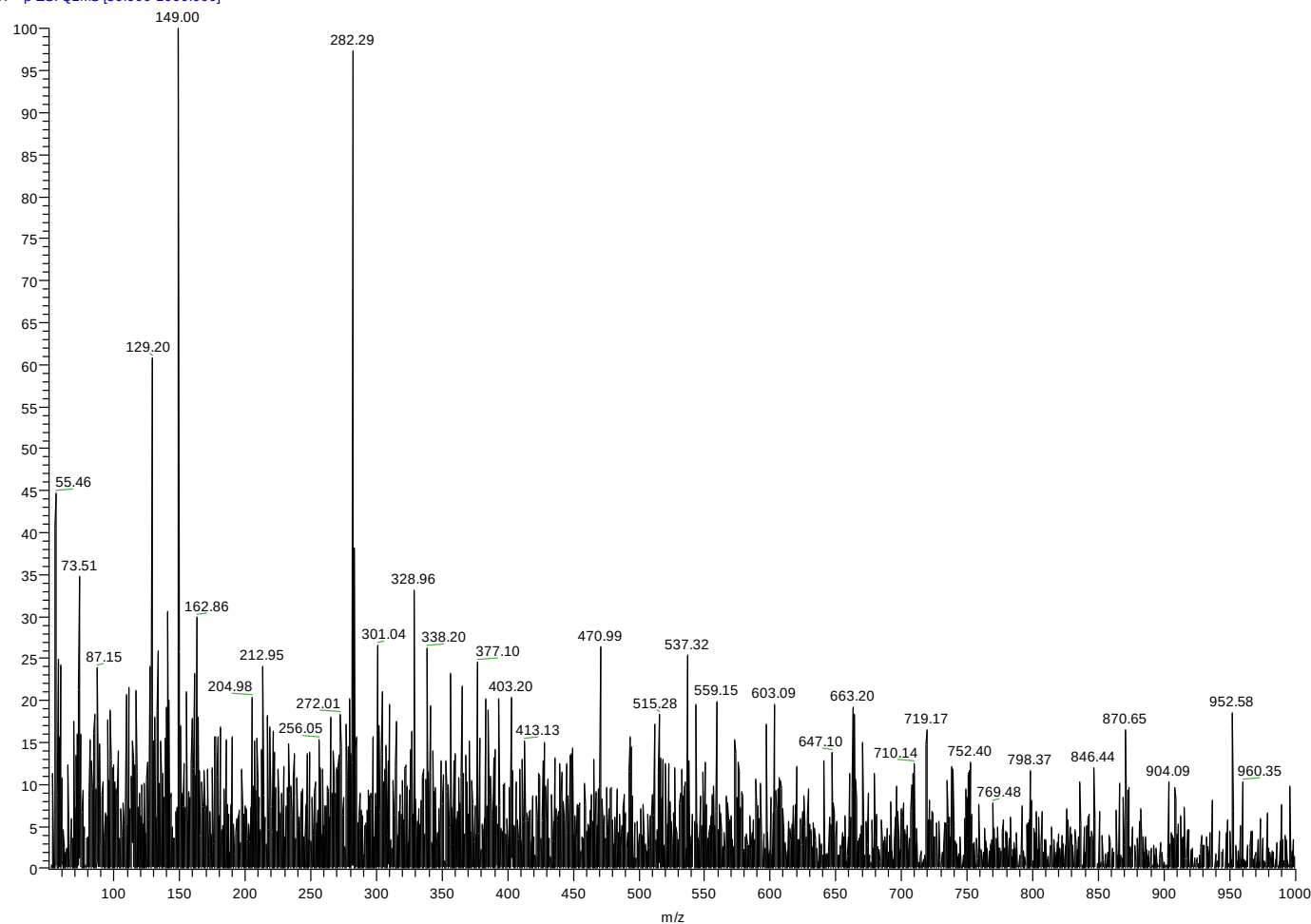
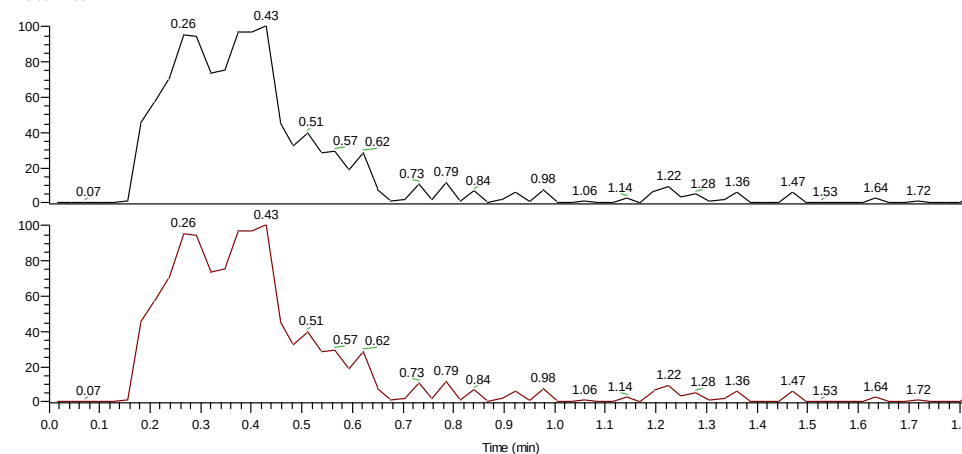


Figure D.18: MS analysis of KJ seed ethanolic extract

Paddy\_Bassam\_SRM\_Apey\_537\_70ev\_20220803

08/03/22 10:22:06

RT: 0.00 - 1.83



NL: 2.54E4

TIC MS Paddy\_Bassam\_SRM\_Apey\_537\_70ev\_20220803

NL: 2.54E4

TIC F: + c ESI SRM ms2 537.000 [270.500-271.500, 284.500-285.500, 300.500-301.500, 312.500-313.500, 324.500-325.500, 338.500-339.500, 354.500-355.500, 366.500-367.500] MS  
Paddy\_Bassam\_SRM\_Apey\_537\_70ev\_20220803

Paddy\_Bassam\_SRM\_Apey\_537\_70ev\_20220803 #5-18 RT: 0.13-0.48 AV: 14 NL: 8.11E3

T: + c ESI SRM ms2 537.000 [270.500-271.500, 284.500-285.500, 300.500-301.500, 312.500-313.500, 324.500-325.500, 338.500-339.500, 354.500-355.500, 366.500-367.500]

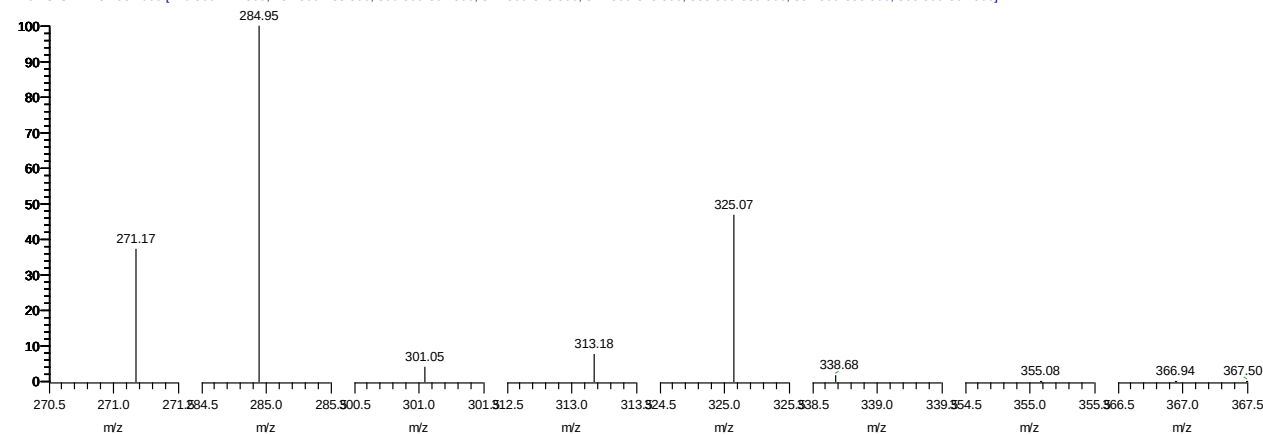
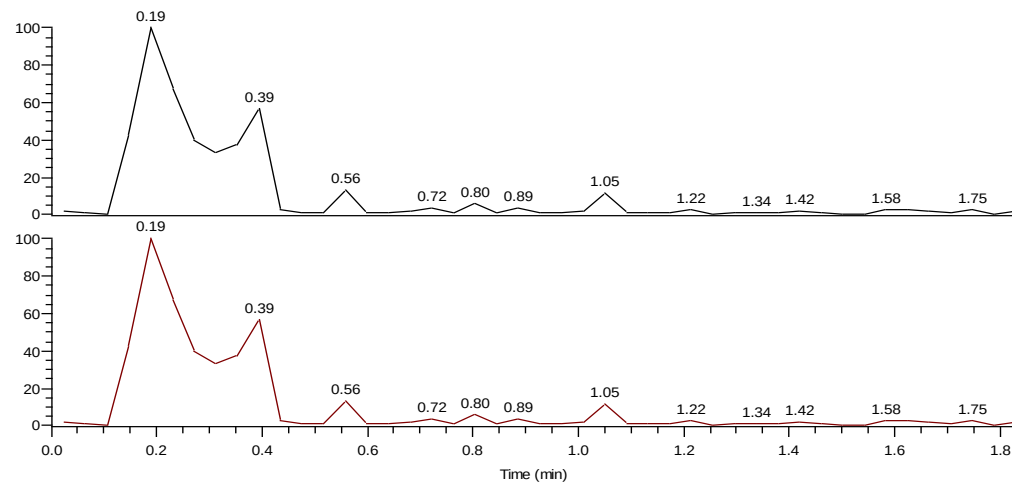


Figure D.19: MSMS fragments for Epoxymurin-A

Paddy\_Bassam\_SRM\_Apey\_553\_70ev\_20220803

08/03/22 10:32:15

RT: 0.00 - 1.83



NL: 8.93E3

TIC: M S Paddy\_Bassam\_SRM\_Apey\_553\_70ev\_20220803

NL: 8.93E3

TIC F: +c ESI SRM ms2 553.000 [272.500-273.500, 274.500-275.500, 284.500-285.500, 286.500-287.500, 300.500-301.500, 302.500-303.500, 312.500-313.500, 314.500-315.500, 342.500-343.500, 354.500-355.500, 370.500-371.500, 382.500-383.500] M S  
Paddy\_Bassam\_SRM\_Apey\_553\_70ev\_20220803

Paddy\_Bassam\_SRM\_Apey\_553\_70ev\_20220803 #2-12 RT: 0.07-0.48 AV: 11 NL: 7.17E2

T: +c ESI SRM ms2 553.000 [272.500-273.500, 274.500-275.500, 284.500-285.500, 286.500-287.500, 300.500-301.500, 302.500-303.500, 312.500-313.500, 314.500-315.500, 342.500-343.500, 354.500-355.500, 370.500-371.500, 382.500-383.500]

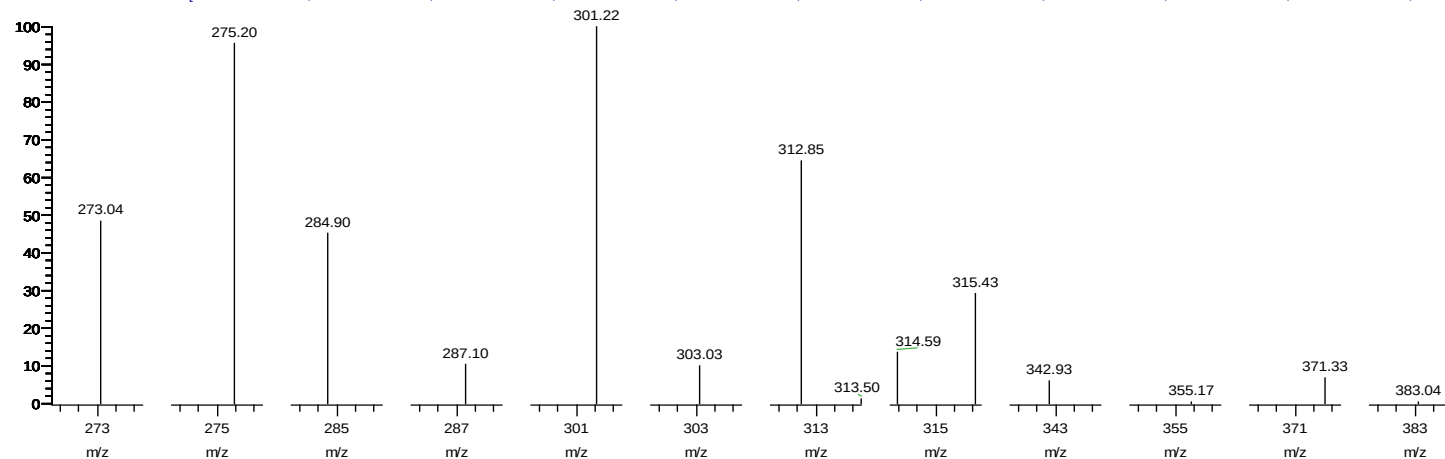


Figure D.20: MSMS fragments for Diepomuricanin A and B

Paddy\_Bassam\_SRM\_Apey\_571\_70ev\_20220803

08/03/22 10:39:38

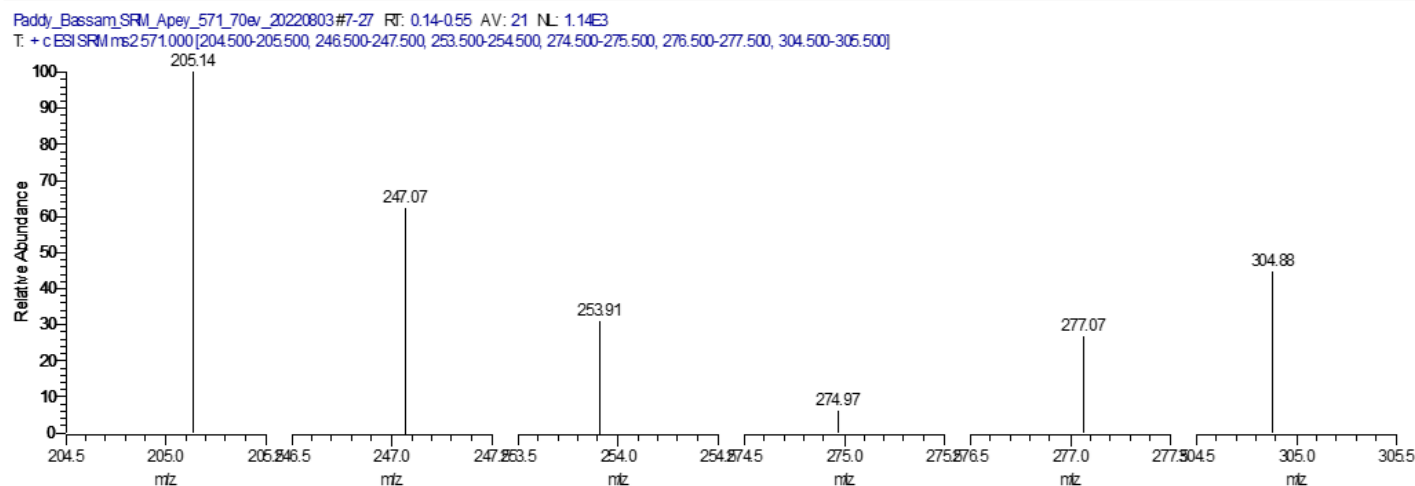
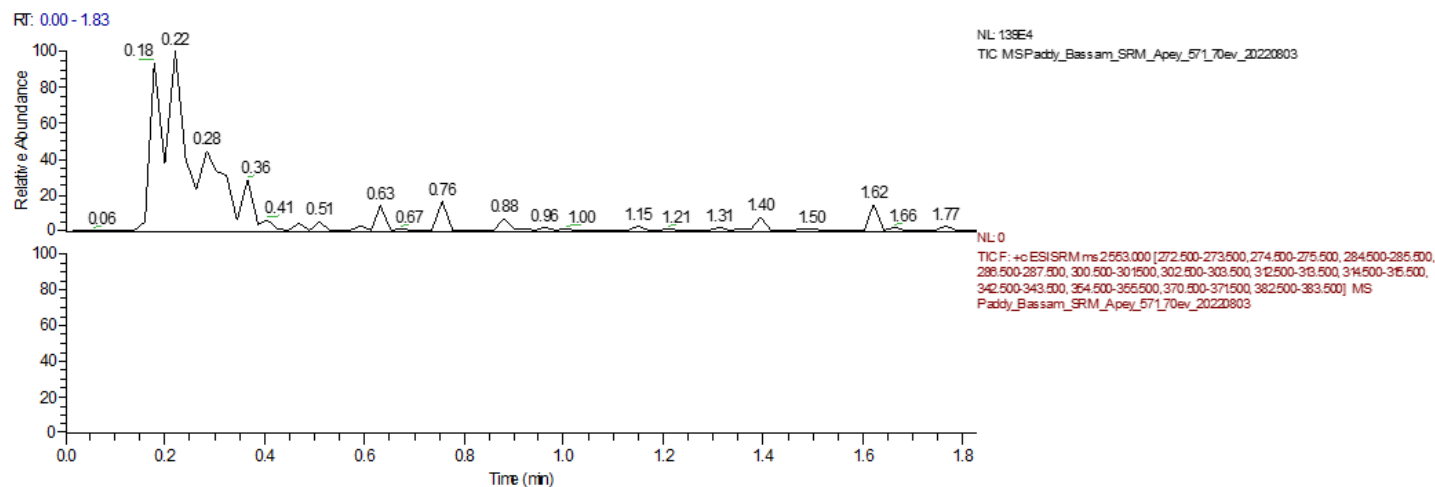


Figure D.21: MSMS fragments for Annotemoyin-1 & 2

Paddy\_Bassam\_SRM\_Apey\_603\_70ev\_20220803

08/03/22 10:54:20

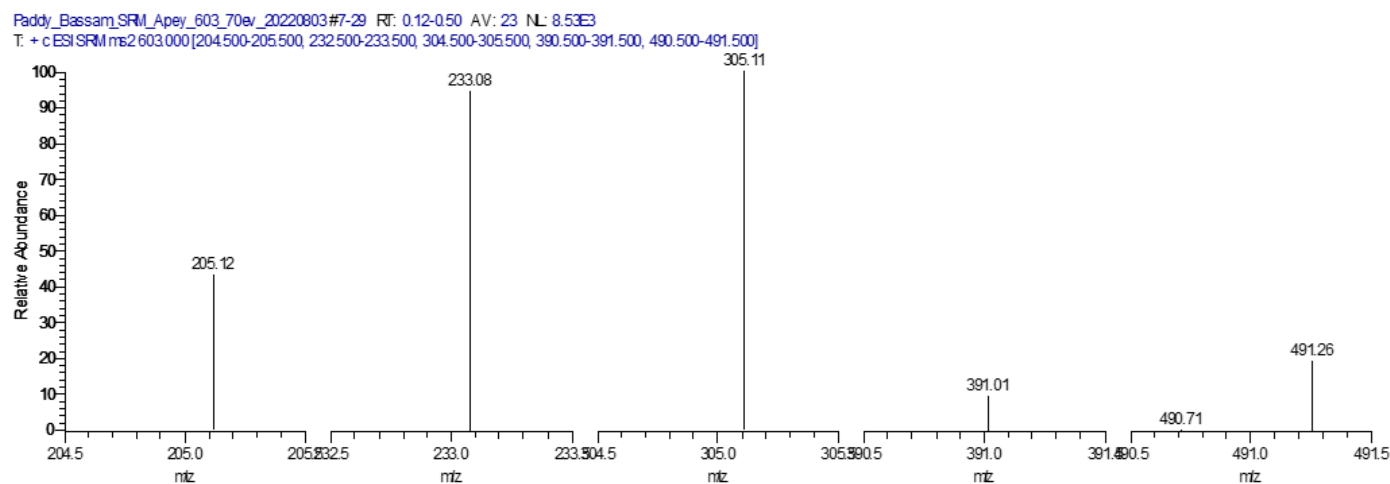
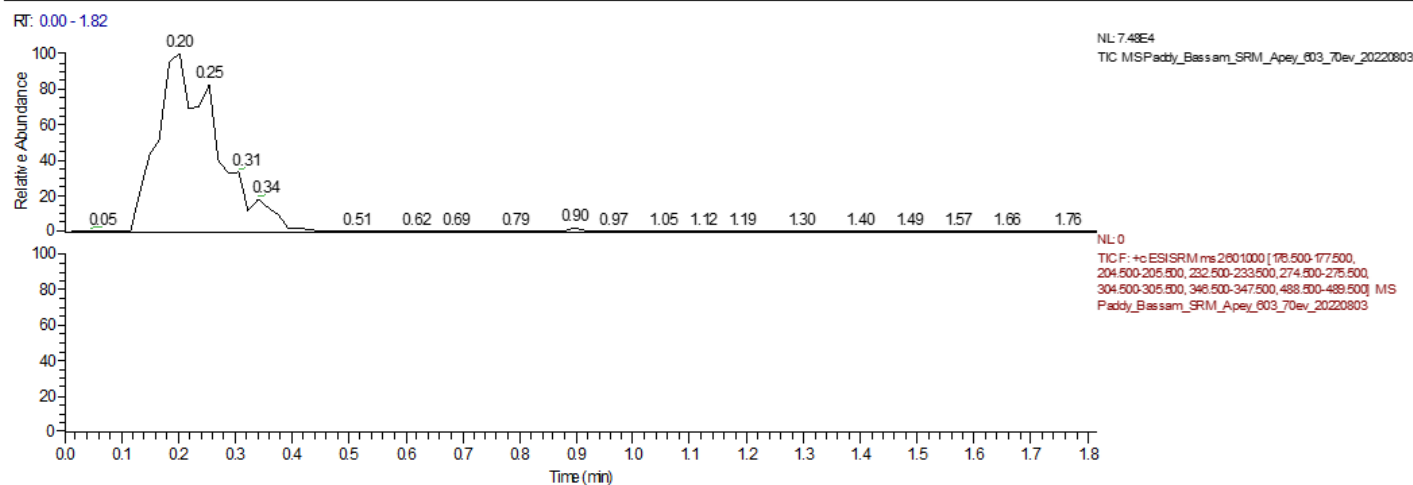


Figure D.22: MSMS fragments for Annonacin

Paddy\_Bassam\_SRM\_Apey\_617\_70ev\_20220803

08/03/22 11:01:50

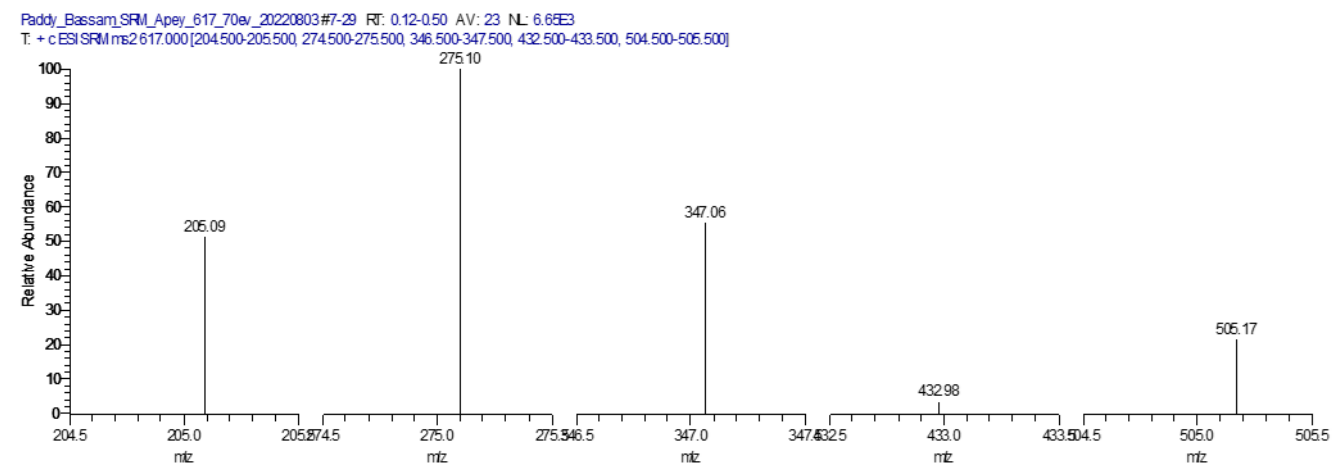
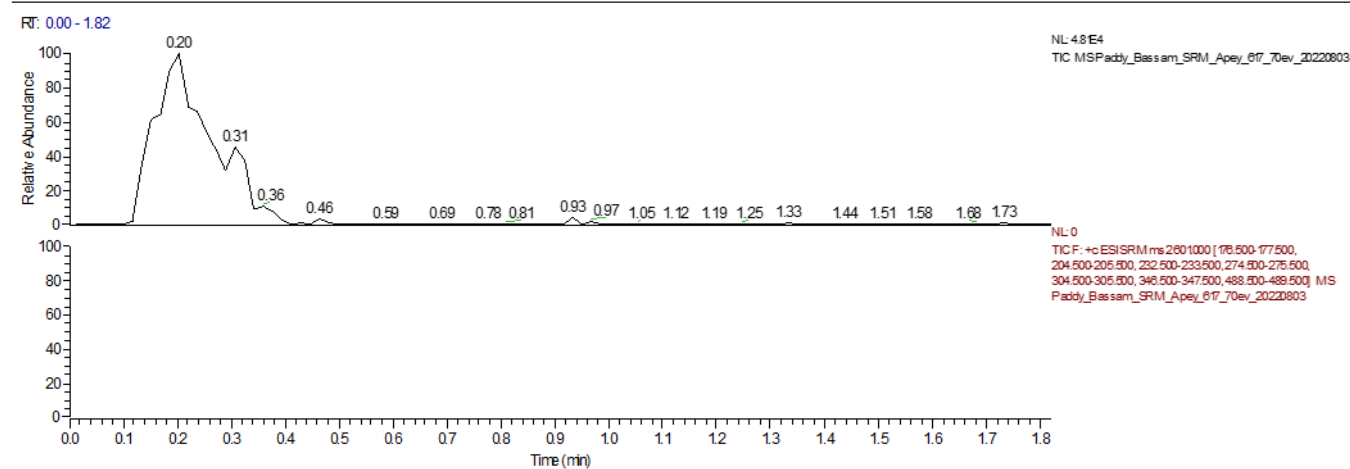
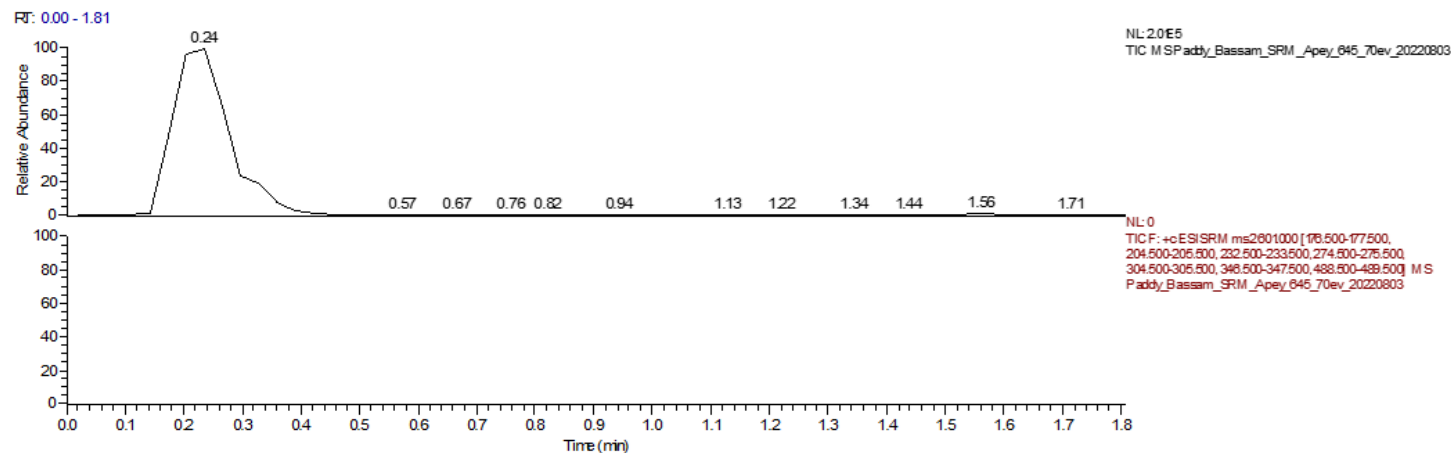


Figure D.23: MSMS fragments for Annonisin

Paddy\_Bassam\_SRM\_Apey\_645\_70ev\_20220803

08/03/22 11:09:13



Paddy\_Bassam\_SRM\_Apey\_645\_70ev\_20220803#7-29 RT: 0.20-0.88 AV: 23 NL: 6.63E3  
T: +cESI SRMms2.645.000[148.500-149.500, 204.500-205.500, 206.500-207.500, 264.500-265.500, 276.500-277.500, 292.500-293.500, 334.500-335.500, 362.500-363.500, 532.500-533.500]

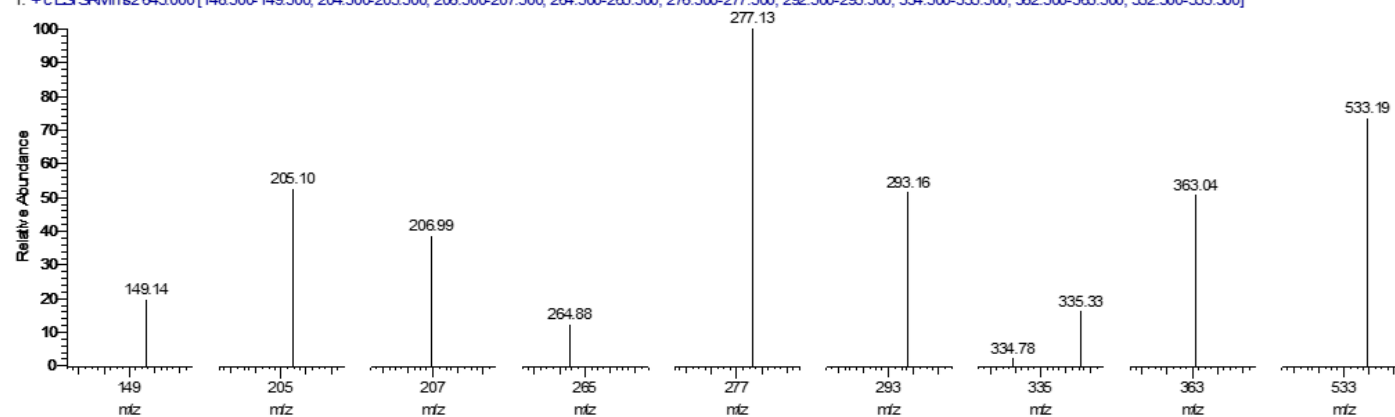
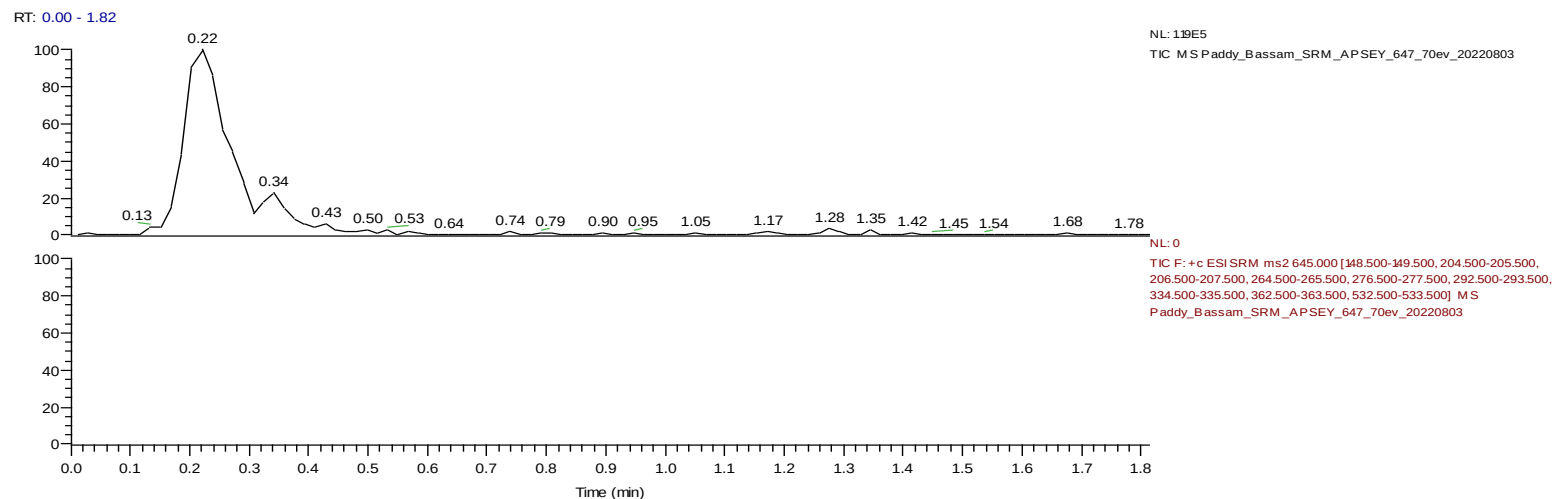


Figure D.24: MSMS fragments for Bullatanocin and Atemoyacin-E



Paddy\_Bassam\_SRM\_APSEY\_647\_70ev\_20220803

08/03/22 14:24:07



Paddy\_Bassam\_SRM\_APSEY\_647\_70ev\_20220803 #8-22 RT: 0.13-0.38 AV: 15 NL: 1.67E4

T: + c ESI SRM ms2 647.000 [204.500-205.500, 276.500-277.500, 376.500-377.500, 462.500-463.500, 534.500-535.500]

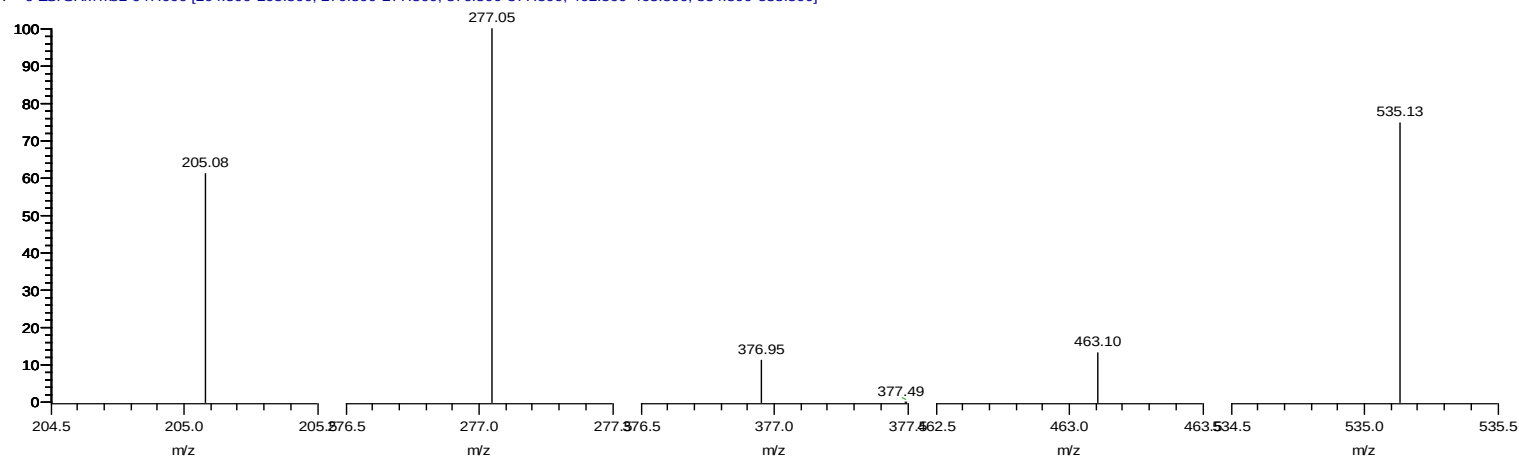
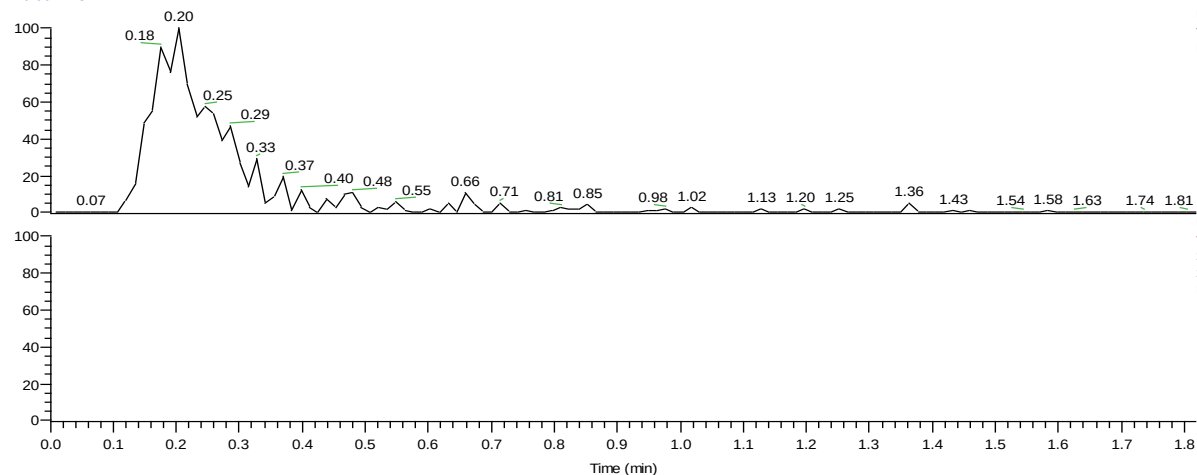


Figure D.25: MSMS fragments for Montanacin A

Paddy\_Bassam\_SRM\_APSH\_613\_70ev\_20220803

08/03/22 13:21:46

RT: 0.00 - 1.82



NL: 4.95E4  
TIC MS  
Paddy\_Bassam\_SRM\_APSH\_613\_70ev\_20220803

NL: 0  
TIC F: +c ESI SRM ms2 571000 [204.500-205.500, 246.500-247.500, 253.500-254.500, 274.500-275.500, 276.500-277.500, 304.500-305.500] MS  
Paddy\_Bassam\_SRM\_APSH\_613\_70ev\_20220803

Paddy\_Bassam\_SRM\_APSH\_613\_70ev\_20220803 #9-28 RT: 0.12-0.38 AV: 20 NL: 1.05E4  
T: +c ESI SRM ms2 613.000 [176.500-177.500, 204.500-205.500, 274.500-275.500, 346.500-347.500]

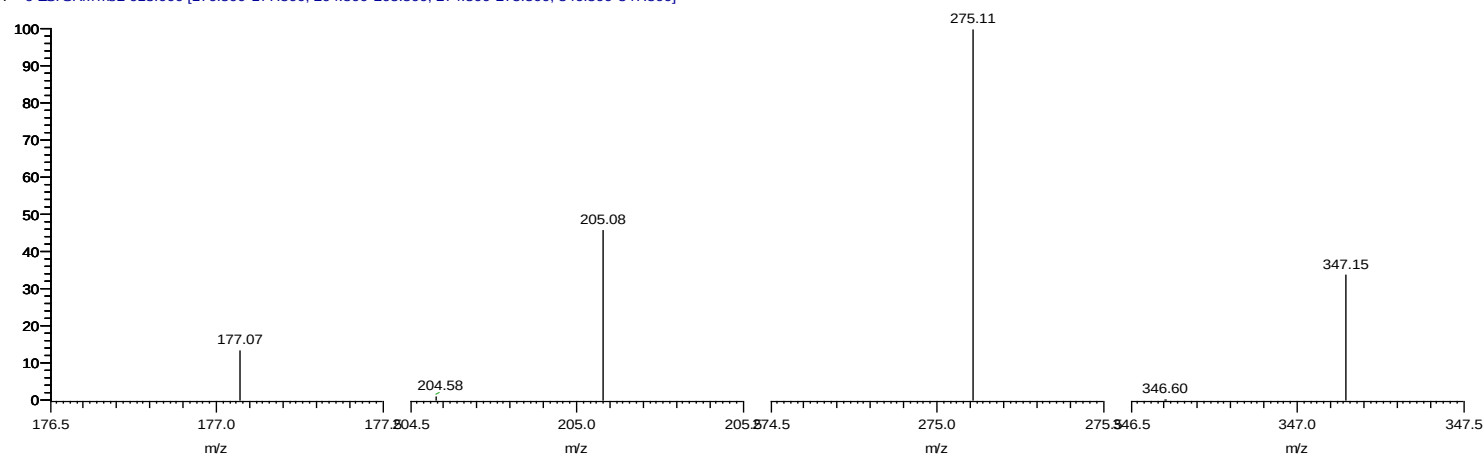
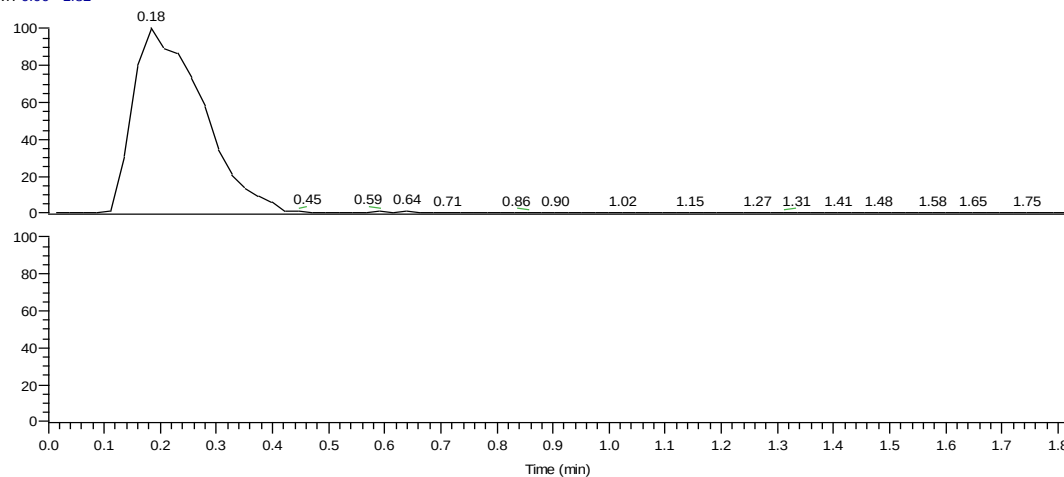


Figure D.26: MSMS fragments for Desacetyluvaricin

Paddy\_Bassam\_SRM\_APSEY\_601\_70ev\_20220803

08/03/22 14:02:01

RT: 0.00 - 1.82



NL: 3.35E5

TIC MS Paddy\_Bassam\_SRM\_APSEY\_601\_70ev\_20220803

NL: 0

TIC F: + c ESI SRM ms2 645.000 [148.500-149.500, 204.500-205.500, 206.500-207.500, 264.500-265.500, 276.500-277.500, 292.500-293.500, 334.500-335.500, 362.500-363.500, 532.500-533.500] MS  
Paddy\_Bassam\_SRM\_APSEY\_601\_70ev\_20220803

Paddy\_Bassam\_SRM\_APSEY\_601\_70ev\_20220803 #6-16 RT: 0.14-0.38 AV: 11 NL: 7.91E4

T: + c ESI SRM ms2 601.000 [176.500-177.500, 204.500-205.500, 232.500-233.500, 274.500-275.500, 304.500-305.500, 346.500-347.500, 488.500-489.500]

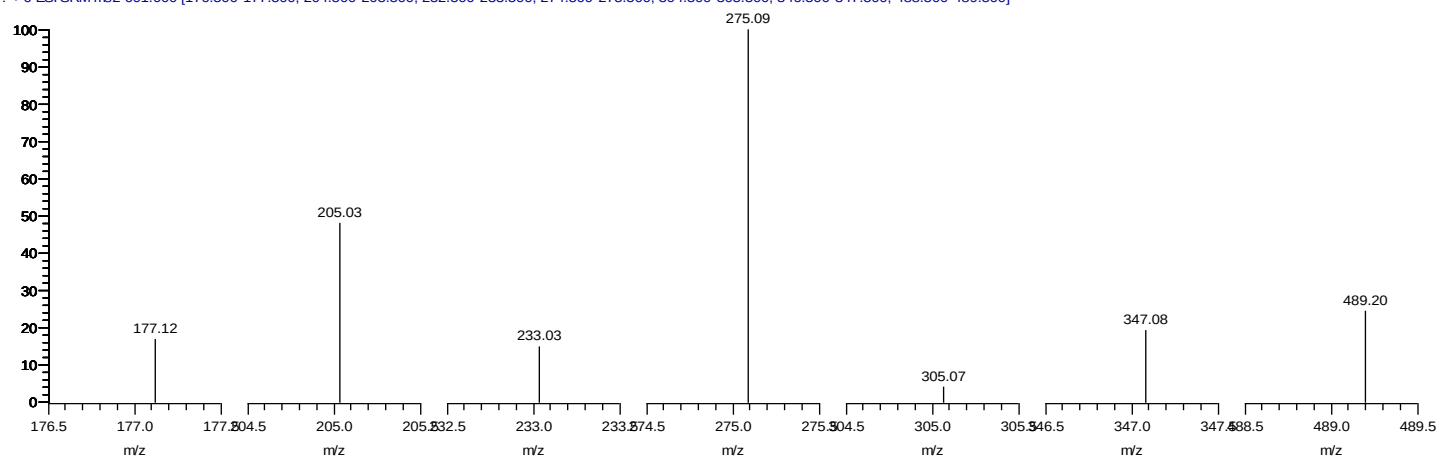
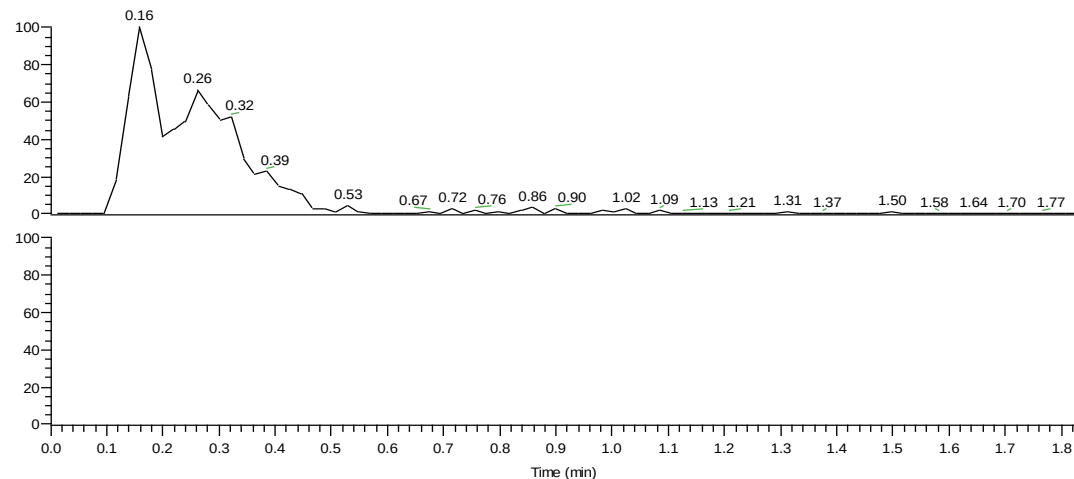


Figure D.27: MSMS fragments for Atemoyacin-A

Paddy\_Bassam\_SRM\_APSEY\_585\_70ev\_20220803

08/03/22 13:54:30

RT: 0.00 - 1.83



NL: 126E5

TIC: MS Paddy\_Bassam\_SRM\_APSEY\_585\_70ev\_20220803

NL: 0

TIC F: +c ESI SRM ms2 645.000 [148.500-149.500, 204.500-205.500, 206.500-207.500, 264.500-265.500, 276.500-277.500, 292.500-293.500, 334.500-335.500, 362.500-363.500, 532.500-533.500] MS  
Paddy\_Bassam\_SRM\_APSEY\_585\_70ev\_20220803

Paddy\_Bassam\_SRM\_APSEY\_585\_70ev\_20220803 #6-19 RT: 0.12-0.39 AV: 14 NL: 2.77E4

T: +c ESI SRM ms2 585.000 [176.500-177.500, 204.500-205.500, 232.500-233.500, 274.500-275.500, 304.500-305.500, 346.500-347.500]

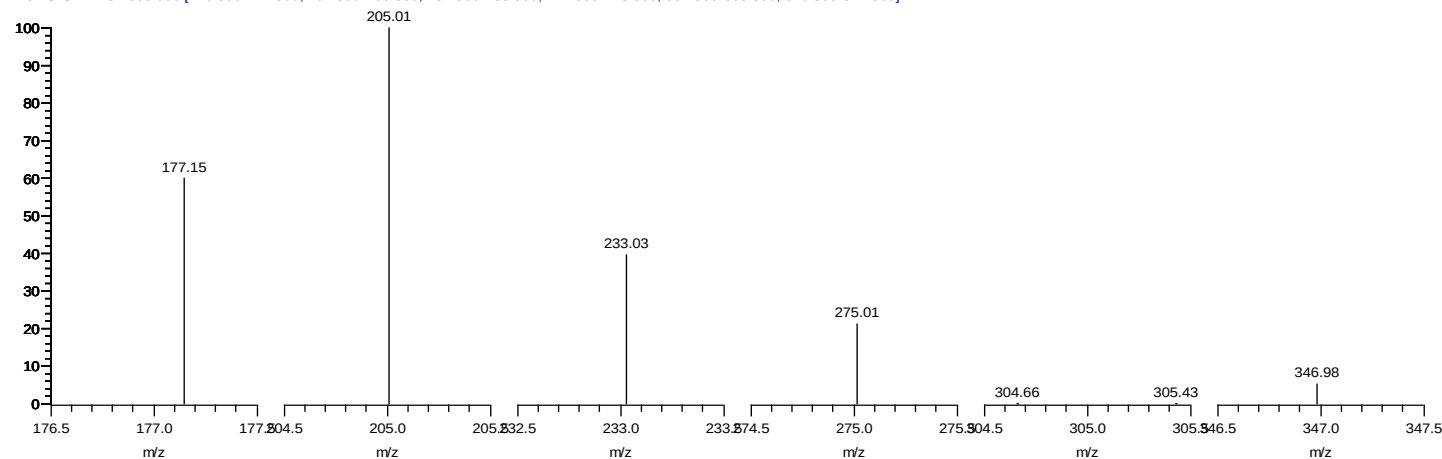
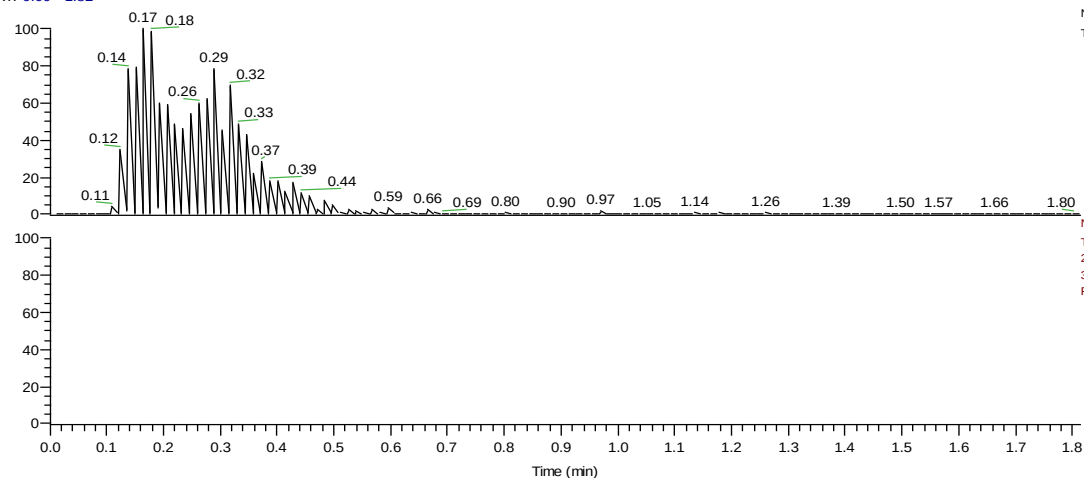


Figure D.28: MSMS fragments for Atemoyin

Paddy\_Bassam\_SRM\_APSEY\_569\_70ev\_20220803

08/03/22 13:46:59

RT: 0.00 - 1.82



NL: 9.66E4

TIC MS Paddy\_Bassam\_SRM\_APSEY\_569\_70ev\_20220803

NL: 0

TIC F: + c ESI SRM ms2 645.000 [148.500-149.500, 204.500-205.500, 206.500-207.500, 264.500-265.500, 276.500-277.500, 292.500-293.500, 334.500-335.500, 362.500-363.500, 532.500-533.500] MS  
Paddy\_Bassam\_SRM\_APSEY\_569\_70ev\_20220803

Paddy\_Bassam\_SRM\_APSEY\_569\_70ev\_20220803 #18-55 RT: 0.12-0.37 AV: 19 NL: 3.57E4

T: + c ESI SRM ms2 569.000 [232.500-233.500, 260.500-261.500, 330.500-331.500]

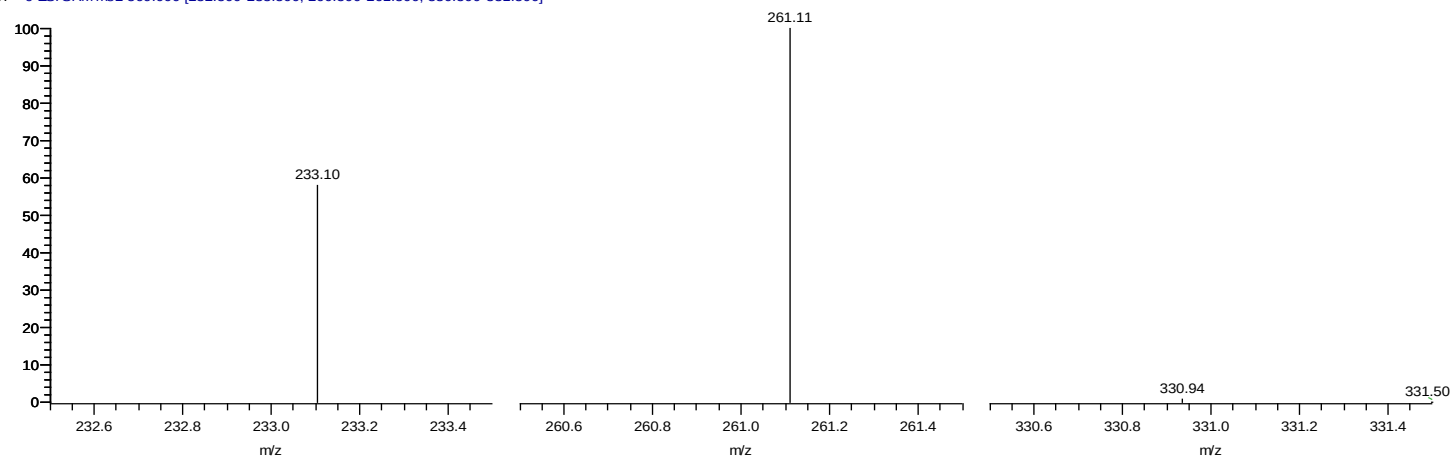


Figure D.29: MSMS fragments for Robustocin

## Appendix E. Publications

Review

# Traditional Uses, Phytochemistry and Pharmacological Activities of Annonaceae

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**Abstract:** In 1789, the Annonaceae family was catalogued by de Jussieu. It encompasses tropical and subtropical plants which are widespread in distribution across various continents such as Asia, South and Central America, Australia and Africa. The genus of *Annona* is one of 120 genera of the Annonaceae family and contains more than 119 species of trees and shrubs. Most species are found in tropical America, where over 105 species have been identified. Due to its edible fruits and medicinal properties, *Annona* is the most studied genus of Annonaceae family. To date, only a limited number of these species have economic value, including *A. squamosa* L. (sugar apple), *A. cherimola* Mill. (Chirimoya), *A. muricata* L. (guanabana or soursop), *A. atemoya* Mabb. (atemoya), a hybrid between *A. cherimola* and *A. squamosa*, *A. reticulata* L. (custard apple), *A. glabra* L. (pond-apple) and *A. macrophyllata* Donn. Sm. (ilama). Phytochemically, several classes of secondary metabolites, including acetogenins, essential oils, alkaloids, terpenoids and flavonoids. The pharmacological activities of *Annona* species leaves and seeds include antibacterial, anticancer, antidiabetic and anti-inflammatory properties.

**Keywords:** Annonaceae; *Annona*; custard apple; phytochemistry; bioactivity; ethnomedicinal pharmacological activity



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## 1. Introduction

In 1789, the Annonaceae family was cataloged by de Jussieu [1,2]. It encompasses tropical and subtropical plants, which are widespread in distribution across various continents such as Asia, South and Central America, Australia and Africa [3]. It is one of the largest Mangnoliidae families and the number of its genera and species is still debated [4–6]. Bailey and Popenoe believe that it has between 40 and 50 genera and from 500 to 600 species [6]; however, many studies have indicated that the Annonaceae family is comprised of more than 2400 species distributed in approximately 120 genera [4,5]. The family of Annonaceae involves trees, lianas and bushes arranged in four large subfamilies: Malmeoideae, Annonoideae, Ambavioideae and Anaxagoreoideae [7,8]. Economically, species of Annonaceae are important as a source of edible fruits, for instance, the pawpaw (*Asimina*), custard apple, sweetsop, soursop and cherimoya [1]. It has also been reported that some oils from the seeds might be used for the production of edible oils and as an ingredient in soaps, and the woods of some species have been reported for alcohol production [3]. Chemical studies of Annonaceae species have reported the isolation of a wide diversity of phytochemical components, including acetogenins, alkaloids and flavonoids from the bark, fruits, leaves, seeds and pulp of Annonaceae [9]. This review aims to provide a comprehensive summary of the botanical features, phytochemistry, pharmacological properties, and the traditional and ethnomedicinal uses of the Annonaceae family and, specifically, *Annona* species.

## 2. Botanical Features of Annonaceae Species

### 2.1. Distribution and Classification

Annonaceae has been listed as a diverse family of aromatic trees, bushes or shrubs, and climbers or lianas, which are predominantly found in the tropical and subtropical regions, with a limited number growing in temperate zones [1,10]. In tropical America, the Annonaceae species are usually shrubby and most grow in open grasslands [1]. In contrast, species that are climbers mostly grow in the tropical area of the old world [1]. In temperate zones like North America, the only genus reported is *Asimina* [1,3]. In Brazil, more than 385 species have been reported, with the majority of them reported in the Amazonian region [2]. According to the Takhtajan system of flowering plant classification, the majority of Annonaceae plants can be found in both Asia and Australasia with approximately 51 genera and more than 950 species, while 40 genera with approximately 450 species are confined to Africa and Madagascar, and about 38 genera and 740 species are native to the American continent [3]. The first classification of the Annonaceae family was described by Dunal in 1817 and was limited to only fruit morphology [11]. Subsequently, a new classification of the Annonaceae family based on flower characteristics was introduced by Diels and Alder in 1932 [11]. However, a later classification by Fries in 1959 was found to be more comprehensive and authentic, using a combination of fruit morphology and flora characteristics [11]. The Annonaceae family are characterised by the presence of a variety of primitive and archaic features, leading to them being described by Darwin as “living fossil” due to their ability to survive the mass extinction [1,11]. Under the Takhtajan system, the Annonaceae family is related to Magnoliaceae, which is one of the largest families of Magnoliales with other families such as Degeneriaceae, Canellaceae, Himantandraceae and Myristicaceae [1,11].

### 2.2. Diagnostic Features

From one species to another, the botanical features of Annonaceae families vary greatly based on their origin, geography, and climate. Based on morphology and habitat, the Annonaceae family is known among the homogeneous plant families [1,4]. The aromatic flowers are commonly open before other parts are entirely developed. The flowers are terminal, axillary, hermaphrodite, singular or grouped and regular [1,11]. The stamens are typically abundant, spirally arranged and hypogenous [1,11]. The leaves are characterised by having a glaucous or metallic sheen, and they are alternate, exstipulate and regular [1,11]. The fruits are typically made up of clusters of berries with an edible fleshy receptacle, particularly in the *Annona* genera and they are extensively consumed due to their high nutritional value [1,11]. Finally, the seeds are enlarged and have a copious, irregular-surfaced endosperm with a minute embryo [1,11].

### 2.3. Traditional Uses

Annonaceae species are famous in tropical regions and used traditionally across tropical regions due to their widespread distribution. Various parts of the species are used traditionally, including leaves, seeds, bark, fruit, stem, roots and twigs. A range of different methods for preparation is reported, such as infusions, pastes and decoctions [11]. For instance, the fresh fruit of *Annona dioica* is used for wound healing in Brazil [11]. The dried leaves of *Annona muricata* are used orally for analgesic effects in some parts of Indonesia [11]. In Burkina-Faso, the bark and roots of *Annona muricata* are used for dysentery and as an anthelmintic medicine, whereas the leaves are utilized for both fever and dysentery [12]. In the northwestern part of Brazil, both leaves and twigs of *Duguetia chrysocarpa* are ground and the extract of this mixture are utilized for treating gastrointestinal ulcers as well as a remedy for bowel disease [11]. A decoction of the stem bark of *Annickia chlorantha* is used orally as a remedy for the treatment of wounds and fever in Cameroon [13]. Further data on the traditional uses of the most widely used Annonaceae species are presented in Table 1.



**Table 1.** Uses of most commonly used Annonaceae family in traditional medicines.

| Annonaceae Species                                              | Region                       | Local Name                                           | Medicinal Uses                                                                                                                   | Part Used                    | Mode of Usage                                                                                         | References |
|-----------------------------------------------------------------|------------------------------|------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------|------------------------------|-------------------------------------------------------------------------------------------------------|------------|
| <i>Alphonsea javanica</i> Scheff.                               | Indonesia                    | Aku Battu                                            | Rheumatism and edema                                                                                                             | Leave                        | Ethanol extract                                                                                       | [14]       |
| <i>Annickia chlorantha</i> (Oliv).                              | Cameroon                     | African yellow wood (c)<br>Moambe Jaune              | Treatment of sores<br>Antipyretic<br>Antiemetic<br>Stimulant<br>Tuberculosis<br>Treatment of jaundice<br>Urinary tract infection | Bark                         | Powder<br>Crushed bark and drink extract<br>Decoction<br>Decoction in baths<br>Decoction<br>Decoction | [12,13]    |
| <i>Annickia affinis</i> (Exell)<br>Versteegh & Sosef            | Cameroon                     | African yellow wood                                  | Wound healing<br>Antiemetic<br>Antipyretic                                                                                       | Stem bark                    | Decoction of stem bark                                                                                | [13]       |
| <i>Anonidium floribundum</i> Pellegr                            | Cameroon                     | Eboum, Libanga Ebom                                  | Poison antidote<br>Dysentery<br>Antipyretic                                                                                      | Roots<br>Root/Bark<br>Leaves | Decoction taken orally                                                                                | [12,15]    |
| <i>Anonidium mannii</i> (Oliv.)                                 | Cameroon                     | Ebome; Npole Wapo'o, Ebome Afan                      | Antipyretic                                                                                                                      | Stem bark                    | Decoction of stem bark                                                                                | [13]       |
| <i>Boutiquea platypetala</i> (Engl.)                            | Cameroon                     | Not reported                                         | To treat fresh wounds                                                                                                            | Leaves                       | Pounded fresh leaves                                                                                  | [12]       |
| <i>Cananga odorata</i> (Lam.) Hook and Thomson                  | Malaysia and India           | Kenanga utan, Perfume tree, Cananga oil, Ylang ylang | Rheumatism<br>Ophthalmic inflammation and wound healing                                                                          | Bark                         | Bark extract eye drops for inflammation and decoction are used to wash fresh wounds                   | [11]       |
| <i>Duguetia chrysocarpa</i> Maas                                | Brazil                       | Pindaiba-da-mata                                     | Bowl and rheumatism inflammation                                                                                                 | Leave and twigs              | Leaves and twigs extract taken to relieve inflammation                                                | [16]       |
| <i>Enicosanthellum pulchrum</i> (King) Heusden                  | Malaysia                     | Disepalum                                            | Rheumatism fever, edema and asthma                                                                                               | Leave                        | Decoction can be used for asthma and rheumatism                                                       | [17]       |
| <i>Enantia chlorantha</i> var. <i>soyauxii</i> Engler and Diels | Africa                       | African yellow wood                                  | Arthritis and wound healing                                                                                                      | Bark                         | Powdered bark with citrus lemon used as dressing                                                      | [11]       |
| <i>Friesodielsia enghiana</i> (Diels.) Verdc                    | Cameroon                     | Lonkosso                                             | Analgesic                                                                                                                        | Bark                         | Decoction of bark is taken orally                                                                     | [15]       |
| <i>Friesodielsia gracilipes</i> (Benth.) Steenis                | Cameroon                     | Ntonda                                               | Treatment of sores, skin infection, ulcers, and jaundice                                                                         | Bark and wood                | Decoction of bark and wood                                                                            | [12]       |
| <i>Fissistigma oldhamii</i> (Hemsl.) Merr                       | Southern China               | Oldhamii                                             | Rheumatoid arthritis                                                                                                             | Stems and roots              | Powdered of stems and roots and orally ingested                                                       | [11]       |
| <i>Greenwayodendron suaveolens</i> (Engl and Diels) Verdc       | Not reported                 | Otunga                                               | Aphrodisiac and Vermifuge<br>Rheumatic pains, fevers, headache, stomach-ache                                                     | Root<br>Leaves and bark      | Chew roots<br>Pulverized leaves or bark and mixed with seeds of <i>Aframomum melegueta</i>            | [15]       |
| <i>Isolona hexaloba</i> (Pierre) Engl & Diels                   | Democratic Republic of Congo | Bodzungu                                             | Malaria                                                                                                                          | Stem bark                    | Decoction of stem bark                                                                                | [18]       |
| <i>Monodora myristica</i> (Gaertn.) Dunal                       | Ivory coast                  | M Kpo. Abidjan district                              | Eye diseases and hemorrhoids, febrile pains and headache                                                                         | Fruits Seed                  | Fruits and seeds consumed whole or ground to be used in soup and stews                                | [19]       |
| <i>Monodora tenuifolia</i> Benth                                | Not reported                 | African nutmeg<br>Ebom osoé<br>Grandes feuilles      | Toothache<br>Dysentery and fevers                                                                                                | Root<br>Bark and root        | Clean the roots, boil and rinse the mouth<br>Prepared as a decoction and used as an enema             | [12]       |
| <i>Polyalthia suaveolens</i> Engl and Diels                     | Cameroon                     | Diels;<br>Otungui; Ntunga                            | Analgesic, Antiepileptic<br>Antipyretic<br>Treatment of jaundice                                                                 | Stem bark                    | Decoction of stem bark                                                                                | [13]       |
| <i>Polyalthia longifolia</i> (Sonn.) Thwaites                   | India                        | Ashoka                                               | Fever                                                                                                                            | Bark                         | Decoction of bark                                                                                     | [20]       |
| <i>Xylopia aethiopica</i> (Dunal) A.Rich                        | Sudan                        | Ethiopia or Negro pepper                             | Rheumatism, colic pain, headache, and neuralgia                                                                                  | Fruits                       | Ethanol fruit extract or dried fruits are used as whole                                               | [21]       |
| <i>Xylopia aromatic</i> Lam. Mart                               | Columbia                     | Monkey pepper                                        | Pulmonary inflammation and hemorrhoids                                                                                           | Roots<br>Leaves              | Insertion of root pieces into rectum and leaves burnt and smoke inhaled                               | [22]       |

Table 1. Cont.

| Annonaceae Species                            | Region                         | Local Name                   | Medicinal Uses                                                          | Part Used | Mode of Usage                 | References |
|-----------------------------------------------|--------------------------------|------------------------------|-------------------------------------------------------------------------|-----------|-------------------------------|------------|
| <i>Xylopia parvifolia</i> Hook.f. and Thomson | East and Central Africa, India | Netawu/Athu ketiya           | Gastrointestinal ulcers<br>Analgesic                                    | Roots     | Decoction Finely dried powder | [23]       |
| <i>Xylopia staudtii</i> Engl & Diels          | Not reported                   | Ntom, Odjobi Bush pepper (c) | Cold and headache treatment                                             | Bark      | Powder                        | [12]       |
| <i>Monodora tenuifolia</i> Benth              | Cameroon                       | Ebome osso                   | Joint and muscle pain, promotion of breast milk production and headache | Stem bark | Decoction of stem bark powder | [13]       |
| <i>Uvaria acuminata</i> Oliv                  | Cameroon                       | Nosonaback                   | Typhoid and Yellow fever<br>Headache and epilepsy                       | Stem bark | Decoction of stem bark        | [13]       |

### 3. Phytochemistry of Annonaceae Family

A wide array of chemical compounds from various parts of Annonaceae plants have been discovered, isolated and characterised. The results of both phytochemical investigations and biological studies on various plants from this family have led to the identification of a wide diversity of compounds such as annonaceous acetogenins, flavonoids, alkaloids and essential oils, as summarized in (Table 2). These phytochemical constituents have been found to exhibit a broad range of biological activities such as immunosuppressive, anti-neoplastic, cytotoxic, antimicrobial, anti-inflammatory effects (Table 3). However, it is the *Annona* genera that are the most widely used as a food source and in traditional medicines.

Table 2. Representative phytochemicals isolated from plants of Annonaceae.

| Species                                            | Part                | Compounds                                                         | Class | References |
|----------------------------------------------------|---------------------|-------------------------------------------------------------------|-------|------------|
| <i>Anaxagoma dolichocarpa</i> Sprague and Sandwith | Fruits              | <i>p</i> -Cymene<br>Spathulenol<br>Caryophyllene oxide<br>Guaiene | ESO   | [5]        |
| <i>Anomianthus dulcis</i> (Dunal) J. Sinclair      | Stem                | (–)-Anolobine<br>(–)-Anonaine                                     | ALK   | [24]       |
| <i>Artabotrys pierreanus</i> Engl. & Diels         | Stem bark           | Cyperene<br>Caryophyllene oxide<br>Cyperermone<br>Cadalene        | ESO   | [5]        |
| <i>Artabotrys hexapetalus</i> (L.f.) Bhandari      | Aerial parts        | 9-Oxo-asimicinone<br>Artapetalin-A<br>Artapetalin-B               | ACT   | [25,26]    |
| <i>Goniothalamus giganteus</i> Hook.f. & Thomson   | Bark                | Pyranicin<br>Pyragonicin<br>Goniotrionin                          | ACT   | [27]       |
| <i>Miliusa balansae</i> Finet & Gagnep             | Leaves and branches | Ombuine<br>Chrysosplenol<br>Pachypodol<br>Chrysosplenol C         | FLA   | [28]       |

ALK (Alkaloids), ACT (Acetogenins), ESO (Essential oils) and FLA (Flavonoids).

**Table 3.** Pharmacological activities of some isolated compounds from Annonaceae species.

| Species                                                        | Part Used                | Isolated Compounds                          | Pharmacological Activity    | Mechanism of Action                                                                                                                                                             | References |
|----------------------------------------------------------------|--------------------------|---------------------------------------------|-----------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>Alphonsea javanica</i><br>Scheff                            | Leaves                   | (+)-Altholactone<br>(+)-Goniothalmin        | Anti-inflammatory           | Inhibited lipopolysaccharide (LPS) induced NO production in RAW 264.7 macrophages with IC <sub>50</sub> = 0.8 µM.                                                               | [14]       |
| <i>Artabotrys hexapetalus</i> (L.f.)<br>Bhandari               | Roots, stems, and leaves | Artabonatine B<br>Squamolone                | Anticancer                  | Exhibited activity against 2,2,15 and Hep G2 cell lines with IC <sub>50</sub> 11.0 and 9.1 µg/mL. Displayed activity against Hep G2 cell lines with IC <sub>50</sub> 2.8 µg/mL. | [29]       |
| <i>Cananga odorata</i> (Lam.)<br>Hook.f. & Thomson             | Fruits                   | Cleistopholine                              | Cytotoxic                   | Exhibited cytotoxicity against both Hep 2,2,15 and Hep G2 cell lines with IC <sub>50</sub> 0.54 and 0.22 µg/mL, respectively.                                                   | [30]       |
| <i>Goniothalamus tamirensis</i><br>Pierre ex<br>Finet & Gagnep | Stem bark                | Dielsiquinone                               | Cytotoxic                   | Displayed cytotoxic activity against U251, RPMI, MCF7, HT029 and A549. with ED <sub>50</sub> 0.37, 0.11, 0.11 1.12 and 0.11, respectively.                                      | [31]       |
| <i>Guatteria blepharophylla</i> Mart                           | Bark                     | Isocoreximine                               | Anti-proliferative activity | Exhibited activity against UACC-62, NCI-H460, HT-29 and MCF-7 with TGI > 764.52 µM.                                                                                             | [32]       |
| <i>Rollinia sylvatica</i><br>A.St.-Hil                         | Leaves                   | Hinesol<br>z-Caryophyllene<br>beta-Maaliene | Anti-inflammatory           | Leukocytes migration was significantly reduced at concentrations of 36.04–45.37 µg/mL.                                                                                          | [33]       |

#### 4. Annona Genera

The genus *Annona* is one of the 120 genera of the Annonaceae family and contains more than 119 species of trees and shrubs, most of them distributed in tropical areas of the Americas and Africa [6]. The majority of these species are found in tropical America, with more than 105 species (26 of them are endemic) and 10 species distributed in tropical Africa [10,34]. It has been reported that this genus is the second or the third largest genus in the Annonaceae family [35]. Its generic name derives from the Latin Hispaniolan Taino “annual harvest” [6,35]. Due to its edible fruits and medicinal properties, *Annona* is the most important genus of Annonaceae family [2]. Numerous *Annona* species furnish edible fruits like *Annona muricata* (“graviola”), *Annona crassiflora* (“araticum”) and *Annona squamosa* (“fruta do conde”) [2]. Most of the fruits are consumed either in fresh form or used in desserts, juices and ice cream preparations [34]. Despite *Annona* having many species, only limited species of this family are economically important such as *A. squamosa* L. (sugar apple), *A. cherimola* Mill. (Cherimoya), *A. muricata* L. (guanabana or soursop), *A. atemoya* Mabb. (atemoya), a hybrid between *A. cherimola* and *A. squamosa*, *A. reticulata* L. (custard apple), *A. glabra* L. (pond-apple) and *A. macrophyllata* Donn. Sm. (ilama) [6]. Phytochemically, several classes of secondary metabolites such as acetogenins,

essential oils, alkaloids, terpenoids and flavonoids have been described in this genus [34,36]. A variety of pharmacological activities have been reported from various parts of *Annona* species specially leaves and seeds including applications against antibacterial [37], antinociceptive [38], anticancer [39], anticonvulsant [40], antidiarrhea [41], antidiabetic [42], anti-malarial [39], anti-inflammatory [43], antioxidant [44], antileishmanial [45], antiulcer [46] and antidepressant [47].

#### 4.1. Botanical Features

Generally, *Annona* species are small trees or shrubs with a height from 5 to 11 m depending on various factors including soil, climate, species, and crop management [2]. In relation to the botanical characteristics of *Annona* species, the majority of them are moderately erect with brown bark that is frequently furrowed (Table 4) [10]. The stems are rust-coloured (ferruginous) and covered with densely matted hairs (tomentose) when young, becoming smooth and hairless (glabrous) as they mature [6,10]. It has thin lateral roots and a taproot that is not generally pronounced [2]. With regard to the flowers, they are hermaphrodites, solitary or fascicle containing from two to four flowers. The flowers are usually fragrant, with six petals and three green sepals, in a circular arrangement of two verticils [6]. Flowering of the plant usually starts at 3 to 4 years and flower opening usually occurs by separation of the apex of external petals [6,10]. Finally, the leaves may be shiny or hairy and have an impressed vein on the upper side, and the fruits are syncarpous and comprised of seeds and many carpels [6,10].

**Table 4.** Botanical information of some *Annona* species.

| Species                  | Synonyms                                                                                                                                               | Local Names                                                                                                        | Geographic Distribution                                                                                                              | References |
|--------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>A. cherimola</i>      | <i>A. tripetala</i> Aiton<br><i>A. pubescens</i> Salisb                                                                                                | Chirimoya<br>Chirimolia<br>Cerimoya<br>Cherimoyer Momona                                                           | South Africa, China<br>Egypt<br>Eritrea<br>Myanmar<br>Philippines<br>India<br>France<br>Italy<br>Mexico, Ecuador<br>Portugal<br>Peru | [6,48]     |
| <i>A. coriacea</i>       | <i>A. coriacea</i> var.<br><i>amplexicaulis</i> S.Moore,<br><i>A. coriacea</i> var. <i>cuneate</i> ,<br><i>A. coriacea</i> var.<br><i>pygmaea</i> Warm | Marolo<br>Araticum<br>Marolino                                                                                     | Brazil (Cerrado,<br>Caatinga)                                                                                                        | [10,49]    |
| <i>A. cornifolia</i>     | <i>A. walkeri</i> S. Moore                                                                                                                             | Araticum-mirim                                                                                                     | Brazil                                                                                                                               | [50]       |
| <i>A. crassiflora</i>    | <i>A. macrocarpa</i> Barb<br><i>A. rodriguesii</i> Barb                                                                                                | Araticum<br>Pinha-docerrado<br>Cerrado pinecone<br>Marolo<br>Cabeça de negro                                       | Brazil                                                                                                                               | [51]       |
| <i>A. macrophyllata</i>  | <i>A. diversifolia</i> Saff                                                                                                                            | Ilama,<br>Papaue<br>Anona blanca                                                                                   | Mexico<br>China<br>India                                                                                                             | [52,53]    |
| <i>A. montana</i> Macfad | <i>A. Montana</i> f. <i>marcgravi</i><br>(Mart.) Porto                                                                                                 | Mountain soursop<br>False graviola<br>jacá do Pará<br>Araticum grande<br>Shan di fan li zhi                        | Southern Asia,<br>South America Amazon<br>Rainforest and<br>Atlantic Forest                                                          | [10]       |
| <i>A. muricata</i>       | <i>A. macrocarpa</i> Barb<br><i>A. muricata</i> Guanabanus<br><i>A. cearensis</i> Morales                                                              | Brazilian pawpaw Soursop, ci guo<br>fan li zhi, Graviola Araticum<br>grande Mullu Raama Phala,<br>Corossol Catuche | Tropical regions of<br>Americas<br>Malaysia, Myanmar,<br>Pakistan, India,<br>Indonesia, China                                        | [6,10]     |

Table 4. Cont.

| Species                     | Synonyms                                                                                                                                                                                                                                                                                                                                                    | Local Names                                                                                                              | Geographic Distribution                                           | References |
|-----------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------|------------|
| <i>A. reticulata</i>        | <i>A. excelsa</i> Kunt<br><i>A. laevis</i> Kunth<br><i>A. longifolia</i> Moc<br><i>A. riparia</i> Kunth                                                                                                                                                                                                                                                     | Custard apple<br>Bullock's heart                                                                                         | Indonesia, West Indies,<br>Bangladesh, China, India               | [54]       |
| <i>A. sclerophylla</i> Saff | <i>Sulcata</i> Urb<br><i>A. spinescens</i> Mart                                                                                                                                                                                                                                                                                                             | Not reported                                                                                                             | Brazil                                                            | [10]       |
| <i>A. senegalensis</i>      | <i>A. senegalensis</i> var.<br><i>arenaria</i> Sillans<br><i>A. senegalensis</i> var. Bail.<br>Wild<br><i>A. senegalensis</i> var.<br><i>glabrescens</i> Oliv<br><i>A. senegalensis</i> var.<br><i>cuneata</i> Oliv<br><i>A. arenaria</i> Thonn<br><i>A. chrysophylla</i><br><i>A. chrysophylla</i> var.<br><i>porpetac</i> Bail<br><i>A. porpetac</i> Bail | Wild soursop<br>Sour soup<br>Abo<br>Uburuoachaand Gwandar                                                                | Nigeria                                                           | [6]        |
| <i>A. squamosa</i>          | <i>A. asiatica</i> L.<br><i>A. squamosa</i> f. <i>Parvifolia</i><br>Kuntze<br><i>A. cinerea</i> Dunal<br><i>Guanabanus squamosus</i> (L.)<br>M.Gómez                                                                                                                                                                                                        | Custard apple<br>Sweetsop<br>Tiep baay<br>Amritaphala<br>Chirimoya<br>Fruta do conde<br>Squamosus<br>Gomez<br>Guanabanus | Egypt, Sudan, Pakistan,<br>Thailand, China, India,<br>Costa Rica, | [6,10]     |

#### 4.1.1. *Annona Cherimola*

*Annona cherimola* Mill (*Cherimoya*) belongs to the genus *Annona* in the Annonaceae family in magnolias order, which means “cold seeds”, and is a small tree that produces heart-shaped and conical edible fruit [55]. It is a steep, semi-momentary and a low bunched tree that is widespread in Ecuador and Peru and distributed throughout Asia, South Europe, America and Africa [56]. In Mexican traditional medicine, this plant has been used to treat various diseases such as diabetes, cough, fever, headache, worms and inflammation either alone or in combination with other plant species [48,57–60]. Recently, various parts of *A. cherimola* have been phytochemically profiled and contain various polyphenols and alkaloids. The leaves were found to be a source of bioactive compounds with potential for use as treatments for skin and eye diseases and gastric, cardiovascular and intestinal disorders [55].

#### 4.1.2. *Annona Squamosa*

*Annon squamosa* L., commonly known as custard apple, is a tropical, endemic species of the West Indies, Ecuador, Peru, Brazil, South and Central America, Mexico, Bahamas, Bermuda, and Egypt [61]. This plant is extensively cultivated in various states of India, including Maharashtra, Gujarat, Madhya Pradesh, Chhattisgarh, Assam, Uttar Pradesh, Bihar, Rajasthan, Andhra Pradesh, and Tamil Nadu. The total area of cultivation has been reported by the Indian Council of Agricultural Research (ICAR) as 40,000 ha [62]. Its tree grows as a small sapling from 3 m to 8 m, with large branches having brownish or light brownish bark and it has thin leaves and is known for its edible fruit [61]. In the Aligarh district village in Uttar Pradesh, *A. squamosa* is well-known for its antidiabetic properties [63]. Its seeds, bark and leaves possess various pharmacological properties, mainly anti-tumour properties [64].

#### 4.1.3. *Annona Muricata*

*Annona muricata* is a commonly known as soursop and graviola and is native to Central and South America. It is a small tree 5–10 m tall and 15–83 cm in diameter; it has low branches and edible fruit that are used commercially for the production of candy, juice and



sherberts [65]. Traditionally, the aerial parts of this plant have been used to treat various diseases like diabetes and malaria and nowadays, it is widely used by people diagnosed with cancer [66]. Moreover, this species possesses several pharmacological properties, including vasodilator, cardio-depressive, antispasmodic, antimutagen, anticonvulsant, antiviral, antidiabetic and antihypertensive effects [67]. Both the leaves and seeds of *A. muricata* have been evaluated for their constituents resulting in the identification and isolation of more than 50 mono-THF acetogenins, alkaloids, terpenoids, saponins, flavonoids, coumarins, cardiac glycosides, phenols, tannins and anthraquinones [67].

#### 4.1.4. *Annona Reticulata*

*Annona reticulata* Linn is a traditionally important plant utilized in traditional medicines [68]. It is indigenous to the West Indies and widely distributed in tropical and subtropical regions of the world [54]. It is a small tree with a height between 6 and 7.5 m and contains numerous lateral branches [54]. It has a cylindrical stem that contains lenticels and very short coffee-colored hairs [54]. The leaves of *A. reticulata* are lanceolate, membranous, oblong, and rounded or curate at the base. Fruits are edible, rough, somewhat heart-shaped and yellow in color that shifts to yellowish-red on ripening, and the seed is smooth and blackish in color [69]. Traditionally, *A. reticulata* has been utilized for the treatment of epilepsy, dysentery, cardiac problem, constipation, haemorrhage, bacterial infection, parasite and worm infestations, fever, ulcers and as an insecticide [68,69]. Its leaves are used for helminthiasis treatment while bark is a powerful astringent and used as a tonic [68,69].

#### 4.1.5. *Annona Coriacea*

*Annona coriacea* Mart. is a species belonging to the *Annona* genera, commonly known as “marolo”, “araticum” and “araticum-liso” [70]. This plant is distributed across Paraguay and Brazil, with little available information about its ethnomedicinal uses [71]. It is a small tree (3–6 m) and its edible fruit consist of an ovoid-obtuse syncarp and weighing up to 1.5 kg [72]. The leaves are glabrous on the ventral surface, obovate, and the base is frequently cordate and margin undulate [73]. The flowers are terminal, thick, solitary and having fleshy petals with colors shifting between orange and pink [73]. The leaves are traditionally used as carminatives, anthelmintics, antirheumatics and in the treatment of stomatitis, headaches, abscesses, neuralgia, rheumatism, ulcers and dermatitis [74,75]. Both seeds and fruits are toxic when crushed and exhibited effects against ectoparasites like lice [75].

#### 4.1.6. *Annona Senegalensis*

*Annona senegalensis* is a small tree 2–6 m tall that is commonly known as wild custard apple and wild soursop [76]. This plant is native to tropical east and northeast, west and west-central, and southern Africa and islands in the western Indian Ocean [76]. Its leaves are simple, alternate, oblong, green to bluish-green, ovate or elliptic, and mainly lack hairs on the upper surface and brownish hairs on the lower surface [77]. This plant has been used in traditional medicine as a pain reliever, antioxidant, antidiarrheal, antitrypanosomal, antimalarial, anti-inflammatory, antimicrobial, antiparasitic, anticonvulsant and as an anti-snake venom [78]. It has been reported that the leaves of *A. senegalensis* are used for the treatment of tuberculosis, yellow fever and smallpox, whereas stem bark is reported for the treatment of injury from venomous animals [79]. The root was also reported for treating erectile dysfunction, tuberculosis, gastritis, reproductive deficiency and in the management of malaria and diabetes [80].

#### 4.1.7. *Annona Vepretorum* Mart

*Annona vepretorum* is commonly recognized as ‘bruteira’, is a small tree of 2.5–10 m high native to the Brazilian biome Caatinga [81]. The fruits of *A. vepretorum* can be consumed either raw or as juice for nutritional purposes [82]. Traditionally, a decoction of the leaves have been used to bathe in for the treatment of allergies, yeast, skin diseases

and microbial infections, whereas the root is traditionally used to treat snake and bee bites, inflammatory conditions and heart pain [81].

#### 4.1.8. *Annona Salzmännii*

*Annona salzmännii* is a tree of 6–20 m high that known as “araticum-da-mata” and “araticum“apé” [83]. It is commonly cultivated in Brazil especially in the States of Bahia, Pernambuco, and Paraíba [83]. Its root, seeds and leaves are used in folk medicine for treating several illnesses like ulcers, dysentery and inflammatory conditions [83]. The leaves and bark of *A. salzmännii* are utilized for the treatment of tumors, diabetes and inflammatory conditions [84].

#### 4.1.9. *Annona Crassiflor*

*Annona crassiflor* is known as araticum of cerrado or cerradão [85]. It is a small tree that bears a typical fruit known as araticum of cerrado or cerradão [86]. The fruits are highly consumed “in natura” by native people and can be used to make juice, jelly and ice-cream [85,86]. In folk medicine, the seeds are used to treat scalp infections, and infusions of the leaves and seeds are utilized for their antidiarrheal and antitumor properties [85]. For more details about the botanical characteristics and traditional uses of *Annona* species, see Tables 4 and 5.

**Table 5.** Traditional uses of common *Annona* species.

| Species                           | Region                                                                 | Local Name                                                                | Medicinal Uses                                                                                                                        | Part Used                                             | Mode of Usage                               | References |
|-----------------------------------|------------------------------------------------------------------------|---------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------|---------------------------------------------|------------|
| <i>A. ambotay</i> Aubl            | French Guiana                                                          | Not reported                                                              | Treating fever                                                                                                                        | Leaves and bark                                       | Leaves and bark crushed and rubbed on body  | [18]       |
| <i>A. cherimola</i> Mill.         | Tropical America<br>Asia<br>Gabon<br>Cultivated in Spain and Australia | Cherimola<br>Chirimoya<br>Chirimoya<br>Custard apple<br>Mao ye fan li zhi | Abortion<br>Anti-anxiety<br>Cough<br>Diarrhea<br>Hypercholesterolemia<br>Infections<br>Painful inflammations<br>Parasitic<br>Sedative | Aerial parts<br>Fruit<br>Leaf<br>Root<br>Seed<br>Stem | Not reported                                | [87,88]    |
| <i>A. coriacea</i> Mart.          | Brazilian (Cerrado, Caatinga)                                          | Araticum<br>Marolino<br>Marolo                                            | Anthelmintic<br>Chronic diarrhea<br>Inflammation<br>Leishmaniasis<br>Malaria<br>Rheuma                                                | Leaves<br>Root<br>Seeds                               | Not reported                                | [89]       |
| <i>A. cornifolia</i> St-Hil       | Bolivian and Brazilian savannah                                        | Not reported                                                              | Antiulcerative (green fruit)                                                                                                          | Seeds                                                 | Not reported                                | [90]       |
| <i>A. crassiflora</i> Mart.       | Brazil (Cerrado)                                                       | Araticum of the Cerrado<br>Araticum-mirim<br>Marolo<br>Panã               | Analgesic<br>Antimicrobial<br>Antirheumatic<br>Carminative<br>Digestive<br>Rheumatism,<br>Anti-inflammatory<br>Wound healing          | Leaves<br>Root bark<br>Root wood<br>Seeds<br>Fruit    | Not reported                                | [43,91,92] |
| <i>A. cuneata</i> (Oliv.) R.E. Fr | Congo                                                                  | Not reported                                                              | Asthenia<br>Female sterility<br>Hernia<br>Parasitic infections<br>Venereal diseases                                                   | Root bark<br>Stem bark                                | Not reported                                | [93]       |
| <i>A. dioica</i> A. St.-Hil.      | Brazil (Cerrado, Pantanal)                                             | Ceraticum and araticum                                                    | Diarrhea<br>Rheumatism                                                                                                                | Fruits<br>Leaves                                      | Dried leave paste and fresh fruit decoction | [11,33]    |

Table 5. Cont.

| Species                              | Region                                                                                                                                                 | Local Name                                                                                              | Medicinal Uses                                                                                                                                                                                                                                               | Part Used                                                                                                  | Mode of Usage                                                                               | References |
|--------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------|------------|
| <i>A. diversifolia</i> Saff          | Tropical forest of Central America                                                                                                                     | Ilama<br>Papausa<br>White anona,<br>Yi ye fan li zhi                                                    | Arthritic pain<br>Anti-spasmodic                                                                                                                                                                                                                             | Leaves<br>Seeds                                                                                            | Not reported                                                                                | [40,53]    |
| <i>A. foetida</i> Mart               | Brazil                                                                                                                                                 | Araticum-da-caatinga                                                                                    | Malaria                                                                                                                                                                                                                                                      | Bark and leaves                                                                                            | Decoction of bark and leaf                                                                  | [94]       |
| <i>A. glabra</i> L                   | Caribbean                                                                                                                                              | Mamain                                                                                                  | Fever                                                                                                                                                                                                                                                        | Leaves                                                                                                     | Not reported                                                                                | [95]       |
| <i>A. glauca</i> Schumacher & Thonn  | West Tropical Africa (Senegal, Ghana, Suriname)                                                                                                        | Dangan<br>Mampihege,<br>Mandé sunsun<br>Tangasu                                                         | Arachnoides<br>Blennorrhoea<br>Diuretic<br>Fish-poisons<br>Insecticides                                                                                                                                                                                      | Roots<br>Seeds                                                                                             | Not reported                                                                                | [96]       |
| <i>A. haematantha</i> Miq            | French Guiana<br>South American tropical rainforest                                                                                                    | Not reported                                                                                            | Fever                                                                                                                                                                                                                                                        | Leaves<br>Bark<br>Roots                                                                                    | Leaves and bark crushed rubbed on body                                                      | [18,97]    |
| <i>A. montana</i> Macfad.            | South America<br>Southern Asia<br>The Amazon<br>Brazil (Mata Atlântica, Pantanal)                                                                      | Mountain soursop<br>Shan<br>Di fan li zhi false<br>Graviola<br>Araticum grande<br>Jacá do Pará          | Against snake bite<br>Against obesity                                                                                                                                                                                                                        | Leaf<br>Pulp juice<br>Seed<br>Stem<br>Twig                                                                 | Not reported                                                                                | [98,99]    |
| <i>A. muricata</i> Linn              | Brazil                                                                                                                                                 | Araticum<br>Condessa<br>Graviola                                                                        | Anthelmintic<br>Analgesic, neuralgia, rheumatism, arthritis pain                                                                                                                                                                                             | Fruit, juice, and crushed seeds<br>Fruit and leaves                                                        | Juice of fruit<br>Water extraction of the leaf                                              | [66]       |
| <i>A. pickelii</i> (Diels) H. Rainer | Mexico<br>Caribbean<br>Central America<br>Venezuela<br>Colombia<br>Belize<br>Central America<br>South America<br>Southern Asia<br>Africa<br>Madagascar | Sincollo<br>Soncoyo<br>Bullock's-heart<br>Custard apple<br>Anona blanca<br>Anona<br>Niiu xin fan li zhi | Contraceptive<br>Blood dysentery<br>Cold<br>Stomachache<br>Fainting spinal disorders<br>Fever<br>Hysteria<br>Influenza<br>Mental depression<br>Skin diseases<br>Unhealthy ulcers<br>Wounds                                                                   | Leaf<br>Leaf<br>Root<br>Stem<br>Seed<br>Aerial parts<br>Bark<br>Fruit<br>Leaf<br>Root<br>Seed<br>Stem bark | Not reported                                                                                | [100,101]  |
| <i>A. reticulata</i> Linn            | West Indies                                                                                                                                            | Ramphal                                                                                                 | Bronchitis<br>Asthma<br>Bowel inflammation                                                                                                                                                                                                                   | Fruit<br>Seeds<br>Leaves                                                                                   | Decoction of fruit<br>Oral ingestion of powdered seeds<br>Oral ingestion of the leaf powder | [102]      |
| <i>A. senegalensis</i> Persoon       | Nigeria                                                                                                                                                | Ukopko (Idoma)                                                                                          | Anti-inflammatory<br>Analgesic<br>Anthelmintic<br>Cancer<br>Diarrhea<br>Epilepsy<br>Infectious diseases<br>Inflammations<br>Sleeping sickness<br>Snakebite<br>Cardiovascular diseases<br>Diabetes<br>Febrile seizures<br>Gout<br>Mental disorders<br>Painful | Leaves<br>Seed<br>Stem<br>Bark<br>Root bark                                                                | Roots and bark are ground together and their decoction is used                              | [103]      |
| <i>A. squamosa</i> Linn              | Cameroon                                                                                                                                               | Sugar apple (English);<br>Kedahan (Yambetta)                                                            | Vomiting, abscesses, muscle aches, fever, and skin disease                                                                                                                                                                                                   | Leaves                                                                                                     | Decoction of leaves                                                                         | [13]       |
| <i>A. vepretorum</i> Mart            | Brazil                                                                                                                                                 | Araticum<br>Bruteira                                                                                    | Analgesic and anti-inflammatory                                                                                                                                                                                                                              | Leaves                                                                                                     | Methanolic leaf extract                                                                     | [104]      |



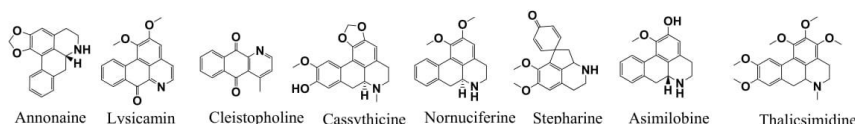
#### 4.2. Traditional and Ethnomedicinal Uses of *Annona* Genus

Traditionally, the *Annona* species have been used widely. For instance, antidiarrheal effects have been reported for *A. reticulata*, *A. muricata* and *A. salzmannii*, whereas *A. cherimola*, *A. squamosa* and *A. reticulata* have been reported for their antiparasitic effects (Table 5) [10]. Moreover, both *A. vepretorum* and *A. salzmannii* have been also reported for anti-inflammatory effects [10]. *A. purpurea* and *A. reticulata* have been used to treat fever, while anticancer effects have been reported for *A. senegalensis* and *A. muricata* [105,106]. Furthermore, *A. foetida*, *A. muricata* and *A. glabra* have been traditionally used to treat rheumatism [107], while *A. reticulata*, *A. salzmannii*, *A. foetida* and *A. squamosa* have been described for treating ulcers [4]. In Indonesia, the fruit juice of *A. muricata* has used as a diuretic and to treat liver ailments and leprosy [108], whereas leaves was used to treat spasms, boils and as an aphrodisiac [36]. The leaves of *A. diversifolia* have been used as anti-inflammatory, anticonvulsant and analgesic agents [52]. Ethnobotanically, despite reports of the toxicity of *A. muricata* seeds, the powder of toasted seeds has been reported to be used as an emetic and cathartic in the traditional Mexican pharmacopeia [36]. To South-east Asian people, the immature fruit of *A. reticulata* was used to treat both dysentery and diarrhea, and a decoction of roots was used to cure toothache and as an antipyretic [109]. Additionally, a decoction of leaves has been used internally against worms and topically to treat abscesses and boils [109]. Finally, the leaves of *A. squamosa* have been used as tonic and cold remedy in tropical America and systemically to cure dysentery in India [108].

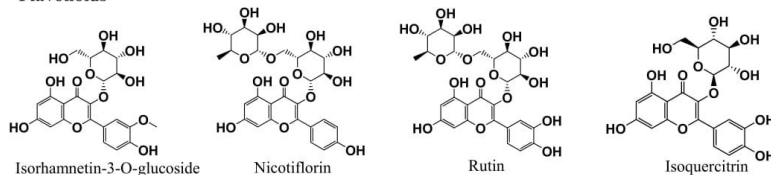
#### 5. Phytochemistry of *Annona* Species

A wide range of secondary metabolites, including acetogenins, flavonoids, alkaloids and essential oils (Figure 1), from nearly every part of *Annona* plants, have been discovered, isolated and characterised (Table 6). The plants of the *Annona* genera are also found to be rich in minerals and vitamins, for instance, calcium, potassium, magnesium, sodium, copper, zinc, selenium, phosphorus, iron, vitamin C, pantothenic acid B<sub>5</sub>, thiamine and riboflavin [6].

##### Alkaloids



##### Flavonoids



##### Terpenoids

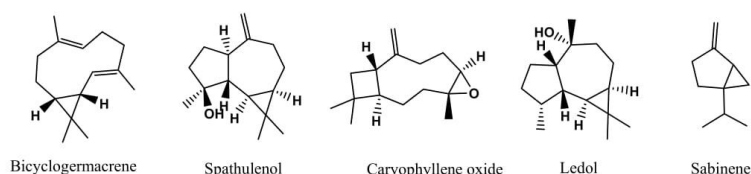
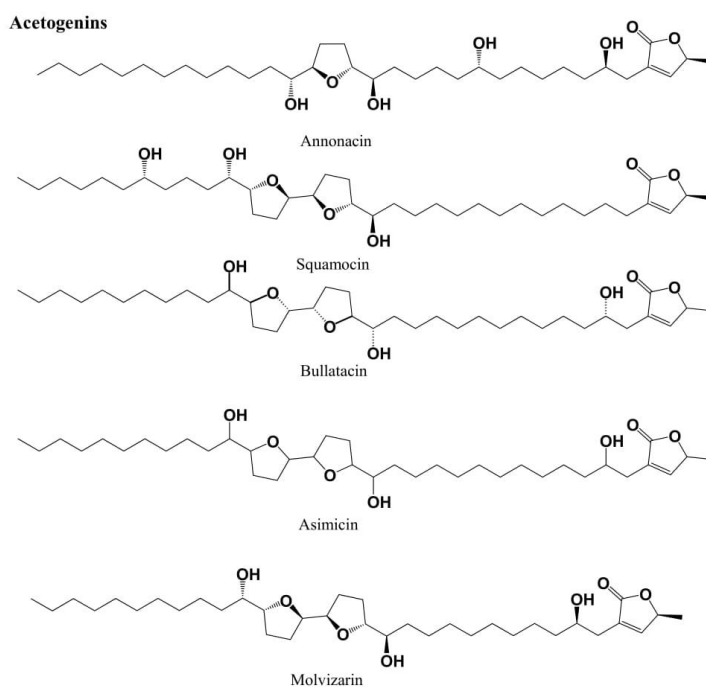


Figure 1. Cont.



**Figure 1.** Structure of selected compounds identified in *Annona* species.

**Table 6.** Compounds isolated from plants of *Annona* genus.

| Species                        | Part  | Isolated Compounds                                                                                                                                                                                                                                                                                                                                                                          | References |
|--------------------------------|-------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>A. amazonica</i> R.E. Fries | Stems | Cassythicine<br>Liriodenine (ALK)                                                                                                                                                                                                                                                                                                                                                           | [110]      |
|                                | Root  | Corytenchine, Isocoreximine (ALK)                                                                                                                                                                                                                                                                                                                                                           |            |
|                                | Fruit | $\alpha$ -Pinene, $\alpha$ -Thujene, Terpinen-4-ol, Germacrene D (ESO)                                                                                                                                                                                                                                                                                                                      |            |
| <i>A. cherimola</i>            | Seed  | 2,4- <i>cis</i> -Annocherinones, Annocherin, 2,4- <i>trans</i> -Isoannonacins, Annocherimolin, Annomolin, Annomocherin, Annomontacin, Annonacin, Asimicin, Tucumanin, 2,4- <i>trans</i> -Annocherinones, 2,4- <i>cis</i> -Isoannonacins, <i>cis</i> -Annonacin, Annogalene, Annosenegalin, Annomolon A, Annomolon B, Cherimolacyclopeptide C (ACT)                                          | [111–116]  |
|                                | Stem  | Annocherine A and B, Artabonatine B, Romucosine H, Cherianoine (ALK)                                                                                                                                                                                                                                                                                                                        |            |
|                                | Bulb  | Crolechinic acid, Crolechinic acid (methyl ester), Annonene, Annonalide (ESO)                                                                                                                                                                                                                                                                                                               |            |
|                                | Seed  | Gigantecin, Coriapentocin A and B, Bullacin (ACT)                                                                                                                                                                                                                                                                                                                                           |            |
| <i>A. coriacea</i> Mart.       | Leaf  | Quercetin-3-O- $\beta$ -(6''-O- $\beta$ -glucosyl)-glucoside, Quercetin-3-O- $\beta$ -(6''-O- $\alpha$ -rhamnosyl)-galactoside, Trigonelline, Rutin, Hyperin, Hyperin, Isorhamnetin-3-O- $\beta$ -glucoside, Isorhamnetin-3-O- $\beta$ -galactoside, Isoquercitrin, Isoquercitrin, Nicotiflorin, Biorobin, Keioside, Cacticin, Isorhamnetin-3-O- $\beta$ -glucoside, Narcissin, Rutin (FLA) | [117–123]  |
|                                | Root  | Coriacin, Coriadienin, Coriaheptocin A and B, Coriacyclodienin, Coriacycloenin, 4-Deoxycoriacin, Annoheptocin A and B (ACT)                                                                                                                                                                                                                                                                 |            |
| <i>A. crassiflora</i>          | Leaf  | Kaempferol-3-O- $\beta$ -diglucoside, Kaempferol-3-O- $\beta$ -glucoside, Quercetin-3-O- $\beta$ -D-galactopyranoside, Epicatechin, Quercetin-3-O- $\beta$ -L-arabinopyranoside (FLA)                                                                                                                                                                                                       | [124]      |

Table 6. Cont.

| Species                                       | Part                | Isolated Compounds                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          | References |
|-----------------------------------------------|---------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>A. foetida</i>                             | Bark                | Annomontine, <i>N</i> -Hydroxyannomontine, Liriodenine, <i>O</i> -methylmoschatoline (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | [125–127]  |
|                                               | Leaf                | ( <i>E</i> )-caryophyllene, Bicyclogermacrene, $\alpha$ -Copaene (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |            |
|                                               | Branch              | Atherospermidine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                      |            |
| <i>A. glabra</i>                              | Fruit               | 16 $\alpha$ -17-Dihydroxy-ent-kauran-19-oic acid, 16 $\alpha$ -Hydro-ent-kauran-17-oic acid, 16 $\beta$ -Hydroxy-17-acetoxy-ent-kauran-19-oic acid, 16 $\alpha$ -Hydro-19-al-ent-kauran-17-oic acid, 16 $\beta$ -Hydro-ent-kauran-17-oic acid, 19-nor-ent-Kauran-4 $\alpha$ -ol-17-oic acid, Annoglabin A and B, ent-Kaur-15-ene-17,19-diol, ent-Kaur-16-en-19-ol, ent-Kaur-16-en-19-oic acid, Methyl-16 $\alpha$ -hydro-19-al-ent-kauran-17-oate (ALK)                                                                                                                                                                                                                                                                                                                                                                                     | [128–132]  |
|                                               | Fruit & stem        | Annoglabin A, B, C, D, E and F, (–)-Anonaine, (–)-Asimilobine, (–)-Kikemanine, (–)-Nornuciferine, (+)-Stepharine, Blumenol A, Liriodenine, <i>N</i> - <i>p</i> -Coumaroyltyramine, (–)- <i>N</i> -Formylanonaine, (+)-Nordomesticine, Annobrine, Dehydrocorydalmine, Lysicamine, <i>N</i> -trans-Feruloyltyramine (ALK), 6- <i>O</i> -Palmitoyl- $\beta$ -sitosteryl-D-glucoside, $\beta$ -Sitosteryl-glucoside, Stigmasteryl-D-glucoside, $\beta$ -Sitosterol, Stigmasterol (STE)                                                                                                                                                                                                                                                                                                                                                          |            |
|                                               | Seed                | Isodesacetyluricin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |            |
|                                               | Leaf                | Bullatanocin, Glabracins A and B, Javoricin, Glacins A and B (ACT), 3- <i>O</i> - $\alpha$ -L-Arabinopyranoside, 3- <i>O</i> - $\beta$ -D-Glucopyranoside (GLU) (–)-Actinodaphnine, (–)-Asimilobine, (–)-Anolobine, (–)- <i>N</i> -Methylactinodaphnine, (–)-Roemeroline, (+)-Boldine, (+)-Norisodomesticine, (+)-Stepharine, Liriodenine, (–)-Pallidine, (+)-1S,2S-Reticuline <i>N</i> -oxide, (+)-Magnoflorine, (+)-Reticuline (ALK) Quercetin, Quercetin–3- <i>O</i> - $\beta$ -D-galactopyranoside (FLA)                                                                                                                                                                                                                                                                                                                                |            |
| <i>Annona leptopetala</i> (R.E.Fr.) H. Rainer | Leaves and branches | Laurotetanine, Nornuciferine, Corypalmine, Norannuradhapurine Anonaine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                | [133]      |
| <i>A. montana</i>                             | Leaf                | Annolatin, Annoretine, Liriodenine, Argentinine (ALK), $\beta$ -Sitosterol- $\beta$ -D-glucoside, $\beta$ -Sitosterol (STE), Montanacin-K, L, C, D, B and E, Annonacin-10-one, Annonacin-A, <i>cis</i> -Annonacin-10-one, Annonacin, <i>cis</i> -Annonacin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | [134–137]  |
|                                               | Seeds               | Montalicens G, Montalicens H Monlicins A & B, Murisolin, 4-Deoxyannomontacin, Muricatacin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |            |
|                                               | Stem                | <i>N</i> -trans-Feruloyltyramine, <i>N</i> - <i>p</i> -Coumaroyltyramine, <i>N</i> -trans-Caffeoyltyramine (PHE)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |            |
| <i>A. muricata</i>                            | Seed                | 2,4- <i>cis</i> -Gigantetrocinone, 2,4- <i>trans</i> -Isoailinonacin, 2,4- <i>trans</i> -Gigantetrocinone, 2,4- <i>trans</i> -Isoannonacin-10-one, Gigantetrocin-A, Muricatenol, Annomontacin, Gigantetronenin, Annonacin A, Annoreticum-9-one, <i>cis</i> -Annomontacin, Murisolin, Muricin H, Xylomaticin, Muricin I, <i>cis</i> -Annonacin, <i>cis</i> -Goniothalamycin, <i>cis</i> -Annonacin-10-one, Arianacin, Javoricin, Donhexocin, Murihexol, Cohibins C, Cohibins D, Gigantetrocin B, Longifolicin, Muricin A, B, C, D, E, F and G, Annomuricin B and C (ACT)                                                                                                                                                                                                                                                                     | [138–146]  |
|                                               | Stem bark           | Muricin A, B and C (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                    |            |
|                                               | Fruit               | Epomuricenins-A and B, Epomuricins-A and B, Epomusenins-A and B, Muricin J, K and L (ACT) Asimilobine, Nornuciferine, Annonaine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |            |
|                                               | Fruit & Root        | Sabadelin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                             |            |
|                                               | Leaf                | Annonacin, Annomuricin C, Muricatocin C, (2,4- <i>cis</i> )-10R-annonacin-A-one, (2,4- <i>trans</i> )-10R-annonacin-A-one, Annohexocin, Annomutacin, Annopentocins A, B and C, Annomuricine, Muricapentocin, Annomuricins A and B, <i>cis</i> -annonmuricin-D-ones, trans-annonmuricin-D-ones, Muricatocins A and B, Murihexocin A and B, Muricoreacin, Murihexocin C (ACT) (R)-4- <i>O</i> -methylcoclaurine, (R)- <i>O</i> , <i>O</i> -dimethylcoclaurine, (R)-Anonaine, Annonamine, (S)-Norcorydine, Anonaine, Isolaureline, Xylopin (ALK) Catechine, Epicatechine, Gallic acid, Chlorogenic acid, Kaempferol, Kaempferol-3- <i>O</i> -rutinoside, Quercetin-3- <i>O</i> -rutinoside, Quercetin-3- <i>O</i> -glucoside, Quercetin-3- <i>O</i> -neohispidoside, Quercetin-3- <i>O</i> -robinoside, Annoionols A and B, Annoionoside (FLA) |            |
|                                               | Leaf & seed         | Annonacin, Annocatacin A and B, Annonacinone, Annocatalin, <i>cis</i> -Corossolone, Goniothalamycin, Isoannonacin, Corossolone (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |            |
|                                               | Pericarp            | Annonacin, Annonacin A, Annomuricin A (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 |            |
| <i>A. muricata</i>                            | Root                | Annonacin, Muridienins-1, 2,3 and 4, Chatenaytrienins-1, 2 and 3, Muricadienin, Montecristin, <i>cis</i> -Panatellin, <i>cis</i> -Reticulatacin-10-one, <i>cis</i> -Uvariamicin IV, Coronin, <i>cis</i> -reticulatacin, <i>cis</i> -Solamin, Cohibins A and B (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                         |            |

Table 6. Cont.

| Species                     | Part         | Isolated Compounds                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | References   |
|-----------------------------|--------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------|
| <i>A. purpurea</i>          | Leaf         | Lirinidine, 7-Formyl-dehydrothalicimidine, 7-Hydroxy-dehydrothalicimidine, N-Methylasimilobine, N-Methylaurotetanine, Thalicsimidine, Norpurpureine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | [105,147]    |
|                             | Root         | Annomontine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |              |
| <i>A. reticulata</i>        | Leaf         | Dopamine, Salsolinol, Spathenelol, Muurolene, Coclaurine, Copaene, Eudesmol (ESO), Squamone, Solamin, Rolliniastatin 2, Anoreticu-9-one, Annonomicin, Annonaretin A (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           | [54,148–152] |
|                             | Stem bark    | Dopamine, Salsolinol (ESO), Reticullacinone, Rolliniastatin-2, (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |              |
|                             | Root         | Liriodenine, Norushinsunine, Neoannonin, Reticuline (ALK) Spathenelol, Copaene, Eudesmol, Muurolene (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                           |              |
|                             | Bark         | Reticulatacin, Liriodenine, Copaene, Coclaurine (ALK) Patchoulane (ESO) Molvizarin, Bullatacin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                |              |
|                             | Seed         | Squamocin, <i>cis-/trans</i> -isomurisolenin, Bullatacin, <i>cis-/trans</i> -Bullatacinone, Anoreticu-9-one, Solamin, Annonomicin, Isoannonaretin, Rolliniastatin-1, 2 Squamone, Annonaretin, 2, 4- <i>cis</i> -Isoannonaretin, Solamin, Murisolin, Reticullacinone, Annonomicin, Sitosterol, Anoreticu (ACT), Myrcene, Limonene, Germacrene D (ESO)                                                                                                                                                                                                                                                                                                                |              |
|                             | Fruit        | Terpinen-4-ol, Germacrene D, Limonene, Pinene, Myrcene (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        |              |
|                             | Root bark    | Anonaine, Michelalbine, Reticuline, Oxoushinsunine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            |              |
|                             | Leaf         | (–)-Roemerine, $\alpha$ -Humulene, $\gamma$ -Cadinene, Germacrene D, $\beta$ -Caryophyllene (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                   |              |
| <i>A. senegalensis</i>      | Aerial parts | (–)-Anonaine, (–)-Asimilobine, (+)-Normantenine (ALK) (+)-Catechin (FLA)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                            | [153,154]    |
|                             | Seed         | Annogalene, Annosenegalin (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     |              |
| <i>Annona sericea</i> Dunal | Leaf         | Normantenine, Normuciferine, Isoboldine, Lysicamine, Hydroxynormuciferine (ALK)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                     | [155]        |
|                             | Leaf         | (–) Anonaine, O-Methylarmepavine, $\beta$ -Caryophyllene, $\beta$ -Cedrene, (E)-Caryophyllene, Germacrene D, Bicyclogermacrene, Quercetin-3-O-glucoside (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |              |
|                             | Bark         | 2,4- <i>cis</i> -Mosinone A, 2,4- <i>trans</i> -Mosinone A, Anoreticu-9-one, Mosin B and C, Bullatacin, Bullatacinone, Squamone (ACT)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |              |
|                             | Pulp fruit   | $\alpha$ -Pinene, Limonene, Sabinene (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                          |              |
| <i>A. squamosa</i>          | Stem         | 11 <i>ent</i> -Kauranes, 10- <i>nor-ent</i> -Kaurane-4 $\alpha$ ,16 $\beta$ ,17-triol, 16 $\alpha$ ,17-Dihydroxy- <i>ent</i> -kauran-19-oic acid, 16 $\beta$ ,17-Dihydroxy- <i>ent</i> -kauran-19-al, 16 $\beta$ -Hydro- <i>ent</i> -kauran-17,19-dioic acid, 17-Hydroxy-16 $\beta$ - <i>ent</i> -kauran-19 oic acid, <i>ent</i> -Kaur-16-en-19-oic acid, 16 $\alpha$ ,17-Dihydroxy- <i>ent</i> -kauran-19-al, 16 $\alpha$ -Hydro-19-al- <i>ent</i> -kauran-17-oic acid, 16 $\beta$ ,17-Dihydroxy- <i>ent</i> -kauran-19-oic acid, 16 $\beta$ -Hydroxy-17-acetoxy- <i>ent</i> -kauran-19-oic acid, 4 $\alpha$ -Hydroxy-19- <i>nor-ent</i> -kauran-17-oic acid (ALK) | [156,157]    |
|                             | Seed         | Neoannonin-B, Annosquamins A, B and C, Annosquacin-I, Annosquamin A, B and C, Annosquatin A and B, Annotemoyin-1 and 2, Cherimolin-1 and 2, Diepomuricanin A and B, Dieporeticenin, Dieposabadelin, Squadiolin A, B and C, D, E, F, G, H, I, J, K, L, M and N, Squamostanin A, B, C, D, E and F, Cyclosquamosin A, B, C, D, E, F, G, H and I, Squamin A and B (ACT)                                                                                                                                                                                                                                                                                                 |              |
| <i>A. vepretorum</i>        | Leaf         | Spathulenol, Bicyclogermacrene, $\alpha$ -Phellandrene (ESO)                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                        | [116,158]    |

ALK (Alkaloids), ACT (Acetogenins), ESO (Essential oils), STE (Sterols), FLA (Flavonoids), PHE (Phenolics).

## 6. Pharmacological Properties of *Annona* Species

The species of the *Annona* genera have a been reported to elicit a diversity of biological activities such as antitumor, anti-inflammatory, antioxidant, antinociceptive, antiprotozoal, antipyretic, antiulcer, antihyperglycemic, anthelmintic, antileishmanial, antimalarial, antidiarrheal, antifungal and antimicrobial promoted by whole extracts, fractions, or pure compounds (Table 7).



Table 7. Pharmacological activities of *Annona* species.

| Species                | Biogeographical Distribution                      | Used Part                                                       | Traditional Use                                                                                                                                             | Pharmacological Activities                                                                                                                                                              | Extract/Compound Evaluated                                                                                                                                                                        | References |
|------------------------|---------------------------------------------------|-----------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>A. ambotay</i>      | South American tropical rainforest                | Trunkwood                                                       | Antipyretic                                                                                                                                                 | Antimicrobial                                                                                                                                                                           | Alkaloids                                                                                                                                                                                         | [159]      |
| <i>A. bullata</i>      | Endemic of Cuba                                   | Bark                                                            | Not reported                                                                                                                                                | Antitumoral                                                                                                                                                                             | 32-Hydroxybullatacinone                                                                                                                                                                           | [160]      |
| <i>A. cherimola</i>    | Tropical America, Asia, Spain<br>Australia, Gabon | Aerial partsFruit<br>Leaf<br>Root<br>Seed<br>Stem               | Abortion<br>Anti-anxiety<br>Cough<br>Diarrhea<br>Hypercholesterolemia<br>Infections<br>Painful inflammations<br>Parasitic<br>Sedative                       | Antidepressant<br>Antifungal<br>Antiprotazoal<br>Antitumoral<br>Antihypercholesterolemic<br>Antiulcerative<br>Antiviral<br>Insecticidal<br>Vasodilator                                  | Acetogenins: Molvizarin, Squamocin, Cherimolin-1, Motrilin, Aherradurin, Tucumanin, Annonoherin, Annonacin, Annonotacin, Alkaloids: Roemerine, Anonaine, Dehydroroemerine                         | [37,161]   |
| <i>A. coriacea</i>     | Brazilia (Cerrado and Caatinga)                   | Leaf<br>Root<br>Seed                                            | Leishmaniasis<br>Malaria<br>Rheuma<br>Anthelmintic<br>Chronic diarrhea<br>Inflammation                                                                      | Antifungal<br>Anti-inflammatory<br>Antitumoral<br>Insecticidal<br>Leishmanicidal<br>Trypanocidal                                                                                        | Acetogenins: Annoheptocins A-B, coriacin, 4-Deoxycoriacin, Coriaheptocins A-B, Coriadienin, Gigantecin                                                                                            | [10]       |
| <i>A. muricata</i>     | America, Asia, Africa                             | Bark<br>Leaf<br>Fruit<br>Root<br>Root bark<br>Seed<br>Stem bark | Anthelmintic<br>Antiscorbutic<br>Asthma<br>Cancer<br>Cough<br>Cystitis<br>Diabetes<br>Diuretic                                                              | Anti-arthritis<br>Antidepressant<br>Antidiabetic<br>Anti-inflammatory<br>Antimicrobial<br>Antimalarial<br>Antiviral<br>Hepatoprotective                                                 | Acetogenins: Solamin, Muricin H, Muricin I, cis-Annonacin, Muricins A-G, Muricoreacin, Muricapentocin, Gigantetrocin A, Annonopentocins A-C                                                       | [162,163]  |
| <i>A. salzmanii</i>    | Brazil                                            | Bark<br>Leaf                                                    | Not reported                                                                                                                                                | Antioxidant<br>Antimicrobial                                                                                                                                                            | Alkaloids: Reticuline, Anonaine, Laurelliptine, Isoboldine                                                                                                                                        | [101]      |
| <i>A. senegalensis</i> | Madagascar, Comoros, Cape Verde, Tropical Africa  | Leaves<br>Seed<br>Stem<br>Bark<br>Root bark                     | Anti-inflammatory and Analgesic<br>Anthelmintic<br>Cancer<br>Diarrhea<br>Epilepsy<br>Infectious diseases<br>Inflammations<br>Sleeping sickness<br>Snakebite | Anticonvulsant<br>Antidiabetic<br>Antidiarrheal<br>anthelmintic<br>Anti-inflammatory<br>Antimalarial<br>Antimicrobial<br>Antioxidant<br>Insecticidal<br>Hepatoprotective<br>Antitumoral | Aqueous extract. Ethanolic extract<br>Terpenoids, coumarins, flavonoids, tannins, alkaloids, quinones. Methanolic extract containing Annonosengalin, Annonalene.                                  | [10,164]   |
| <i>A. squamosa</i>     | Tropical America, Asia Australia                  | Seed<br>Stem<br>Bark<br>Root bark                               | Analgesic<br>Anthelmintic<br>Antirheumatic<br>Cancer<br>Digestive<br>Headache<br>Anti-inflammatory<br>Antimicrobial<br>Carminative                          | Antibacterial<br>Antidiabetic<br>Antilipidemic<br>Antioxidant<br>Antimalarial<br>Antigenotoxicity<br>Antileishmanial                                                                    | Acetogenins: Squadiolins A and B, Squafosin B, Bullatacinona, Squamona, Tetrahydrosquamone<br>Monoterpenes: Limonene, $\beta$ -Cubebene, $\beta$ -Caryophyllene, Spathulenol, Caryophyllene oxide | [10]       |

### 6.1. Antibacterial Activity

The antibacterial activities of *Annona* species have been reported in many studies, for example, both methanolic and ethanolic leaf extracts of *A. muricata* exhibited antimicrobial activity against *Staphylococcus aureus*, and this activity was attributed due to the presence of flavonoids, alkaloids and steroids in the extract [165,166]. In contrast, an aqueous extract of the peel of *A. muricata* did not show any activity [165,166]. The root of *A. reticulata* has also been investigated for its antibacterial activity against Gram-positive *Staphylococcus aureus*, *Bacillus cereus* and *Bacillus subtilis*, and Gram-negative *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella typhi* [54]. The root extract was found to possess pronounced activity against *Bacillus cereus* as well as notable inhibition against all tested strains [54]. Moreover, the leaves of *A. cherimola* have been reported for antibacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* with growth inhibition zone diameters of 11 mm and 14 mm, respectively [37]. The aqueous and methanolic seed extracts of *A. squamosa* have reported activity against *Staphylococcus aureus* with Minimum Inhibitory Concentrations (MIC) of 50 mg/mL and Minimum Bactericidal Concentrations (MBC) of 100 mg/mL [167]. The activity of the isolated compounds from *Annona* species has been reported in various studies; for instance, the fatty alcohol 11-hydroxy-16-hentriacontanone

isolated from leaves of *A. squamosa* has a reported activity against Gram-positive and Gram-negative bacterial strains, with MIC values of 25–50 µg/mL [168]. Additionally, the alkaloids liriodenine, annonaine, asimilobine, reticuline and cleistopholine isolated from *A. salzmannii* demonstrated activity against a range of Gram-positive bacteria, including *Kocuria rhizophila*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis* with MIC values from 25 to 500 µg/mL [36]. Notably, annonaine and asimilobine had activity equal to or better than the control chloramphenicol (MIC 50 µg/mL) against many of the species tested [36].

### 6.2. Anticancer and Antiproliferative Activity

Various studies have reported the anticancer activity of either crude extracts or isolated compounds from *Annona* species. For example, the leaves extract of both *A. squamosa* and *A. reticulata* exhibited potent antiproliferative effects against two human T-lymphotropic virus type = 1 infected cell lines (MT-1 and MT-2) with EC<sub>50</sub> values from 0.1 to 1 µg/mL [169]. In in vitro studies, the ethanolic extract of *A. muricata* leaves was reported for its cytotoxicity against promonocytic leukemic cells (U-937) with an LC<sub>50</sub> = 7.8 µg/mL [170]. Isocoreximine isolated from *A. cherimola* demonstrated cytotoxicity against multiple cancer cell lines. At a concentration of 50 µg/mL, isocoreximine inhibited cell viability of the breast cancer cell line (MCF-7) by 85.76%, human colorectal carcinoma cell line (HCT-15) by 63.05%, human prostate tumor cell line (PC-3) by 78.71%, human astrocytoma cell line (U-251) by 65.23% and human leukemia cell line (K-562) by 94.15% [112].

The antiproliferative activity of methanolic extracts from the leaves and seeds of *A. coriacea* was tested in vitro against a range of human tumor cell lines; including melanoma (UACC-62), non-small cell lung cancer cells (NCI-H460), colon cancer cell line (HT29), breast cancer (MCF-7) and leukemia (K-562). The seed extracts displayed potent antitumor activity with GI<sub>50</sub> values between 0.02 and 3.83 µg/mL, and the leaf extracts exhibited anticancer activity at concentrations ranging from 0.02 to 0.08 µg/mL [35,171]. The cytotoxicity of annonacin, found in many *Annona* species, has been reported against various cell lines derived from cervical cancer (HeLa and HeLa S3) with IC<sub>50</sub> 0.219 and 0.426 µg/mL, and ovarian cancer (PA-1 and SKOV3) with IC<sub>50</sub>s of 0.452 and 0.411 µg/mL [172]. The cytotoxicity of annonacin has also been demonstrated against bladder cancer (T24), breast cancer (MCH7) and skin cancer (BCC-1) with IC<sub>50</sub>s 0.324, 0.433 and 0.427 µg/mL, respectively [172]. The cytotoxicity of five other acetogenins (squamocin M, annofolin, isolongimicin B, glaucanisin, and annotacin) isolated from *A. cornifolia* against human breast cancer (MCF-7) was reported, with IC<sub>50</sub>s of approximately 0.3 µM [173]. In an in vitro study, the annoaceous acetogenins laherradurin and cherimolin-2 isolated from *A. diversifolia* were shown to have ED<sub>50</sub>s of 0.015 and 0.05 µg/mL, respectively, against the cervical cancer cell line (HeLa) [174].

In clinical studies, the anticancer activity of *A. muricata* has been reported in a small number of studies. A patient diagnosed with breast cancer has maintained stable disease activity with no reported side effects after using an aqueous extract of *A. muricata* leaves for more than five years [175]. Another patient with metastatic ovarian cancer experienced disease stability after starting to take a complementary medication containing *A. muricata* as a tablet [176]. Finally, the effect of *A. muricata* leaves extract revealed higher cytotoxicity in the supplemented group with colorectal cancer compared with the placebo group in a randomized controlled trial [177].

### 6.3. Antidiabetic and Antilipidemic Activity

Multiple studies have investigated the antidiabetic activity of various extracts from *Annona* plants such as *A. cherimola*, *A. squamosa*, *A. muricata* and *A. reticulata*. The ethanolic leaf extract of *A. cherimola* (300 mg/kg) was administered to alloxan-induced type 2 diabetic rats, and four hours later, blood glucose level had decreased from 331.5 mg/dL to 149.2 mg/dL [58]. The young leaves of *A. squamosa*, often in combination with black pepper (*Piper nigrum*), have been used in northern Indian traditional medicine as an anti-diabetic, and are

still in use today. Administration of aqueous *A. squamosa* leaf extract to streptozotocin-nicotinamide type-2 diabetic rats resulted in decreased blood glucose and increased levels of serum insulin [178]. Another traditional Indian medicine used as an antidiabetic and anti-lipidemic is a polyherbal formulation of *A. squamosa* fruits and *Nigella sativa* seeds. The polyherbal formulation administered over a one-month period, dose-dependently decreased blood glucose and increased insulin in streptozotocin-induced diabetic rats, with a dose of 200 mg/kg showing similar to the effects of a dose of 250 mg/kg tolbutamide [179]. A single dose of 100 or 200 mg/kg of aqueous leaf extract of *A. muricata* did not inhibit blood glucose levels in normal rats; however, the same doses administered to the diabetic rats effectively lowered blood glucose levels by 31.77% and 45.77%, respectively [180]. Finally, a dose of 100 mg/kg of both methanolic extract and the residual fractions of *A. reticulata* leaves decreased blood glucose levels from 432.33 to 371.67 mg/dL and 417.83 to 402.50 mg/dL, respectively, in streptozotocin-induced diabetic rats [181]. *A. cherimola* leaf extract was also found to decrease HbA1c by 7% and lead to a significant decrease in urine glucose over a 28-day subchronic study in streptozotocin-induced diabetic mice [182]. These studies support the traditional use of *Annona* species as antidiabetics, suggesting that further identification of the active constituent(s) with antidiabetic properties and clinical studies of a longer duration are warranted.

Limited studies have also investigated the antilipidemic activity of some *Annona* species. The polyherbal formulation of *A. squamosa* fruits and *Nigella sativa* seeds (200 mg/kg) administered to streptozotocin-induced diabetic rats for one month also resulted in significant inhibition of the formation of both lipid peroxide and tissue lipids [179]. Administration of an extract of *A. muricata* extracts resulted in reductions in the serum total cholesterol, low-density lipoprotein cholesterol and triglycerides in diabetic rats [183]. The tea infusion of leaves from *A. cherimola* (1.5 g) also elicited a reduction in the total cholesterol, triglycerides, and low-density lipoprotein by 15.4, 21.9 and 63.2%, respectively, in streptozotocin-induced diabetic rats [182].

#### 6.4. Anti-Inflammatory Activity

The anti-inflammatory effects of *Annona* plants have been reported in many studies; for instance, after one day of orally administered doses of 200 and 400 mg/kg of *A. squamosa* root extract in an acute carrageenan-induced rat paw edema model, significant inhibition was produced with 24% and 47% inhibition respectively compared to diclofenac sodium inhibiting inflammation by 72% [184]. In an in vitro study, the chloroform extract of *A. muricata* leaves significantly inhibited activity of phospholipase A<sub>2</sub> [185]. With doses of 0.2–0.6 mg/mL, the enzyme activity was inhibited by 23.91%–43.48% [185]. In the same study, the chloroform extract of *A. muricata* leaves at 0.5 and 1.0 mg/mL also inhibited prostaglandin synthase activity by 87.46% and 82.92%, respectively, compared to the positive control indomethacin at 1 mg/mL which reduced enzyme activity by 87.46% [185].

Extracts of *A. senegalensis* roots were assessed for anti-inflammatory activity through in vitro inhibition of protein denaturation, hyaluronidase and xanthine oxidase. The ethyl acetate fraction was found to have the greatest activity inhibiting protein denaturation (70.6%), hyaluronidase (72.2%) and xanthine oxidase (78.7%) at a concentration of 100 µg/mL [186]. The ethanolic extract of *A. muricata* leaves exhibited anti-inflammatory effects in the carrageenan-induced rat paw acute edema model. Paw edema was reduced after orally administered doses of 200 and 400 mg/kg with (23.16% and 29.33%) and (29.50% and 37.33%), respectively, after 60 and 90 min of treatment [187]. Additionally, *A. muricata* fruit has been shown to exert an anti-inflammatory effect in a xylene-induced ear edema test [188]. Additional information regards pharmacological activities of *Annona* species, see (Table 7).

The lyophilized fruit extract at 50 mg/kg and 100 mg/kg inhibited the xylene-induced ear edema by 82.35% and 76.47%, respectively, compared to prednisolone, which reduced ear edema by 47.06% [188]. At intraperitoneal doses of 25, 50 and 100 mg/kg, the ethanolic extract of *A. vepretorum* leaves, inhibited carrageenan-induced leukocyte migration to the



peritoneal cavity by 62%, 76% and 98%, compared to dexamethasone (2 mg/kg, i.p.). which reduced leukocyte migration by 89% [104]. The flavonoids, quercetin and kaempferol isolated from leaves of *A. dioica* exhibited potent dose- and time-dependent anti-inflammatory activity in a carrageenan-induced paw oedema model with  $IC_{50}$ s of 8.53 and 10.57  $\mu\text{g/mL}$ , respectively. The crude methanolic extract of *A. dioica* also reduced myeloperoxidase activity 6 h after the induction of paw oedema with a maximal inhibition of 51% at a dose of 300 mg/kg. [189]. Finally, hinesol, z-caryophyllene and beta-maaliene isolated from leaves of *A. sylvatica* also inhibited leukocyte migration at concentrations from 36.04 to 45.37  $\mu\text{g/mL}$  in both carrageenan- and complete Freund's adjuvant-induced mouse paw edema [33].

#### 6.5. Antioxidant Activity

An extract of *A. coriacea* seeds was investigated for its antioxidant activity using free radical 2,2-diphenyl-1-picrylhydrazil (DPPH) and bleaching of  $\beta$ -carotene, and a moderate antioxidant effect was reported of 31.53% in the DPPH test and 51.59% for the  $\beta$ -carotene bleaching test [190]. The pulp of *A. coriacea* fruit displayed a weaker antioxidant activity compared to the seeds, with 13.49% for the DPPH test and 32.32% for the  $\beta$ -carotene assay [190]. Additionally, various parts of *A. muricata*, including bark, leaves and stem, exhibited antioxidant activity using DPPH assay and the  $EC_{50}$  value was recorded as 90 mg/g for bark, 290 mg/g for leaves, and 116 mg/g for the stem, compared to ascorbic acid with 157.5 mg/g [44]. Finally, an ethanolic extract of *A. squamosa* leaves was also reported as having antioxidant activity when evaluating DPPH, nitric oxide and superoxide radical assays. The activity was reported as 75.12%, 34.69% and 10.29%, respectively, at a concentration of 100  $\mu\text{g/mL}$  [191].

#### 6.6. Antileishmanial Activity

Many extracts and pure compounds from *Annona* plants have been tested against *Leishmania*, such as methanolic seed and leaf extracts of *A. squamosa*, for activity against *L. amazonensis*, with resulting showing  $IC_{50}$ s of 46.54  $\mu\text{g/mL}$  and 28.32  $\mu\text{g/mL}$ , respectively [192]. Alkaloids and acetogenins isolated from both leaves and seeds of *A. squamosa* were also reported for their activity against promastigote forms of *L. chagasi*, with the  $EC_{50}$  value reported as 23.3  $\mu\text{g/mL}$  for alkaloids and from 25.9–37.6  $\mu\text{g/mL}$  for acetogenins [192]. Alkaloids like liriodenin isolated from the leaves of *A. mucosa* exhibited antileishmanial activity against promastigote forms of *L. braziliensis*, *L. guyanensis* and *L. amazonensis* with  $IC_{50}$ s of 55.92  $\mu\text{g/mL}$ , 0.84  $\mu\text{g/mL}$  1.43  $\mu\text{g/mL}$  respectively [193]. Finally, O-methylarmepavine isolated from leaves *A. squamosa* displayed antileishmanial activity against both promastigote and amastigote forms of *L. chagasi* with  $EC_{50}$ s of 23.3  $\mu\text{g/mL}$  and 25.3  $\mu\text{g/mL}$ , respectively [45].

#### 6.7. Antiviral Activity

The antiviral activity of various *Annona* species was reported in several studies using either whole extract or pure compounds. For instance, 16 $\beta$ ,17-dihydroxy-ent-kauran-19-oic acid was isolated from the fruits of *A. squamosa* and showed significant activity against human immunodeficiency virus (HIV) replication using H9 lymphocyte cell assay with  $EC_{50}$  value of 0.8  $\mu\text{g/mL}$  [194]. The ethanolic extract of *A. squamosa* seeds at 0.15  $\mu\text{g/mL}$ , 0.25  $\mu\text{g/mL}$  and 0.35  $\mu\text{g/mL}$  also exhibited dose-dependent antiviral activity against the Avian influenza virus with the percentage of antiviral activity at 33.33%, 43.06% and 59.72%, respectively [195]. The leaves of *A. squamosa* extract were also tested against dengue virus type-2 (DENV-2) in Vero cells using Viral ToxGLO™ assay. At a concentration of 6.25  $\mu\text{g/mL}$ , DENV-2 replication was reduced with  $IC_{50}$  73.78  $\mu\text{g/mL}$  in Vero cells [196]. The methanolic extracts from the peels of *A. squamosa* and *A. reticulata* demonstrated antiviral activity against human immunodeficiency virus 1 (HIV-1) using a non-radioactive immune/colorimetric assay. Both *A. squamosa* and *A. reticulata* revealed high antiviral activity by inhibition of HIV-1 reverse transcriptase with values of 96.45% and 78.63% [197].



Moreover, *A. cherimola* was also evaluated for its antiviral activity against herpes simplex virus type 2 (HSV-2) using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. The leaf extract inhibited HSV-2 replication and showed antiherpetic activity with a therapeutic index 8.40 [198]. Finally, the ethanolic stem extraction of *A. muricata* demonstrated antiviral activity against herpes simplex virus type 1 (HSV-1) with a minimum inhibitory concentration (MIC) of 1 mg/mL [199].

## 7. Pharmacological Activity of Isolated Compounds from *Annona* Species and their Mechanism of Action

The in vitro and in vivo biological activity of compounds that have been isolated from various parts of *Annona* plants will be discussed. Squamins C–F were isolated from the seeds of *A. globiflora* and tested in vitro against trophozoites of *Acanthamoeba* spp. strains such as *A. castellanii* Neff, *A. polyphaga*, *A. griffin* and *A. quina* (Table 8) [200]. All tested compounds exhibited antiameoboid activity against the strains by inducing programmed cell death [200]. The same compounds were also tested for their cytotoxicity against murine macrophage cell line J774A.1 (ATCCTIB-67) and showed no cytotoxicity effect with CC<sub>50</sub> values greater than 200 µM [200].

**Table 8.** Antiameobic activity of squamins C–F versus *Acanthamoeba* spp. Strains.

| Compounds | <i>A. castellanii</i> Neff IC <sub>50</sub> (µM) | <i>A. polyphaga</i> IC <sub>50</sub> (µM) | <i>A. griffin</i> IC <sub>50</sub> (µM) | <i>A. quina</i> IC <sub>50</sub> (µM) |
|-----------|--------------------------------------------------|-------------------------------------------|-----------------------------------------|---------------------------------------|
| Squamin C | 20.77 ± 3.48                                     | 71.78 ± 0.41                              | 38.81 ± 7.34                            | 24.28 ± 0.64                          |
| Squamin D | 18.38 ± 1.14                                     | 71.57 ± 0.14                              | 39.53 ± 5.90                            | 26.52 ± 0.87                          |
| Squamin E | 21.00 ± 0.86                                     | 62.19 ± 15.52                             | 44.75 ± 2.06                            | 25.82 ± 0.99                          |
| Squamin F | 18.02 ± 3.28                                     | 64.08 ± 12.42                             | 50.49 ± 6.92                            | 30.32 ± 0.27                          |

Rollinacin and rolliniastatin-1 isolated from the seed of *A. mucosa* were also reported for their larvicidal effect against *Aedes aegypti* and *Aedes albopictus* larvae [201]. Rolliniastatin-1 exhibited the best larvicidal effect against both *Aedes aegypti* and *Aedes albopictus* with LC<sub>50</sub>s of 0.43 and 0.20 µg/mL<sup>−1</sup>, respectively. Rollinacin displayed similar activity against *Aedes aegypti* and *Aedes albopictus* with LC<sub>50</sub>s of 0.78 µg/mL and 1.128 µg/mL, respectively [201]. However, the larvicidal mechanism action of these compounds was not reported. Annonacin isolated from the seed of *A. muricata* was evaluated for its larvicidal activity on *Aedes aegypti* and *Aedes albopictus* larvae [202]. The greater larvicidal activity was reported against *Aedes aegypti* with a LC<sub>50</sub> of 2.65 µg/mL compared to *Aedes albopictus* with LC<sub>50</sub> of 8.34 µg/mL. The mechanism of action was reported as being inhibition of their metabolic enzymes, particularly proteases and amylases that are important for the development of *Aedes* spp. larvae [202]. Twelve acetogenins isolated from the seed of *A. cornifolia* were tested for their antioxidant activity against DPPH [90]. These acetogenins were identified as 9-hydroxyfolianin, 4-desoxylongimicin, squamocin M, squamocin L, folianin A, folianin B, annofolin, isolongimicina B, bullatacin, asimicin, cornifolin and anotacin, and showed a strong DPPH radical scavenging with IC<sub>50</sub>s ranging from 0.99 ± 0.18 to 1.95 ± 0.34 µg/mL compared to ascorbic acid with an IC<sub>50</sub> 1.62 ± 0.35 µg/mL [90]. It has been suggested that the antiradical activity of acetogenins may be related to the α,β-unsaturated lactone ring moiety, which is also present in ascorbic acid [90]. Furthermore, the antioxidant activity of pure compounds from the bark of *A. salzmanni* after isolation of five alkaloids identified as liriodenine, anonaine, asimilobine, reticuline and cleistopholine [203]. The antioxidant activity was assessed through the Oxygen Radical Absorbance Capacity (ORAC) assay and asimilobine was found to be the most active alkaloid with ORAC value of 2.09 [203]. The rest of the compounds exhibited antioxidant activity with ORAC values ranging from 0.25 to 0.85 [203]. These same compounds were also examined for their antimicrobial activity against *Kocuria rhizophila*, *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Enterococcus faecalis* with MIC values from 25 to 100 µg/mL [203]. In an in vitro study, the antimicrobial activity of isolated alkaloids from aerial parts of *A. senegalensis* was assessed in a microdilution assay [154].

These alkaloids were identified as anonaine and asimilobine, and demonstrated against *Streptococcus mutans* with MIC values of 0.12 and 0.25 mg/mL, respectively [154]. For trypanocidal activity, three alkaloids lirioidenine, annomontine, and *O*-methylmoschatoline were isolated from the branch of *A. foetida* and tested against both epimastigote and trypomastigote forms of *Trypanosoma cruzi* [101]. A potent trypanocidal effect was demonstrated against epimastigote forms with IC<sub>50</sub>s ranging from 92.0 ± 18.4 to 198.0 ± 4.2 µg/mL, and from 3.8 ± 1.8 to 4.2 ± 1.9 µg/mL for trypomastigote forms [101]. Additionally, *N*-hydroxyannomontine isolated from the bark of *A. foetida*, demonstrated antileishmanial activity versus *Leishmania. braziliensis* and *L. guyanensis* with IC<sub>50</sub> values of 252.7 ± 2.2 and 437.5 ± 2.5 µM, respectively [204].

Many compounds isolated from various *Annona* species have demonstrated cytotoxicity against different cancer cell lines (Table 9). Three alkaloids were isolated from leaves of *A. crassiflora* identified as crassiflorine, xylopine and stephalagine and tested for their activity against colon carcinoma cells (HCT-116) using MTT assay [204]. The cytotoxicity activity for the tested compounds was reported with IC<sub>50</sub> values of 143.4 µM, 30.2 µM and 48.5 µM, respectively [204]. Muricin J–K isolated from the fruit of *A. muricata* exhibited anticancer activity against human prostate cancer cell lines (PC-3) through inhibition of the mitochondrial complex I in an in vitro study [205]. The anticancer activity was also reported for the alkaloid coclaurin isolated from aerial parts of *A. squamosa*. Cytotoxicity studies against human breast cancer cells (MCF-7), human colon cancer cells (HCT116) and human liver cancer cells (HEPG-2) reported IC<sub>50</sub> values of 15.345 µg/mL, 8.233 µg/mL and 1.674 µg/mL, respectively [206]. Bullatacin isolated from *A. cherimola* demonstrated inhibition of tumor growth at a dose of 15 µg/kg in mice bearing HepS and S180 xenografts and tumor growth was reduced by 63.4% and 65.8%, respectively [207]. In the same study, annonacin administrated orally (10 mg/kg) to hybrid mice (BDF-1) models significantly reduced lung cancer by 57.9% [207]. However, the mechanism of action of these acetogenins was not described in this study. Finally, stephalagin, an alkaloid isolated from the peel of *A. crassiflora* fruit, was reported as pancreatic lipase inhibitor with an IC<sub>50</sub> of 8.35 µg/mL<sup>−1</sup> in vitro study [208].

**Table 9.** Anticancer activity of isolated compounds from *Annona* species and their mode of action.

| <i>Annona</i> Species | Plant Part    | Isolated Components                           | Cell Line or Animal Model                                                                      | Mechanism of Action                                                      | References |
|-----------------------|---------------|-----------------------------------------------|------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------|------------|
| <i>A. cherimola</i>   | Seeds         | Annomolin and Annocherimolin                  | Prostate tumor cell line (PC-3), breast (MCF-7) and colon (HT-29) cancer cell lines            | Exhibited potent cytotoxicity                                            | [209]      |
|                       | Leaves        | Asimilobine                                   | Acute myeloid leukemia cell line                                                               | Upregulation of Bax, downregulation of Bcl2, and cleavage of PARP        | [210]      |
| <i>A. crassiflor</i>  | Crude extract | Catechin                                      | Cervical cancer cell                                                                           | Apoptosis via intrinsic pathway                                          | [211]      |
| <i>A. glabra</i>      | Fruits        | Annoglabasin H                                | Lung adenocarcinoma cell line (LU-1), human breast carcinoma (MCF-7), human melanoma (SK-Mel2) | Exhibited significant cytotoxic activity                                 | [212]      |
|                       |               | Annoglabayin                                  | Human liver cancer cell line (Hep G2)                                                          | Apoptosis via mitochondrial pathway                                      | [132]      |
|                       | Leaves        | Cunabic acid and ent-kauran-19-al-17-oic acid | Liver cancer (HLC) cell line SMMC-7721                                                         | Apoptosis via down-regulation of BCL-2 gene and upregulation of bax gene | [213]      |
|                       |               | Asinicin                                      | Human monocytic leukemia cells (CRL-12253)                                                     | Mitochondria mediated anticancer and antiproliferative effects           | [214]      |
|                       |               | Annoglacin A and B                            | Human breast carcinoma (MCF-7) and Pancreatic carcinoma (PACA-2) cell lines                    | Suppressed proliferation                                                 | [215]      |
|                       |               | Icariside D2                                  | Human leukemia cell line (HL-60)                                                               | Induced apoptosis and decreased phosphorylation of AKT in cells          | [216]      |

Table 9. Cont.

| <i>Annona</i> Species           | Plant Part | Isolated Components                          | Cell Line or Animal Model                                                                  | Mechanism of Action                                                                                                                | References |
|---------------------------------|------------|----------------------------------------------|--------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------|------------|
| <i>A. muricata</i>              | Leaves     | Annomuricin                                  | Breast cancer cell                                                                         | Suppressed breast cancer proliferation and induced apoptosis                                                                       | [217]      |
|                                 |            | Muricoreacin, Murihexocin                    | Colon cancer cell (HT-29, HCT-116)                                                         | Up-regulation of Bax, downregulation of Bcl-2 proteins and activated initiator and executioner caspases                            | [218]      |
|                                 |            | Annomuricine, Muricapentocin                 | Pancreatic carcinoma (PACA-2) and colon adenocarcinoma (HT-29) cell                        | Exhibited repressive effect                                                                                                        | [219]      |
|                                 |            | Muricatocins A and B                         | Lung tumor cell line (A-549)                                                               | Enhanced cytotoxic activity                                                                                                        | [146]      |
|                                 | Fruits     | Muricin M and Muricin N                      | Prostate cancer (PC-3) cells                                                               | Exhibited cytotoxicity                                                                                                             | [220]      |
| <i>A. purpurea</i>              | Roots      | Annopurpurici-ns A–D                         | HeLa and HepG2 cells                                                                       | Mitochondrial membrane depolarization and apoptosis                                                                                | [221]      |
| <i>A. reticulata</i>            | Fruits     | Catechin                                     | Breast cancer cell line (MCF-7)                                                            | Inhibition via apoptosis                                                                                                           | [222]      |
|                                 | Seeds      | Annonacin                                    | T24 bladder cancer cells                                                                   | Bax expression was induced, caspase-3 activity enhanced and caused apoptosis                                                       | [172]      |
|                                 |            | Bullatacin                                   | Leukemia cell line (K562) and breast cancer cell line (MCF-7)                              | Cell death via apoptosis                                                                                                           | [223]      |
|                                 | Leaves     | Annomonicin                                  | Colon cancer (HCT15), human lung cancer (Hop65) and human hepatoma (HEPG2) cell lines      | Exhibited cytotoxic effect                                                                                                         | [224]      |
|                                 |            | Rolliniastatin                               | Breast cancer cell (T-47D)                                                                 | Caspases dependent apoptosis                                                                                                       | [225]      |
| <i>A. senegalensis</i>          | Leaves     | (–) Roemerine                                | Breast cancer MDA-MB-231 cells                                                             | Exhibited dose-dependent cytotoxicity via targeting the ribosomal protein eL42 and arresting the crosslinking reaction with tRNAox | [226]      |
|                                 | Bark       | Kaurenoic acid                               | Pancreatic tumor (PANC-1) cell lines and Henrietta Lacks' cervical cancer cell line (HeLa) | Exhibited significant cytotoxic activity                                                                                           | [227]      |
|                                 | Stem       | Ent-kaurenoids                               | Breast cancer (MCF-7) cells, prostate cancer (PC-3) cells                                  | Exhibited significant cytotoxic activity                                                                                           | [228]      |
| <i>A. squamosa</i>              | Leaves     | Annoreticuin                                 | Breast cancer cell (MCF-7)                                                                 | Induced Apoptosis                                                                                                                  | [229]      |
|                                 | Seeds      | Dieporeticenin B, Squamocin, Annosquatin III | Nasopharyngeal cancer (KB) cells, breast cancer (MCF-7) cells                              | Exerted inhibitory activity                                                                                                        | [230]      |
|                                 |            | Asimilobine                                  | Human colon cancer cell (WiDr)                                                             | Increased expression of caspase-3                                                                                                  | [231]      |
|                                 |            | Annosquatin A, B                             | Human leukemia cell line (K-562), human colon carcinoma (COLO-205)                         | Reduced intracellular glutathione levels and regulation of Bcl-2 and PS externalization                                            | [232]      |
|                                 |            | Annosquacins A–D, Annosquatin A, B           | Human breast cancer cell line (MCF-7), human lung adenocarcinoma cell line (A-549)         | Exhibited cytotoxic activity                                                                                                       | [233]      |
|                                 |            | (–)-Anonaine                                 | H22 solid tumor cell                                                                       | Inhibition of IL-6/Jak/Stat3 pathway                                                                                               | [234]      |
|                                 | Bark       | Coclaurine                                   | DMBA painted hamsters                                                                      | Enhanced lipid peroxidation                                                                                                        | [235]      |
| <i>A. sylvatica</i><br>A.St-Hil | Leaves     | Quercetin<br>Kaempferol                      | Anti-inflammatory                                                                          | Leukocytes migration was significantly reduced at IC <sub>50</sub> 8.53 and 10.57 µg/mL, respectively.                             | [189]      |
| <i>A. vepretorum</i><br>Mart.   | Leaves     | Bicyclogermacrene                            | Antimicrobial                                                                              | Against <i>Candida tropicalis</i> with a MIC value of 100 µg · mL <sup>−1</sup> .                                                  | [237]      |



## 8. Toxicity and Interactions

Generally, the safety of natural medicines can be assessed according to their effects and drug-drug interactions. An epidemiological study has reported that consumption of fruits of Annonaceae led to the prevalence of atypical parkinsonism in Guadeloupe due to the presence of acetogenins in the plant fruits [238]. According to Champy et al. 2005, the amount of annonacin per a single fruit is approximately 15 mg, and an adult who consumes a daily intake of one fruit for one year is equivalent to the amount of annonacin injected into rats, which induced the brain lesions [239]. It has been suggested that the toxicity might be related to the capacity of the tetrahydrofuran ring to chelate calcium ions [35]. Moreover, the fruit of *A. squamosa* has been analysed for its quantity of squamocin using HPLC-MS and reported 13.5–36.4 mg of squamocin for each fruit, and that long-term consumption of *A. squamosa* fruit may be a risk factor in the development of neurodegenerative disorders [240]. Additionally, the use of a dietary supplement sold in the USA containing an extract of *A. muricata* has been found to exhibit neurotoxic effects in human neuron cultures [241].

The interactions of *Annona* species with other drugs have been reported in other studies; for instance, administration of capsules of *A. muricata* leaves in combination with glibenclamide resulted in improved glycaemic control compared to patients who received only glibenclamide [242]. An additional study reported that a combination of aqueous custard apple leaf extract and glipizide enabled a decrease in the dose of glipizide by up to half in rats with type-2 diabetes and reduced the risk of requiring insulin therapy [243]. These outcomes suggest the potential use of certain *Annona* species in conjunction with antidiabetic medications to maximize the efficacy of a lower therapeutic dose.

## 9. Conclusions

This review provides a comprehensive summary of the botanical features, ethnomedical uses, pharmacology and phytochemistry of the main species of Annonaceae family and, in particular, the *Annona* genera used in traditional medicine practices. Of the many members of the Annonaceae family, the *Annona* species is heavily used in traditional medicines across the world. Among the 30 reviewed *Annona* species, six species *A. squamosa*, *A. muricata*, *A. cherimola*, *A. senegalensis*, *A. reticulata* and *A. coriacea* are the most widely studied for their pharmacological activities and phytochemical profiles of their bark, leaves, fruits and seeds. Various pharmacological properties have been reported, including antidiabetic, hepatoprotective, anti-inflammatory, antiprotozoal, antitumor, antioxidant, antimicrobial and anticonvulsant activity.

With regard to the phytochemistry of *Annona* species, the main classes of constituents identified to date are acetogenins, alkaloids, phenols and essential oils. The alkaloids are mainly present in the leaves, whereas acetogenins are present in the seeds and found in smaller quantities in the pulp and leaves of *Annona* species. The chemical profiles of the acetogenins present in different species have been extensively studied and their anticancer activity investigated, with low concentrations exhibiting chemotoxicity against several cancer cell lines. These preclinical results, along with the reported case studies, suggest that further clinical studies evaluating the role of acetogenins in the treatment of various types of cancers are warranted. Importantly, formulations, including the parts of the *Annona* species used, agricultural practices and the extraction methods vary considerably, leading to likely variations in the phytochemical and pharmacological profiles. In this respect, further characterization of standardized formulations of *Annona* species is required to predict likely clinical effects. Additional interesting results on the antidiabetic effects of fruits from *Annona* species also warrant further investigation as nutraceuticals to assist in the therapy of diabetes.

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Review

# The Phytochemical Constituents and Pharmacological Activities of *Annona atemoya*: A Systematic Review

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**Abstract:** *Annona atemoya* also known as the custard apple is a hybrid between two Annonaceae species: Cherimoya (*Annona cherimola*) and the sugar apple (*Annona squamosa*). It is widely cultivated in tropical and subtropical continents including north and south America, Asia, Africa and Australia. Despite becoming an increasingly important commercial fruit plant due to its' creamy succulent flesh, compared to other Annonaceae species relatively few studies have investigated the phytochemistry and bioactivities of *A. atemoya*. Studies that evaluated *A. atemoya* extracts and its constituents were searched through the databases Scopus, Pubmed and Embase from inception to June 2020. Constituents of *A. atemoya* include alkaloids, flavonoids, terpenes and acetogenins. The results indicate that the constituents of *A. atemoya* possess cytotoxic, anti-angiogenic, hypolipidemic, antioxidant, anti-inflammatory and neuroprotective activities. However, many of these studies are currently limited in quality and further phytochemical and pharmacological studies are required.

**Keywords:** *Annona atemoya*; custard apple; nutraceutical; phytochemistry; bioactivity; pharmacological activity

## 1. Introduction

*Annona atemoya* is a commercially important fruiting plant belonging to the Annonaceae family [1]. *A. atemoya*, is widely cultivated in tropical and subtropical continents including southern and northern America, Asia, Africa and Australia [2], Spain and Israel [3]. *A. atemoya* is a hybrid between two Annonaceae species: Cherimoya (*Annona cherimola*) and the sugar apple (*Annona squamosa*). Hybridization was conducted by P. J. Webster in Florida (USA) between 1907 and 1908 [2,3]. However, natural hybridization is also believed to have occurred between 1850 and 1930 in Australia and Palestine [3]. The cultivars vary between countries e.g., United States of America (Bradley, Page, Keller, Priestly, Stremmer and Caves) [2]; Brazil (African Pride, Pink's Mammoth, Thompson and Gefner) [4]; Australia (Island Gem, Nielsen, Hillary White, Maroochy Gold, Pink's Mammoth and African Pride) [2,5]; Israel (Jennifer, Kabri, Malalai, African Pride and Gefner) [2] and Thailand (Golden Flesh and Pet Pakchong) [5].

*A. atemoya* grows to approximately 7.5–10 m, it is a short-bunked with low drooping branches [2,3]. The leaves of *A. atemoya* are alternate, leathery, deciduous and less hairy than those of the parent cherimola, averaging 15 cm in length [2]. The flowers are triangular, long stalked and yellow in colour, approximately 6 cm long and 4–5 cm in width. The fruit is fleshy, pale to bluish green, and variable shape from heart-shaped to round (2–2.5 kg), with a bumpy skin and its surface covered with prominent angular areoles that have some protuberances [2,3]. The fruits are also characterized by a pleasant flavour, the flesh is white and the pulp can be easily separable from the seeds which are



smooth and brown to black in colour [1,3,5,6]. In Australia, this hybrid is commonly known as the “custard apple” and the increasingly popular fruit is available in retail food outlets [3,7].

Various *Annona* species such as *Annona muricata* have documented indications for use in traditional and complementary medicine practice [8]. As a relatively recent hybrid, traditional uses of *A. atemoya* are lacking, with some limited anecdotal evidence that the leaves of *A. atemoya* are purchased from growers for making teas. Several phytochemical constituents have been isolated from different parts of the *A. atemoya* plant and assessed for their biological potential in both *vivo* and *vitro* studies [9–12]. The leaves, fruits and seeds of *A. atemoya* are the most widely studied for their chemical and pharmacological properties. Although *A. atemoya* is a hybrid of *A. squamosa* and *A. cherimola* and both species have reported uses in traditional and folkloric medicine [10,13–15], to date only limited studies have investigated the phytochemistry and bioactivities of *A. atemoya*. The aim of this review was to systematically search the literature to identify studies reporting on the phytochemical constituents and bioactivity of *A. atemoya*.

## 2. Methodology

### 2.1. Search Strategy

A systematic search of the literature published from inception to 9 June 2020 was performed by using the three electronic databases Scopus, Pubmed and Embase using the search terms: “*Annona atemoya*” OR “custard apple” OR acetogenins OR bullatacin AND phytochemistry OR “phytochemical constituents” AND “pharmacological effects” OR “biological activities” OR anti-cancer OR anti-bacterial OR antioxidant OR anti-angiogenic AND toxicity OR toxicology.

### 2.2. Inclusion and Exclusion Criteria

Studies reporting on the pharmacological properties of isolated compounds or whole extracts of *A. atemoya*, and/or studies which examined the phytochemical constituents of any parts of the plant (aerial part, leave, fruit, seed, stem and root) were considered for inclusion in this review. Finally, regards language, both English and non-English language (one Portuguese paper) were included. Articles that discussed isolation or biological activity of acetogenins, synthetic acetogenins and bullatacin were not included unless they had been isolated from *A. atemoya*.

### 2.3. Data Extraction

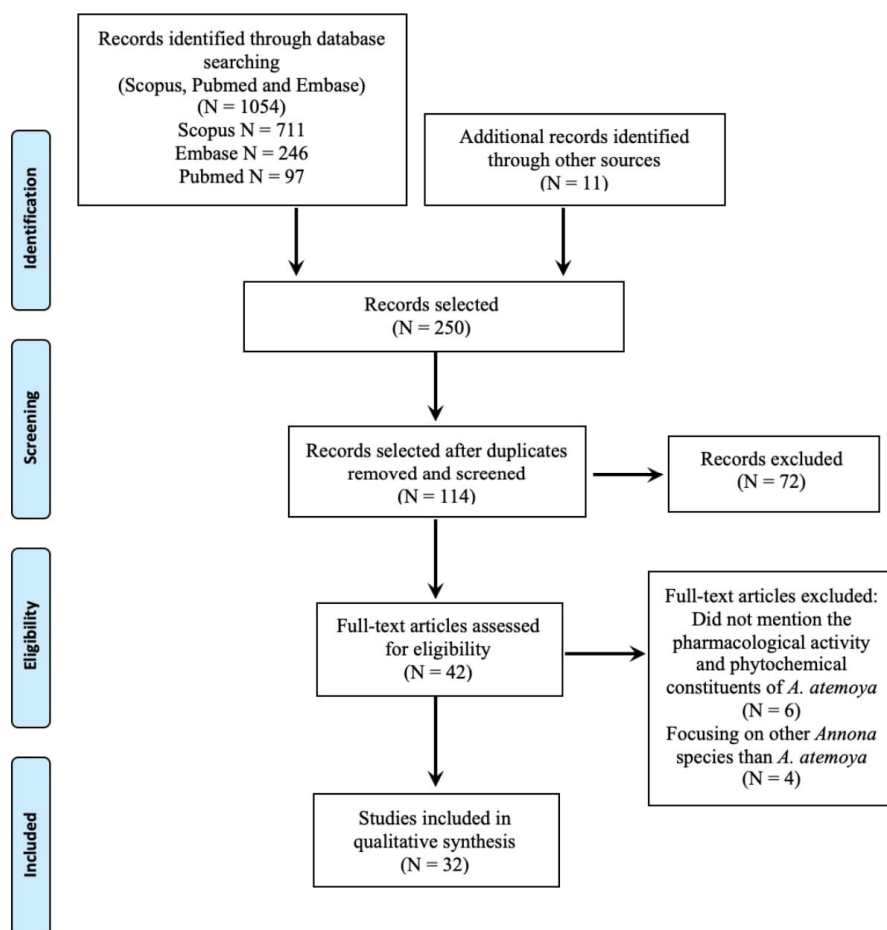
Data extracted included information relating to the pharmacological and phytochemistry of *A. atemoya*: method of extraction, solvent of extraction, analytical techniques used for identifying pure compounds, structure of pure compounds, pharmacological activity of both crude extracts and pure compounds, animal tested, cell line tested, dosage range, route of administration, duration of treatment, in vivo or in vitro study, and the reported outcomes.

## 3. Results

### 3.1. Eligible Studies

As presented in Figure 1, a total of 1054 abstracts were identified in the initial search. After title and abstract screening and removal of duplicates, 114 studies were further evaluated for key terms and relevance to the aims of the review. Of the 114 abstracts identified, a further 72 articles were removed due to not meeting the inclusion criteria. A total 42 full text articles were evaluated resulting in a further 10 articles being removed due to not reporting pharmacological activity or phytochemical constituents or focusing on an *Annona* species other than *A. atemoya*. A total of 32 articles were included in this review. Of the 32 selected articles, 18 studies examined the phytochemical constituents of various parts of *A. atemoya* and 14 studies investigated the biological activities in vivo ( $n = 5$ ), in vitro ( $n = 7$ ) and two studies discussed its in vitro toxicity.





**Figure 1.** Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flowchart of literature search strategy and results.

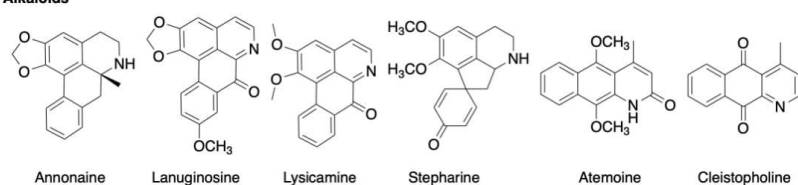
The quality assessment of animal studies was undertaken using the Animal Research: Reporting of In Vivo Experiments (ARRIVE) quality assessment criteria including ethical statement, study design, experimental procedure, experimental animals, housing and husbandry, sample size, allocating animals to experimental groups, experimental outcomes, statistical methods, baseline data, number of analysed, outcomes and estimation and adverse events [16]. Each ARRIVE quality assessment criteria was assessed as being met or not. Two authors agreed on the inclusion, exclusion criteria and search strategy. This systematic review was conducted by one author and reviewed by two authors.

### 3.2. Phytochemicals in *A. atemoya*

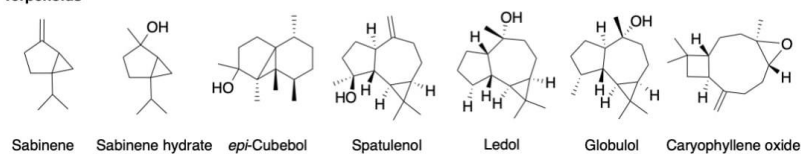
As presented in Table S1, an extensive number of phytochemicals have been isolated from the parts of *A. atemoya*, the structures of some representative phytochemicals from *A. atemoya* are shown in Figure 2. The fruit has been reported to contain a wide range of volatile oils and phenolic compounds [17–19]. The seeds contain alkaloids and annonaceous acetogenins, and the leaves contain

alkaloids and phenolic compounds [20–26]. *A. atemoya* is rich in the minerals iron, calcium, magnesium, zinc, and vitamins including thiamine, riboflavin, niacin,  $\alpha$ -carotene,  $\beta$ -carotene, cryptoxanthin and ascorbic acid [19,26–28].

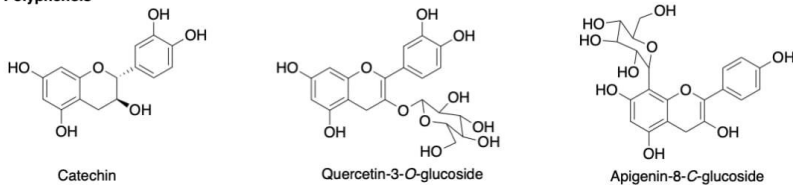
#### Alkaloids



#### Terpenoids



#### Polyphenols



#### Acetogenins

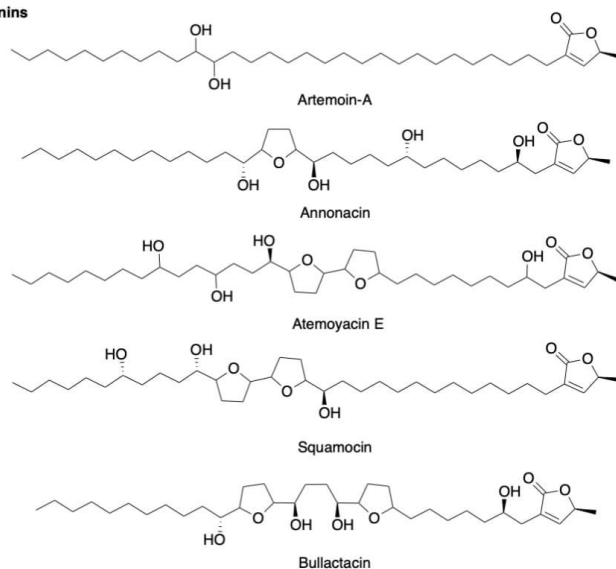


Figure 2. Structures of some representative phytochemical constituents of *Annona atemoya*.

### 3.2.1. Fruits

As the fruit of *A. atemoya* has a unique sweet and custard like aroma when ripe, the volatile constituents of *A. atemoya* have been investigated in many studies and the fruit is found to be the richer in volatiles compared to other parts of the plant [17,19]. Gas chromatography/mass spectrometry (GCMS) analysis of the essential oils from the mature, fresh and ripe fruits collected in South East Queensland Australia and stored at 25 °C for five days identified 40 phytochemical components [17]. They reported that 94% of the GC peak area contained esters, with methyl butanoate, ethyl butanoate and methyl hexanoate being the predominant constituents. The main terpenes from a total of 25 identified, were  $\alpha$ -pinene,  $\beta$ -pinene, (*E*)-ocimene and germacrene D [17]. In a separate study, mono and sesquiterpene hydrocarbons were the main class of aromatic volatiles identified making up 77% and 80% of total volatile constituents identified [17]. The main compounds identified in these three studies were,  $\alpha$ -pinene (70.4 ppm), limonene (44.2 ppm),  $\beta$ -pinene (27.3 ppm), germacrene D (20.0 ppm) and bornyl acetate (14.6 ppm) [6,7,17].

Additionally, another study reported the volatile constituents isolated from immature fruits of *A. atemoya* by various drying methods including solar drying and oven drying at 30 °C and at 50 °C [18]. The results of this study showed some similarities to the previously mentioned studies. Oven drying at 50 °C led to more volatile compounds being identified compared to the other methods. Both mono and sesquiterpene hydrocarbons were the largest group of phytochemicals identified [18].

Using liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS), the fruit of *A. atemoya* was shown to contain phenolic compounds including epicatechin and catechin at concentrations of 211  $\mu\text{g/g-dw}$  and 38.6  $\mu\text{g/g-dw}$ , respectively [27]. Other compounds were identified at minor concentrations: 3,4-dihydroxybenzoic acid ( $7.75 \pm 0.18 \mu\text{g/g-dw}$ ), chlorogenic acid (1.79  $\mu\text{g/g-dw}$ ) and *p*-coumaric acid (1.77  $\mu\text{g/g-dw}$ ). Caffeic acid and ferulic acid, rutin and quercetin were not detected in the samples tested in this study [27].

One study on the antitumour effects of bullatacin states that bullatacin is isolated from the fruit of *A. atemoya* [29], yet reference cited the paper by Chang et al. (1999) reporting bullatacin as being isolated from the seeds [20]. Given that acetogenins have not been reported as being isolated from *A. atemoya* fruit, it is most likely that the bullatacin was isolated from the seeds of the fruit.

### 3.2.2. Leaves

The alkaloid constituents isolated by extraction with hexane and methanol from the leaves of *A. atemoya* collected in Petrolina in Brazil have been reported [30]. The phytochemical investigation of the methanol extract led to the identification of seven alkaloids: anonaine, asimilobine, lanuginosine, liriodenine, lysicamine, pronuciferine and stepharine were identified by mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. This was the first time that these alkaloids have been identified in the leaves of *A. atemoya* [30]. A recent study also reported similar compounds isolated from an 3:1 ethanol:water extract of the leaves of a commercial cultivar of *A. atemoya* grown in Sicily, however, the alkaloids anonaine and asimilobine, were not detected [31]. *A. atemoya* was also found to have comparable amounts of both oxoaporphine (liriodenine and lanuginoside) and proaporphine (stepharine and pronuciferine) alkaloids in the leaves [31]. In the same study, a number of phenolic compounds were isolated from the leaves of *A. atemoya* such as quercetin-3-*O*-rutinoside-7-*O*-glucoside, quercetin-3-*O*-rutinoside-7-*O*-pentoside, quercetin-3-*O*-rutinoside, kaempferol-3-galactoside-7-rhamnoside, quercetin-3-*O*-glucoside, kaempferol-3-*O*-glucoside, luteolin-3-galactoside-7-rhamnoside, luteolin-3-glucoside-7-rhamnoside, apigenin-8-*C*-glucoside, catechin and epicatechin [31].

### 3.2.3. Seeds

There have also been limited studies on the phytochemical constituents from the seeds of the *A. atemoya*. According to many studies, the annonaceous acetogenins are the main phytochemical

components of seeds compared to both phenolic and alkaloid constituents [20–25,32,33]. The seeds of *A. atemoya* collected in Taiwan, were crushed and extracted with EtOAc and then partitioned to yield aqueous and chloroform (CHCl<sub>3</sub>) extracts [20]. The CHCl<sub>3</sub> layer was concentrated and partitioned between *n*-hexane and MeOH. A waxy residue (140 g) was collected from the MeOH extract and acetogenins were identified by testing with Kedde's reagent [20]. The MeOH fraction was subjected to column chromatography and eluted with CHCl<sub>3</sub>, *n*-hexane and CHCl<sub>3</sub> + MeOH resulted in 30 fractions. These fractions were re-chromatographed and then recrystallized using various solvent such as *n*-hexane: EtOAc (2:1) and MeOH:CHCl<sub>3</sub> (20:1) [20]. The study identified 17 annonaceous acetogenins present in the seeds (12,15-*cis*-squamosatin-D, 12,15-*cis*-squamosatin-A, squamosatin-A, squamosatin-D, neoannonin, artemoin-A, artemoin-B, artemoin-C, artemoin-D, squamocin, bullatacin, bullatanocin, bullatalicin, 12,15-*cis*-bullatanocin, 12,15-*cis*-bullatalicin, desacetyluvaricin and isodesacetyluvaricin) and their structures were established [20].

A study of the seeds of *A. atemoya* collected in China and then extracted with 95% EtOH was carried out [32]. The residue was partitioned between H<sub>2</sub>O and EtOAc, to yield 12.5 g of a waxy compound from the organic phase. The wax was subsequently purified using a column chromatography with an eluent of cyclohexane and acetone (1:1) [32]. One fraction collected and then purified via preparative thin layer chromatography afforded a whitish wax (Atemoyacin A) [32].

Additionally, four studies examined the phytochemical constituents of seeds collected in Australia [21–25]. A total of 22 compounds were identified (annonisin, parviflorin, asimicin, cherimolin-1 and -2, mortilin, molvizarin, rolliniastatin-1, annonacin, almunequin, atemoyin, desacetyluvaricin, squamocin, rolliniastatin-2, neoannonin, isodesacetyluvaricin, uvariamicin-III, annotemoyin-1 and 2, reticulatin-1, bulladecin and atemotetrolin) and their structures determined spectroscopically [21–25]. Finally, chromatographic purification of the ethanolic extracts of the seeds of *A. atemoya* afforded atemoyacin E [33].

As would be expected from a hybrid species, comparing the phytochemical constituents of *A. atemoya* with those identified and isolated from both *Annona cherimola* and *Annona squamosa*, indicate that all three species have some similarities in their constituents regardless of the parts of the plant from which they isolated from. For instant, various annonaceous acetogenins, alkaloids and volatile components have been isolated from *A. atemoya* and *A. squamosa* such as 12,15-*cis*-squamosatin-A, bullatacin, anonaine,  $\alpha$ -Pinene, camphene,  $\beta$ -pinene, myrcene, spathulenol, germacrene D, squamosatin D,  $\beta$ -caryophyllene, uvariamicin-III, squamocin-G, squamocin-H, squamocin-J, squamocin-K, squamocin-L, squamocin-M; squamosatin-A, squamocin, annotemoyin-1, annotemoyin-2, lirioidenine, annonacin, squamocin and molvizarin [34–40]. Anonaine, asimilobine, lysicamine, lirioidenine, stepharine,  $\alpha$ -pinene, camphene,  $\beta$ -pinene, myrcene, germacrene D, molvizarin, rolliniastatin-2, squamocin were also isolated from both *A. atemoya* and *A. cherimola* [13,41–43].

Both the fruits and seeds of *A. atemoya* contain a greater range and concentration of phytochemical constituents, and have been studied more extensively compared to the leaves. However, this should be interpreted within the context that there a fewer studies evaluating the *A. atemoya* leaves. The volatile constituents are the major components of the fruit, whereas the annonaceous acetogenins are the main components of the seed. Although the annonaceous acetogenins have been isolated from the seeds, no studies have reported the isolation of annonaceous acetogenins from the fruits or leaves. Alkaloids have been identified in both leaves and seeds, and phenolic components have been isolated from fruits and seeds. Therefore, it can be deduced from the studies included in this review, that the seed is the most diverse and richest source of phytochemicals including annonaceous acetogenins, alkaloids and phenolic compounds.

Despite the fact that many pure compounds have been isolated from *A. atemoya* and various extracts of different plant parts have been examined for their activity, knowledge about the pharmacological activities of *A. atemoya* remains relatively limited. Only fourteen studies included in this review evaluated the pharmacological activities of *A. atemoya*. Of the fourteen studies, only two studies reported the cytotoxic activities of pure compounds against various cancer cell lines [20,44]. These compounds



were isolated from the seeds and identified as annonaceous acetogenins which have been reported in other *Annona* species [20]. The remaining twelve studies described below, discuss the biological activity of crude extracts of different plant parts such as seed, leave, stem and fruit.

### 3.3. Pharmacological Properties of *A. atemoya*

Different extracts of *A. atemoya* and the phytochemical constituents isolated from several parts of the plant exhibit pharmacological activities such as anti-inflammatory and antinociceptive activity [45], antioxidant [11,31,46,47], antibacterial [46], anti-angiogenic [12], neuroprotective and anti-Alzheimer's [9,11], anticancer [20,31,44], hypolipidemic [48] and antiobesity activity [49], in both in vivo and in vitro studies (Table 1).

**Table 1.** Plant part, extraction methods and biological activities and results.

| Plant Part | Extraction Method/Type | Bioactivity      | Model                                                        | Main Results                                                                                                                         | Ref. |
|------------|------------------------|------------------|--------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------|------|
| Leaves     | Ethanol extract        | Neuroprotective  | In vitro: anti-oxidant activity                              | Dose-dependently (6.25–100 mg/mL) enhanced scavenging activity against ABTS and DPPH radicals                                        | [9]  |
|            |                        | Neuroprotective  | In vitro: HT22 neuronal cell death                           | Extract significantly reversed H <sub>2</sub> O <sub>2</sub> -induced neuronal cell death at 25 or 50 µg/mL                          | [9]  |
|            |                        | Neuroprotective  | In vivo: Aβ-injected AD like mouse-model                     | Increased expression of NeuN and BDNF in hippocampus reversing the effects of intracerebroventricular injection of Aβ aggregates     | [9]  |
|            |                        | Neuroprotective  | In vivo: Aβ-injected AD like mouse-model                     | Reduced the Aβ-mediated phosphorylation of EGFR and GRK2                                                                             | [9]  |
|            |                        | Anti-Alzheimer's | In vitro: Aβ aggregation                                     | Dose-dependently inhibited Aβ aggregation by 91.35% at 100 mg/mL                                                                     | [9]  |
|            |                        | Anti-Alzheimer's | In vivo: Aβ-injected AD like mouse-model                     | At 100 µg/mL extract significantly attenuated the effects of Aβ aggregation in the passive avoidance task and Y-maze test            | [9]  |
|            |                        | Neuroprotective  | In vivo: SCO-induced hippocampal neuronal damage             | Prevented scopolamine-induced neuron damage in SCO-mediated memory deficit mice as shown by cresyl violet staining                   | [11] |
|            |                        | Neuroprotective  | In vivo: cholinergic function in scopolamine-treated Mice    | Increased acetylcholine content, choline acetyltransferase, and acetylcholinesterase activity in the hippocampus of SCO-treated mice | [11] |
|            |                        | Neuroprotective  | In vivo: oxidative Stress in scopolamine-treated Mice        | Attenuated the SCO-induced increase in reactive oxygen species (ROS) levels in the hippocampus                                       | [11] |
|            |                        | Neuroprotective  | In vivo: neuronal apoptosis in SCO-treated mice              | Significantly decreased apoptotic activation in hippocampus of SCO-treated mice                                                      | [11] |
|            |                        | Anti-Alzheimer's | In vivo: SCO-induced cognitive deficit mouse model           | Significantly attenuated the memory deficits from scopolamine treatment in passive avoidance task and Y-maze test                    | [11] |
|            |                        | Antioxidant      | In vitro: ABTS and DPPH free radical scavenging assays       | At 100 µg/mL, AALE dose-dependently enhanced scavenging activity against ABTS and DPPH radicals by 97% and 82% respectively.         | [11] |
|            |                        | Antioxidant      | In vitro: ABTS, DPPH and FRAP free radical scavenging assays | ABTS 5.01 TE g <sup>-1</sup><br>DPPH 13.51 TE g <sup>-1</sup><br>14.79 TE g <sup>-1</sup>                                            | [31] |

Table 1. Cont.

| Plant Part | Extraction Method/Type      | Bioactivity              | Model                                                                                                                                                        | Main Results                                                                                                           | Ref.    |
|------------|-----------------------------|--------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------|---------|
|            |                             | Anticancer               | In vitro: cytotoxicity HeLa, HepG2 cells                                                                                                                     | GI <sub>50</sub> ~ 2 µg/mL                                                                                             | [31]    |
|            |                             | Antinociceptive activity | In vivo: acetic acid-induced writhing and formalin mouse models                                                                                              | AAIW 100 mg/kg inhibited writhing 63.48%<br>FMM 100 mg/kg inhibited pain response 63.48%                               | [45]    |
|            |                             | Anti-inflammatory        | In vivo: air pouch mouse model<br>In vivo: carrageenan-induced peritonitis mouse models                                                                      | 100 mg/kg inhibited leukocyte migration in to air-pouch by 73.16%<br>100 mg/kg inhibited leukocyte migration by 63.85% | [45]    |
|            | Methanolic extract          | Antibacterial            | In vitro: against strains of <i>S. epidermidis</i> , <i>B. cereus</i> , methicillin-resistant <i>S. aureus</i> , <i>K. pneumoniae</i> and <i>S. aureus</i> . | MBC range 3125 to 12,500 µg/mL.                                                                                        | [46]    |
|            | Hexane extract              | Antioxidant              | In vitro: inhibition of β-carotene-linoleic acid bleaching assay                                                                                             | 41.12 ± 4.35% inhibition                                                                                               | [46]    |
| Seeds      | Ethanol extract             | Anti-angiogenic          | In vitro and in vivo models, involving cell proliferation, HUVEC and tumour-induced angiogenesis.                                                            | EEAA dose-dependently inhibited HUVEC proliferation at conc. ≥ 100 µg/mL.                                              | [12]    |
|            |                             | Anticancer               | In vitro: cytotoxicity Hep G <sub>2</sub> , Hep 2.2.15, KB, CCM <sub>2</sub> and CEM cells                                                                   | Isolated acetogenins ED <sub>50</sub> from 2.2 × 10 <sup>-4</sup> to > 500 µg/mL                                       | [20,44] |
|            |                             | Neurotoxicity            | In vitro: LUHMES cells                                                                                                                                       | 0.1 µg/mL reduced cell viability to 4.0% ± 0.8%                                                                        | [50]    |
|            | Methanolic                  | Antioxidant              | ABTS and DPPH free radical scavenging assays                                                                                                                 | 46.14 ± 1.25 and 4.82 ± 0.32 µmol TE g <sup>-1</sup>                                                                   | [47]    |
|            |                             | Larvicidal               | In vitro: <i>Trichoplusia ni</i>                                                                                                                             | Topical LC <sub>50</sub> 197.7 µg/larva<br>Oral LC <sub>50</sub> 382.4 ppm                                             | [51]    |
| Stem       | Ethanol extract             | Antioxidant              | In vitro: ABTS and DPPH free radical scavenging assays                                                                                                       | DPPH; IC <sub>50</sub> = 10.44 ± 1.25 µg/mL<br>ABTS; IC <sub>50</sub> = 24.81 ± 0.49%                                  | [46]    |
|            |                             | Antibacterial            | In vitro: against <i>S. epidermidis</i> , <i>B. cereus</i> , methicillin-resistant <i>S. aureus</i> , <i>K. pneumoniae</i> , <i>S. aureus</i> .              | MBC range 781–6250 µg/mL.                                                                                              | [46]    |
| Fruits     | Ethanol extract             | Hypolipidemic Effect     | In vivo: oral administration of extracts to Female KKAY mice (5 weeks of age) fed a high fat diet for 4 weeks                                                | Significantly lowered the plasma triglyceride (TG) concentration at doses of 125 and 500 mg/kg.                        | [48]    |
|            | Ethanol and hexane extracts | Anti-Obesity Activity    | In vitro: 3T3-L1 cell line                                                                                                                                   | 50% or more inhibition of adipogenesis in 3T3-L1 cells.                                                                | [49]    |
|            | Ethanol extract             | Neurotoxicity            | In vitro: LUHMES cells                                                                                                                                       | 10 µg/mL decreased cell viability to 12.7% ± 3.7%                                                                      | [50]    |

AAIW acetic acid induced writhing; Aβ β-amyloid; ABTS 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); AD Alzheimer's disease; AALE *A. atemoya* leaf extract; BDNF brain derived neurotrophic factor; DPPH 2,2-diphenyl-1-picrylhydrazyl; EEAA ethanol extract *A. atemoya*; EGFR epidermal growth factor receptor; FMM formalin mouse model; FRAP ferric reducing antioxidant power; GRK2 G protein-coupled receptor kinase 2; HUVEC: human umbilical vascular endothelial cells; MBC minimum bactericidal concentration; NeuN neuronal nuclear protein; ROS reactive oxygen species; SCO scopalamine.

### 3.3.1. Cytotoxic Activity

Annonaceous acetogenins have attracted considerable attention due to their broad biological activities. Compounds isolated from the seeds of *A. atemoya* exhibit potent cytotoxicity against

various cancer cell lines such as Hep G2/2.2.15, CCM2, CEM, KB and Hep G2 [20]. These compounds were identified as 12,15-*cis*-squamosatin-D, squamosatin-D, squamocin, neoannonin, bullatacin and desacetyluricin. Their activity on different cancer cells was assessed using a methylene blue colorimetric assay [20]. The cytotoxic activity of these compounds is presented in Table 2.

**Table 2.** Isolated pure compounds from *A. atemoya* and their cytotoxic activities [20].

| Compound                         | (ED <sub>50</sub> µg/mL) |                       |                       |                       |     |
|----------------------------------|--------------------------|-----------------------|-----------------------|-----------------------|-----|
|                                  | Hep G2                   | Hep 2,2,15            | KB                    | CCM2                  | CEM |
| 12,15- <i>cis</i> -Squamosatin-D | $2.20 \times 10^{-4}$    | $3.10 \times 10^{-3}$ | $4.05 \times 10^{-4}$ | -                     | -   |
| Squamosatin-D                    | $1.50 \times 10^{-4}$    | $1.50 \times 10^{-3}$ | $3.90 \times 10^{-4}$ | -                     | -   |
| Squamocin                        | $8.80 \times 10^{-4}$    | $1.50 \times 10^{-3}$ | $2.70 \times 10^{-1}$ | $1.60 \times 10^{-2}$ | 149 |
| Neoannonin                       | $1.10 \times 10^{-4}$    | $1.26 \times 10^{-4}$ | $1.46 \times 10^{-4}$ | 10.9                  | 520 |
| Bullatacin                       | $9.70 \times 10^{-5}$    | $1.11 \times 10^{-4}$ | $1.17 \times 10^{-4}$ | $1.41 \times 10^{-1}$ | 169 |
| Desacetyluricin                  | $1.02 \times 10^{-4}$    | $1.18 \times 10^{-4}$ | $1.35 \times 10^{-4}$ | 23.5                  | 100 |

Bullatacin isolated from *A. atemoya* demonstrated a potent cytotoxic activity inhibiting the proliferation of Hep G2/2.2.15 cells by inducing apoptosis [29], via a reduction of intracellular cAMP and cGMP levels [44]. Using the methylene blue assay, a significant inhibition in Hep G2/2.2.15 cell proliferation was recorded after treatment with bullatacin ( $10^{-1}$  to  $10^{-5}$  µM), ED<sub>50</sub> values were recorded as  $7.8 \pm 2.5$  nM for 24 h [44].

An aqueous-ethanolic extract of the leaves of *A. atemoya* was shown to be cytotoxic to both HeLa and HepG2 cancer cells. The anti-cancer activity was not found to correlate to either polyphenol content, antioxidant properties or total alkaloid content. However, there was a moderate correlation between antiproliferative activity and aporphine-type alkaloid content [31].

### 3.3.2. Anti-Angiogenic Activity

The ethanolic extract of *A. atemoya* seeds was examined for its anti-angiogenic activity in human umbilical vein endothelial cells (HUVECs). The ethanolic seed extract of *A. atemoya* elicited a significant dose-dependent inhibition of HUVEC mobility at concentration  $\geq 100$  µg/mL without decreasing cell viability [12]. Using the matrigel plug assay, the ethanol extract at concentration  $\geq 25$  µg/mL was also shown to inhibit the formation of new blood vessels in vivo, in a dose-dependent manner. Further investigation indicated that under hypoxic conditions the extract down-regulated the expression of vascular endothelial growth factor (VEGF) and hypoxia-inducible factor (HIF-1α/2α) [12]. These results indicate that the extract modulated angiogenesis both directly through the endothelial cells and indirectly through modulating the tumour-induced angiogenic pathways [12].

### 3.3.3. Hypolipidemic Effect

The ethanolic fruit extract of *A. atemoya* was investigated for its hypolipidemic effects using female KKAY mice at one month of age [48]. For four weeks, the female KKAY mice were fed a high fat diet. Oral administration of ethanolic fruit extract of *A. atemoya* at doses of 125 and 500 mg/kg to the female KKAY mice led to a significant inhibition of plasma triglyceride concentrations and the hypolipidemic effect might be through diminished fatty acid mobilization [48]. The hypolipidemic mechanism of the ethanolic fruit extract is not reported, although the authors claim that in a previous study [49], both the hexane and ethanolic extracts of *A. atemoya* fruit (along with many other popular Okinawa food stuffs) inhibited adipogenesis activity in 3T3-L1 cells by 50% or more. However, this study provides only limited results and does not include the extraction methodology or the concentrations of the extracts used, making it difficult to understand if this is a likely mechanism for the anti-obesity actions of *A. atemoya* [49]. While interesting, the dose of the crude extract used in the hypolipidemic study is high, translating into a dose of approximately 9 g/per day for an average adult. However, the significant issues faced by many western populations due to obesity and related health issues mean that the

hypolipidemic activities warrant further study. In particular, further purification of the extract and isolation of the hypolipidemic constituent(s) is required.

#### 3.3.4. Antioxidant Activity

In an in vitro study, the methanolic seed extract of *A. atemoya* was examined for antioxidant potential [47]. Both 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and oxygen radical absorbance capacity (ORAC) assays were employed. For the ORAC and DPPH assays, the Trolox equivalent antioxidant activity of the methanolic seed extract was recorded at  $46.14 \pm 1.25$  and  $4.82 \pm 0.32 \mu\text{mol TE g}^{-1}$ , respectively [47].

Other studies have reported the free radical scavenging activity of ethanolic extracts of stems and the hexane extract of leaves using DPPH, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) and antioxidant activity  $\beta$ -carotene/linoleic acid assays [46]. The ethanolic extract of stems exhibited the most effective antioxidant in DPPH and ABTS ( $\text{IC}_{50} = 10.44 \pm 1.25 \mu\text{g/mL}$ ) and ( $24.81 \pm 0.49\%$ ), respectively. The hexane leaf extract of *A. atemoya* has also demonstrated antioxidant activity in the  $\beta$ -carotene linoleic acid assay with approximately ( $41.12 \pm 4.35\%$ ) [46]. The aqueous ethanol leaf extracts of Sicilian *A. atemoya* also showed strong antioxidant activity in the ABTS ( $5.01 \text{ TE g}^{-1}$ ), DPPH ( $13.51 \text{ TE g}^{-1}$ ) and metal reducing properties in the ferric reducing antioxidant power (FRAP) assay ( $14.79 \text{ TE g}^{-1}$ ) [31].

#### 3.3.5. Antibacterial Activity

The antimicrobial potential of the hexane and methanolic extract of leaves, and the ethanolic extract of the stems of *A. atemoya* has been investigated against both Gram-positive and Gram-negative bacteria using a serial dilution protocol [46]. All strains were resistant to the hexane extract. However, both the leaf methanolic and stem ethanolic extracts exhibited activity against gram positive *Staphylococcus epidermidis*, *Bacillus cereus*, *Staphylococcus aureus* and a clinical isolate of methicillin-resistant *Staphylococcus aureus* (MRSA), with *Klebsiella pneumoniae* the only Gram-negative strain sensitive to the extract. The minimum bactericidal concentration (MBC) values recorded for the ethanolic stem extract ranged from  $781 \mu\text{g/mL}$  against MRSA to  $6250 \mu\text{g/mL}$  against *K. pneumoniae* and *S. aureus*. The methanolic leaf extract showed weaker activity with MBC values ranging from 3125 against *S. epidermidis* to  $12,500 \mu\text{g/mL}$  (*S. aureus*, *K. pneumoniae* and *B. cereus*) [46]. Although, the MBC was determined, the minimum inhibitory concentration (MIC) was not determined for any of the strains. Therefore, it is not possible to determine whether the mechanism of action is bactericidal or bacteriostatic. From the reported total polyphenol content of these extracts it is suggest that flavonoids are responsible for the activity antibacterial action of both the ethanol stem extract and the methanol leaf extract [46].

#### 3.3.6. Antinociceptive Activities

An in vivo study evaluating the antinociceptive activity of *A. atemoya* was conducted using the acetic acid-induced writhing and formalin mouse models [45]. The ethanolic extract of leaves (25, 50 and  $100 \text{ mg/kg}$ ) inhibited acetic acid-induced writhing by 42.14, 48.88 and 63.48%, correspondingly. The same doses of the ethanolic extract of leaves also exhibited antinociceptive activity with 56.71, 64.35 and 50.09% inhibition, respectively using the formalin test [45].

#### 3.3.7. Anti-Inflammatory Activities

The anti-inflammatory activity of *A. atemoya* was reported in a single study with the evaluation carried out using the air pouch and carrageenan-induced peritonitis models in an in vivo study [45]. In the carrageenan-induced peritonitis model the ethanolic extract of leaves (25, 50 and  $100 \text{ mg/kg}$ ) inhibited leukocyte migration by 35.40, 46.20 and 63.85%, respectively. In the air pouch assay, the leaf extract (25, 50 and  $100 \text{ mg/kg}$ ) also reduced leukocyte migration into the air pouch by 56.17, 62.04 and



73.16% respectively [45]. As this study used only the crude the ethanolic extract it is not possible to identify which constituents may be responsible for the antinociceptive and anti-inflammatory activities.

### 3.3.8. Neurological Activities

Oral administration of an ethanolic extract of *A. atemoya* was found to reduce scopolamine- induced memory impairment in the mouse Y-maze and passive avoidance tests [9,11]. Investigation into the mechanism of action indicated that *A. atemoya* extract inhibits memory impairment by preventing cholinergic dysfunction and decreasing cell death associated with stress induced oxidation [9]. The crude ethanolic leaf extract and to a lesser degree the fruit extract but not the seed extract was also shown to prevent amyloid- $\beta$  ( $A\beta$ ) aggregation and increase free radical scavenging activity, preventing  $H_2O_2$  induced damage to hippocampal HT22 cells in vitro [9]. Immunohistochemical and western blotting studies indicated that the extract inhibited  $A\beta$ -mediated loss of brain derived neurotrophic factor (BDNF) expression and decreased  $A\beta$ -mediated phosphorylation of epidermal growth factor receptor (EGFR) and G Protein-Coupled Receptor Kinase 2 (GRK2) [9]. Further fractionation into hexane, butanol and water led to all fractions inhibiting  $A\beta$  aggregation to some degree, with the ethyl acetate fraction demonstrating the greatest inhibition. However, the majority of the neuroprotective activity was found in the butanol fraction with the hexane and ethyl acetate fractions (the fractions most likely to contain the lipophilic acetogenins) being cytotoxic to the cultured neurons. Phytochemical analysis of the butanol extract suggested that the flavonoids rutin and isoquercitrin were predominantly responsible for the reported bioactivity [9].

However, one study has reported the neurotoxicity of ethyl acetate extracts of the pulp and seeds in an in vitro study using Lund human mesencephalic (LUHMES) cells [50]. This suggests that constituents of the seeds and pulp are neurotoxic as has been found for other members of the *Annona* family [2]. There have been suggestions that a high dietary consumption of *A. muricata* may be linked a Parkinson disease-like syndrome possibly due to the annonacin content [52]. The studies investigating the neurotoxicity of *Annona* species have used ethyl acetate extracts which would predominantly contain the acetogenins, or purified acetogenins such as annonacin. Acetogenins have been identified as highly potent inhibitors of mitochondrial complex I [53], and this has been suggested as the mechanism of neurotoxicity. The neuroprotective effect of crude ethanolic extracts of leaves and fruit of *A. atemoya*, containing both acetogenins and flavonoids, suggests that the presence of the flavonoids may offer protection from the neurotoxic effects of the acetogenins.

### 3.3.9. Toxicity

The neurotoxicity of the ethyl acetate extracts of a range of Brazilian *A. atemoya* pulp and seeds in LUHMES cells was assessed using both MTT assay to determine cell viability and LDH assay to determine cell death [50]. The pulp extract of *A. atemoya* had little effect on cell viability of the LUHMES cells at 1  $\mu$ g/mL, but at 10  $\mu$ g/mL decreased cell viability to  $12.7\% \pm 3.7\%$ . The seed extract of *A. atemoya* exhibited significantly higher toxicity with cell viability reduced to  $4.0\% \pm 0.8\%$  at a concentration of 0.1  $\mu$ g/mL. LDH levels were increased to  $52.8\% \pm 3.9\%$  by treatment of the LUHMES cells with concentration of 10  $\mu$ g/mL of the *A. atemoya* pulp extract and to  $60.8\% \pm 4.3\%$  when treated with 0.1  $\mu$ g/mL of seed extract [50]. When compared to the fruit pulp extract of other *Annona* species in the study *A. atemoya* had a lesser effect on cell viability than *A. squamosa* but resulted in a similar increase in LDH indicating a similar effect on cell death. This is despite *A. atemoya* containing a similar amount of annonacin to *A. squamosa* (3.8 of 2.2  $\mu$ g/g dry weight) and approximately a 100-fold greater amount of squamocin [50]. The ethyl acetate extract used in this study will contain a complex mixture of lipophilic compounds, including many acetogenins, and the neurotoxicity cannot be attributed only to annonacin. It is possible that *A. atemoya* may also contain lipophilic neuroprotective compounds.

A second study reported the toxicity of *A. atemoya* on *Trichoplusia ni* (cabbage looper larva) using topical and oral administration. The methanolic seed extract was applied to *T. ni* larvae and the  $LC_{50}$

was recorded at 197.7 µg/larva [51]. Finally, the methanolic seed extract also showed toxicity to *T. ni* larvae when administered orally with the LC<sub>50</sub> recorded as 382.4 ppm [51].

### 3.4. Quality Analysis

As presented in Table 3, the quality of the in vivo studies none of the studies met all of the ARRIVE criteria [16]. In particular, no studies reported baseline animal data or adverse events. With regards allocating animals to experimental groups, this was reported by only one group [9]. These factors limit the reliability of our findings in terms of the in vivo studies.

**Table 3.** Methodological quality assessment of animal studies using ARRIVE quality assessment criteria.

| Study | * Arrive Quality Items |   |   |   |   |   |   |   |   |    |    |    |    |
|-------|------------------------|---|---|---|---|---|---|---|---|----|----|----|----|
|       | 1                      | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| [9]   | Y                      | Y | Y | Y | Y | Y | Y | Y | Y | N  | N  | Y  | N  |
| [11]  | Y                      | Y | Y | Y | Y | Y | N | Y | Y | Y  | N  | Y  | N  |
| [12]  | Y                      | Y | Y | Y | N | N | N | Y | N | N  | N  | Y  | N  |
| [48]  | N                      | Y | Y | Y | Y | N | N | Y | N | N  | N  | Y  | N  |
| [45]  | Y                      | Y | Y | Y | Y | Y | N | Y | Y | Y  | N  | Y  | N  |

\* Arrive quality items: 1 Ethical statement, 2 Study design, 3 Experimental procedure, 4 Experimental animals, 5 Housing and husbandry, 6 Sample size, 7 Allocating animals to experimental groups, 8 Experimental outcomes, 9 Statistical methods, 10 Number of analysed, 11 Baseline data, 12 Outcomes and estimation, 13 Adverse events.

## 4. Conclusions

To our knowledge, this is the first systematic review to summarize the available literature reporting the phytochemical constituents and pharmacological effects of *A. atemoya*. The systematic review of the included studies affords a comprehensive report of the currently available data regarding both the phytochemical constituents and biological activities of *A. atemoya*. The selected studies in this systematic review were judged to be of medium quality as there are limited animal studies and no human studies reported. The phytochemical components of *A. atemoya* have been examined as extracts and pure compounds in both in vivo and in vitro. However, many studies used only crude ethanolic or methanolic extracts and did not attempt to purify these further in order to identify which constituents may be responsible the activities. In regard to the phytochemical screening of *A. atemoya* plant, several studies have reported a range of constituents isolated from various parts including the leaves, fruits and seeds. However, very few of the studies attempted to profile or quantify the bioactive constituents. Amongst the selected studies, only three studies reported the cultivar of *A. atemoya* (African Pride) one of the most common varieties [19,21,22]. It is also of interest to note that at the current time no acetogenins have been isolated from the fruit or leaves of *A. atemoya*, unlike the many acetogenins isolated from the fruit and leaves of *A. cherimola* and *A. squamosa*.

Although, there is no solid evidence medicinal or nutraceutical uses of *A. atemoya* there is some limited anecdotal evidence reporting the use of leaves for making teas in a traditional manner, or possibly using the leaves as a supplement. As an increasingly important commercial fruit crop with nutraceutical potential, further studies are recommended in order to isolate and investigate the pure components and identify the bioactive compounds responsible for the effects identified.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/1424-8247/13/10/269/s1>, Table S1: Phytochemical constituents found in different parts of the *Annona atemoya* plant.

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Communication

# Identification of Annonaceous Acetogenins and Alkaloids from the Leaves, Pulp, and Seeds of *Annona atemoya*

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**Abstract:** Annonaceae is a large family composed of more than 119 genera and more than 2500 species that are distributed in both tropical and subtropical areas. The *Annona* genus is a member of Annonaceae family, which encompasses about 175 species, most of which are native to Brazil and tropical America. This plant is commonly found on tropical and subtropical continents. *Annona atemoya* is a commercially important hybrid of *A. squamosa* and *A. cherimola*. Phytochemical investigations of *A. atemoya* leaves, fruit, and seeds have been conducted in limited studies. The purpose of this study was to investigate the constituents of the leaves, fruit pulp, and seeds of *A. atemoya* because few studies have reported their constituents. Annonaceous acetogenins were identified in the leaves and pulp of *A. atemoya* for the first time. Twenty compounds were identified: sixteen were acetogenins and four were alkaloids. Additionally, two compounds were isolated, and their structures were confirmed by spectroscopic analysis and compared with the results of previous studies. The concentration of acetogenins in the pulp was very low compared with that in the leaves, whereas the seeds were found to contain the highest concentrations and greatest diversity of compounds.

**Keywords:** *Annona atemoya*; leaves; fruit's pulp; seeds; annonaceous acetogenins; alkaloids



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## 1. Introduction

Annonaceae is a large plant family composed of more than 119 genera and more than 2500 species that are distributed across tropical and subtropical areas. This family is chemically characterised by the existence of various phytochemical constituents such as alkaloids, annonaceous acetogenins (ACs), volatile oils, and phenolic compounds [1]. The *Annona* genus is a member of the Annonaceae family that encompasses approximately 175 species, many of them native of Brazil and tropical America [1–3]. Due to their edible fruits and medicinal properties, *Annona* is the most important genus of the Annonaceae family [1]. *Annona atemoya* (*A. atemoya*) belongs to the family of Annonaceae known as custard apple [1–3]. This plant is commonly found in tropical and subtropical areas such as Asia, Africa, South and North America, and Australia [4]. *A. atemoya* is a hybrid of two *Annona* species: *Annona squamosa* (sugar apple) and *Annona cherimola* (cherimoya), first reported in 1907 [3,4]. Various cultivars have been reported for *A. atemoya*, and the most common ones grown in Australia are African Pride, KJ Pink's Mammoth, Island Gem, Nielsen, Hillary White, and Maroochy Gold [4]. Despite the fact that there are many *Annona* species, only a limited number of this family are economically important, and *A. atemoya* is one of them. In Australia, this plant is commonly known as the “custard apple”, having a sweet and creamy, custard-like fruit. It is increasingly available in most retail food stores [3]. There is also some anecdotal evidence reported by growers of the leaves being purchased for making teas similar to those made with *atemoya* species and used in traditional medicines.



Many *Annona* species have a long history of use in traditional medicines, and the constituents have been well studied and are known to exhibit a range of biological activities [5–8]. However, the phytochemical investigations of the leaves, fruits, and seeds of the newer hybrid *A. atemoya* have been reported only in a limited number of studies. An initial study reported the isolation of alkaloids such as lanuginosine, liriodenine, lysicamine, pronuciferine, anonaine, asimilobine, and stepharine from the leaves [1]. A more recent study reported the isolation of additional alkaloids such as dehydroanonomuricine-*N*-oxide, anomuricine, reticuline, scoulerine, normuciferine, and norisocorydine [9]. The leaves of *A. atemoya* were found to also contain a number of phenolic compounds including quercetin-3-*O*-rutinoside-7-*O*-glucoside, quercetin-3-*O*-rutinoside, quercetin-3-*O*-glucoside, luteolin-3-*O*-galactoside-7-*O*-rhamnoside, apigenin-8-*C*-glucoside, quercetin-3-*O*-rutinoside-7-*O*-pentoside, kaempferol-3-*O*-galactoside-7-*O*-rhamnoside, luteolin-3-*O*-glucoside-7-*O*-rhamnoside, catechin, and epicatechin [10]. The fruits of *A. atemoya* have been reported to contain a wide range of essential oils, with the main compounds identified being  $\alpha$ -pinene,  $\beta$ -pinene, germacrene D, and (*E*)-ocimene [11–13]. In addition, the fruit of *A. atemoya* was reported to contain phenolic compounds such as epicatechin, catechin, 3,4-dihydroxybenzoic acid, chlorogenic acid, and *p*-coumaric acid [14]. ACs have been reported as the main phytochemical constituents of the seeds in comparison with alkaloids, *N*-acyl-tryptamines, and phenolic components [15–22]. Approximately 36 ACs have been isolated and identified from the seeds of *A. atemoya* [15–22], 2 alkaloids including atemoine and cleistopholine [23], and 4 phenolic compounds including chlorogenic acid, ferulic acid, vanillic acid, and myricetin [24].

Despite the potential health benefits of *Annona* fruits, high levels of consumption of the fruit, nectar, and/or herbal teas of *A. muricata* L. (Graviola, soursop) have been associated with a higher incidence of atypical parkinsonism or progressive supranuclear palsy (PSP) [25–27]. This disease has been correlated to the contents of alkaloids and acetogenins present in *A. muricata*, particularly ACs such as annonacin [28,29]. Annonacin was also found to be toxic to dopaminergic and other mesencephalic cells [30] and to induce the accumulation of tau proteins in the cultured neurons [31]. Given the increasing consumption of custard apples and the few studies reporting their phytochemical constituents, the aim of this study was to further investigate the phytochemical constituents of the leaves, fruit pulp, and seeds of *A. atemoya*.

## 2. Results and Discussion

### 2.1. Thin-Layer Chromatography (TLC) and Column Chromatography (CC)

African Pride (AP) and KJ Pinks Mammoth (KJ) are cultivars of *A. atemoya*, which display similarities in their phytochemical components according to TLC analysis. Therefore, the AP cultivar was studied in detail, whereas the KJ cultivar was only studied via mass spectrometry (MS) (Supplementary Materials Figure S1). Based on the TLC results, all AP parts (leaves, pulp, and seeds) showed the presence of ACs as pink bands after reacting with Kedde's reagents. The concentration of low polarity ACs (*R<sub>f</sub>* 0.65) in the hexane extract of the leaves was very low, shown as a very faint spot. The ethyl acetate extract was the richest in ACs, shown as approximately three separate bands with an *R<sub>f</sub>* of between 0.25 and 0.40. The TLC of the pulp suggested that the concentration of ACs was very low, with the ethyl acetate extract displaying two faint spots with *R<sub>f</sub>* values of 0.25 and 0.85. Similarly, the hexane extract also exhibited only a very faint spot of a nonpolar AC with an *R<sub>f</sub>* of 0.85. To the best of our knowledge, this is the first report of the presence of ACs in the leaves and pulp of *A. atemoya*. All three extracts of the seeds indicated the presence of ACs as pink bands after reacting with Kedde's reagents (Supplementary Materials Figure S2). Both the hexane and ethanol extracts displayed similar pink bands at an *R<sub>f</sub>* of 0.25; however, these spots were significantly less intense compared with those of the ethyl acetate extract. The ethyl acetate extract exhibited the richest diversity of ACs compared with the hexane and ethanol extracts.

Column chromatography under gravity of the crude leaf extract (6.57 g) afforded a total of 163 fractions (10 mL); fractions (110–129; 145.2 mg) were collected in one flask (fraction 1H), as TLC indicated the presence of the lowest polarity ACs in the extract along with other constituents. The TLC of the fractions (130–131; 33.3 mg) indicated the presence of ACs containing some contaminants visible under UV light, and these fractions were combined (fraction 2H). Finally, the TLC of six fractions (138–143; 24 mg) also indicated the presence of very polar ACs, so these fractions were combined (fraction 3H).

Column chromatography of the ethanol extract (12.6 g) eluted with DCM:MeOH (100:0 *v/v*) to DCM:MeOH (0:100 *v/v*) yielded a total of 276 fractions, which were collected; separation resulted in five fractions, namely: A (240 mg), B (80 mg), C (2.27 g), D (5.9 g), and E (2.60 g). Of the five fractions, only fractions A and B exhibited four orange spots, indicating alkaloids with an *R<sub>f</sub>* ranging from 0.42 to 0.85 after visualisation with Dragendorff's reagent (Supplementary Materials Figure S3). Due to the small amount of the ethyl acetate crude extract, it was directly submitted to flash chromatography for further purification. Both the pulp and seed crude extracts were submitted to NMR and MS analysis due to the small amount of these crude extracts.

## 2.2. Flash Chromatography

The ethyl acetate extract of the leaves was directly purified by flash chromatography due to small amount of the crude extract (2.991 g). Gradient elution of DCM (100:0 *v/v*) to DCM:MeOH to MeOH: 100 *v/v* yielded 158 fractions (25 mL), which were analysed by TLC. Similar fractions were combined as follows: fraction 1E (46–66) (121.4 mg) and fraction 2E (67–79) (66.7 mg) containing the lowest polarity ACs; fraction 3E (80–100) (119.4 mg), fraction 4E (101–132) (164.2 mg) and fraction 5E (133–144) (190.9 mg) (Supplementary Materials Figure S4). The further purification by flash chromatography of fractions 1E to 5E using similar gradient methodology is summarised in the Supplementary Materials (Table S1). After the final chromatographic separation, eight fractions were identified as containing ACs.

## 2.3. Nuclear Magnetic Resonance (NMR), Mass Spectrometry (MS), and Tandem Mass Spectrometry (MS/MS)

### 2.3.1. Leaves

The concentration of ACs in the hexane extract was extremely low compared with that in the ethyl acetate extract, and the four compounds identified were common to the other extracts according to the NMR and MS results. Both NMR and MS indicated the presence of the same ACs in the hexane and ethyl acetate extracts, and the TLC also confirmed a very weak pink spot. The MS fragmentation pathway used to identify ACs has previously been reported [10,32]. The molecular mass was determined as 530 by ESI-MS ( $[M+H]^+$   $m/z = 531$ ) suggesting a molecular formula  $C_{35}H_{62}O_3$ . The MSMS indicated the product ion of  $[M + Li]^+$  ( $m/z = 537$ ) with intense fragment peaks at  $m/z$  313, 301, 285, and 271, allowing localisation of the epoxy ring at C-15/C-16, with the double bond location at C-19/C-20. In the  $^1H$ -NMR spectrum, the peak at 6.98 ppm was indicative of the deshielded ethylenic proton, and the presence of resonances at  $\delta$  2.90 and 2.93 were suggestive of an epoxy ring indicated epoxymurin-A (1). The concentration of this compound was lower in ethyl acetate extract than the hexane concentration. Both the NMR and MS results are consistent with two studies that have reported this compound in the pulp and stem bark of *A. muricata* [33,34].

Two epoxy ACs were identified as a mixture of isomeric compounds in both the hexane and ethyl acetate extracts as diepomuricanin A and B (2 and 3). The  $^1H$ -NMR spectrum showed a peak at 6.96 ppm and a molecular mass of 564  $m/z$ , corresponding to the molecular formula  $C_{35}H_{62}O_4$ . The MSMS indicated the product ion of  $[M + Li]^+$  ( $m/z = 553$ ), and the location of the epoxy rings in the alkyl chain for diepomuricanin A at the C-15/C-16 and C-19/C-20 was indicated by the presence of two pairs of peaks at  $m/z$  383 and 371 and  $m/z$  313 and 301. For diepomuricanin B, epoxy rings were observed at



C-13/C-14 and C-17/C-18 by the presence of two pairs of peaks at  $m/z$  355 and 343 and  $m/z$  285 and 273. These compounds were identified via a comparison of the spectral data with the only study reporting these compounds from the hexane fraction of the methanolic extract of *A. muricata* seeds [35].

Annotemoyin-1 and -2 (**4** and **5**) were identified in the hexane and ethyl acetate extracts, showing peaks in the  $^1\text{H-NMR}$  at 6.98 ppm and a molecular mass of 564  $m/z$ . The MSMS analysis revealed intense fragments at  $m/z$  277, 247, and 205, suggesting the localisation of a mono-THF ring at C-18/C-21. These mono tetrahydrofuran ACs were previously reported in the seeds of *A. atemoya* (African Pride cultivar) [21]. An additional four compounds were identified in the ethyl acetate extract; two of them were isolated and structurally characterised. The isolation and identification of ACs from *A. atemoya* leaf has not been previously reported.

The structures of the pure compounds were elucidated based on NMR experiments and are consistent with those previously reported for these compounds containing two adjacent bis-tetrahydrofuran rings: squamocin G (**6**) (11.2 mg) and squamocin C (**7**) (9 mg) [36–38]. Compound **6** ( $\text{C}_{37}\text{H}_{66}\text{O}_7\text{Na}$ ;  $m/z$  645.4  $[\text{M} + \text{Na}]^+$ , ESI-MS) was determined to possess an adjacent bis-bis-tetrahydrofuran ring system located between C-15 and C-24 according to both MS and  $^1\text{H-NMR}$  data. The presence of the  $\alpha, \beta$ -unsaturated  $\gamma$ -lactone was supported by peaks in the  $^1\text{H-NMR}$  spectrum at 7.18 (1H, d,  $J = 1.6$ , H-35), 5.06 (1H, dq, H-36), 3.85 (1H, m, H-4), 2.50 (1H, m, H-3a), 2.40 (1H, m, H-3b), and 1.44 (3H, d,  $J = 6.7$ , H-37), indicating the absence of a hydroxyl substitution at C-3/4/5 and confirmed by  $^{13}\text{C-NMR}$  data 174.33 (C-1), 151.77 (C-35), 131.12 (C-2), 77.96 (C-36), 69.99 (C-4), 33.2 (C-3), and 19.05 (C-37). Compound **7** ( $\text{C}_{37}\text{H}_{66}\text{O}_7\text{Na}$ ;  $m/z$  645.4  $[\text{M} + \text{Na}]^+$ , ESI-MS) was determined to possess an adjacent bis-tetrahydrofuran ring system located between C-15 and C-24, according to both MS and NMR data. The occurrence of the  $\alpha, \beta$ -unsaturated  $\gamma$ -lactone was indicated by the peaks in the  $^1\text{H-NMR}$  at 6.98 (1H, q, H-35), 4.99 (1H, qq, H-36), 2.25 (2H, t, H-3), and 1.40 (3H, d, H-37), indicating the presence of a hydroxyl substitution at C-34. Additionally, the spectrum revealed the presence of a terminal methyl group as a triplet at 0.87 (C-34).  $^{13}\text{C-NMR}$  data confirmed the existence of  $\alpha, \beta$ -unsaturated  $\gamma$ -lactone by the presence of seven oxygenated carbons at 83.30 (C-16), 82.21 (C-19), 82.52 (C-20), and 82.78 (C-23). The remaining three hydroxylated carbons, C-15, C-24, and C-29, gave rise to peaks at 74.12, 71.39, and 71.77 respectively (Table 1).

Additionally, annonacin (**8**) and annonisin (**9**) were identified in the ethyl acetate extract of the leaves by the existence of product ions in the MSMS of  $[\text{M} + \text{Li}]^+$   $m/z = 603$  and 617, respectively. Both compounds **8** and **9** revealed intense peaks for fragments at  $m/z$  491, 391, 305, 233, and 205, and 505, 433, 347, 275, and 205 respectively, and were previously been reported in *A. atemoya* seeds [18].

The most concentrated ACs in the leaves according to TLC analysis gave rise to a product ion  $[\text{M} + \text{Li}]^+$  ( $m/z = 645$ ), leading to the molecular formula  $\text{C}_{37}\text{H}_{66}\text{O}_8$ , indicating bullatanocin (**10**) and atemoyacin-E (**11**). A series of fragment ions at  $m/z$  533, 363, 335, 277, and 205, and 533, 363, 293, 265, 207, and 149 in the MSMS confirmed the presence of compounds **10** and **11**, respectively. Both compounds have previously been reported in *A. atemoya* seeds [15,22]. For compound **10**, the presence of  $\alpha, \beta$ -unsaturated  $\gamma$ -lactone was confirmed by peaks in the  $^1\text{H-NMR}$  at 7.18 (1H, q, H-35), 5.06 (1H, qq, H-36), 3.86 (1H, m, H-4), 2.54 (1H, m, H-3a), 2.40 (1H, m, H-3b), and 1.42 (3H, d, H-37). Peaks in the  $^{13}\text{C-NMR}$  at 174.46 (C-1), 151.90 (C-35), 131.11 (C-2), 78.01 (C-36), 69.88 (C-4), and 19.07 (C-37) confirmed the presence of  $\alpha, \beta$ -unsaturated  $\gamma$ -lactone, and the presence of two nonadjacent THF rings was indicated by proton resonances at 3.86 (H-12) and 3.81 (H-15, 20, 23).

**Table 1.**  $^1\text{H}$  and  $^{13}\text{C}$  NMR data for compounds **6** and **7** recorded in  $\text{CDCl}_3$ .

| Position | 6                         |                    | 7                         |                    |
|----------|---------------------------|--------------------|---------------------------|--------------------|
|          | $\delta \text{ H (J Hz)}$ | $\delta \text{ C}$ | $\delta \text{ H (J Hz)}$ | $\delta \text{ C}$ |
| 1        |                           | 174.33             |                           | 173.9              |
| 2        |                           | 131.18             |                           | 134.3              |
| 3        | 2.40, 2.50                | 33.2               | 2.25                      | 25.15              |
| 4        | 3.85                      | 69.99              | 1.52                      | 27.37              |
| 5        | 1.42                      | 37.39              | 1.2–1.7                   | 29.16              |
| 6        | 1.25–1.4                  | 25.54              | 1.2–1.7                   | 29–30              |
| 7        | 1.25–1.4                  | 29.10–29.67        | 1.2–1.7                   | 29–30              |
| 8        | 1.25–1.4                  | 29.10–29.67        | 1.2–1.7                   | 29–30              |
| 9        | 1.25–1.4                  | 29.10–29.67        | 1.2–1.7                   | 29–30              |
| 10       | 1.25–1.4                  | 29.10–29.67        | 1.2–1.7                   | 29–30              |
| 11       | 1.25–1.4                  | 29.10–29.67        | 1.2–1.7                   | 29–30              |
| 12       | 1.25–1.4                  | 29.10–29.67        | 1.2–1.7                   | 29–30              |
| 13       | 1.25–1.4                  | 25.61              | 1.2–1.7                   | 25.64              |
| 14       | 1.33–1.56                 | 33.32              | 1.2–1.7                   | 33.28              |
| 15       | 3.39                      | 74.04              | 3.40                      | 74.12              |
| 16       | 3.84–3.93                 | 83.2               | 3.82–3.96                 | 83.3               |
| 17       | 1.97–1.63                 | 28.34              | 1.52–1.9                  | 28.36              |
| 18       | 1.97–1.63                 | 28.88              | 1.52–1.9                  | 28.9               |
| 19       | 3.93                      | 82.51              | 3.82–3.96                 | 82.21              |
| 20       | 3.84                      | 82.26              | 3.82–3.96                 | 82.52              |
| 21       | 1.97–1.63                 | 28.93              | 1.52–1.9                  | 28.9               |
| 22       | 1.92–1.90                 | 24.49              | 1.52–1.9                  | 24.80              |
| 23       | 3.84–3.93                 | 82.77              | 3.82–3.96                 | 82.78              |
| 24       | 3.84–3.93                 | 71.33              | 3.82–3.96                 | 71.39              |
| 25       | 1.33–1.56                 | 32.42              | 1.25–1.4                  | 32.45              |
| 26       | 1.25–1.4                  | 26.03              | 1.25–1.4                  | 22.0               |
| 27       | 1.25–1.4                  | 29.10–29.67        | 1.25–1.4                  | 25.64              |
| 28       | 1.25–1.4                  | 29.10–29.67        | 1.25–1.4                  | 37.48              |
| 29       | 1.25–1.4                  | 29.10–29.67        | 3.59                      | 71.77              |
| 30       | 1.25–1.4                  | 29.10–29.67        | 1.25–1.4                  | 37.23              |
| 31       | 1.25–1.4                  | 29.10–29.67        | 1.25–1.4                  | 25.31              |
| 32       | 1.25–1.4                  | 31.89              | 1.25–1.4                  | 31.9               |
| 33       | 1.25–1.4                  | 22.67              | 1.25–1.4                  | 22.6               |
| 34       | 0.87                      | 14.10              | 0.78                      | 14.1               |
| 35       | 7.18                      | 151.77             | 6.98                      | 148.87             |
| 36       | 5.06                      | 77.97              | 4.99                      | 77.4               |
| 37       | 1.44                      | 19.10              | 1.40                      | 19.2               |

The other chemical constituents identified from the leaves of *A. atemoya* were alkaloids, with TLC and MS confirming their presence in the ethanolic extract. The positive ESI-MS  $m/z$ : 291  $[\text{M} + \text{H}]^+$  indicated lysicamine (**12**), and the MS data were in agreement with

those in the literature [1,39]. The other oxoaporphine alkaloid found in both ethyl acetate and ethanol extracts was lanuginosine (**13**), with a positive ESI-MS reported at  $m/z$ : 304  $[M + H]^+$ . The MS data agreed with those in the literature. Both compounds (**12** and **13**) were previously been reported in the leaves of *A. atemoya* [1]. Roemerine (**14**), an aporphine alkaloid, was identified in the ethanol extract with an ESI-MS  $m/z$ : 279  $[M + H]^+$ . This compound has not previously been reported in *A. atemoya* but has been reported in the leaves of *A. squamosa* and the stem of *A. cherimola* [7,40,41]. Finally, another aporphine alkaloid, corydine (**15**), was confirmed by ESI-MS  $m/z$ : 341  $[M + H]^+$  and was previously reported in the leaves of *A. squamosa* [40], but not in *A. atemoya*.

### 2.3.2. Pulp

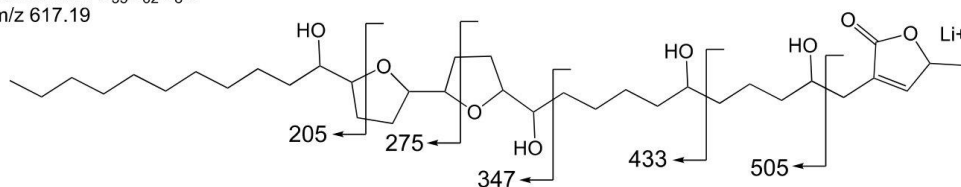
The concentration of ACs in the pulp was very low compared with that in the leaves and seeds. However, the presence of ACs could be detected in all three extracts (hexane, ethyl acetate, and ethanol) by NMR and MS analysis. Compound **1** was identified in all three extracts of pulp, and the MSMS confirmed the presence of the product ion of  $[M + Li]^+$  ( $m/z = 537$ ) and intense fragments at  $m/z$  313, 301, 285, and 271. A compound identified only in the ethyl acetate extract of the pulp was montanacin A (**16**), which showed a peak in the  $^1H$  NMR at 7.20 ppm, and a  $[M + Li]^+$  ( $m/z = 647$ ), leading to the molecular formula  $C_{37}H_{68}O_8$ . The MSMS analysis revealed fragments at  $m/z$  535, 463, 337, 277, and 205, suggesting the localisation of the mono-THF ring at C-20 and C-23. This compound was previously reported in *A. montana* seeds, and our result is consistent with those previously reported [42].

### 2.3.3. Seeds

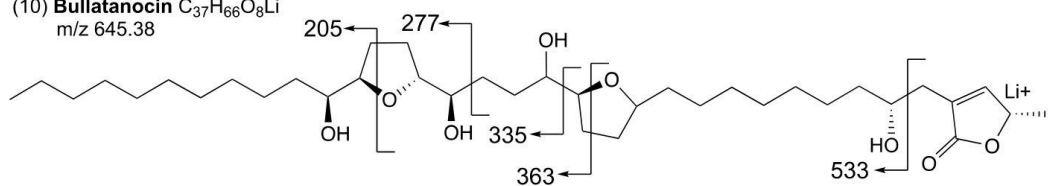
All three solvent extracts of the seeds contained similar ACs, with a greater diversity of compounds in the ethyl acetate extract. Compound **1** was found only in the hexane extract of the seeds with a molecular mass of 530 by ESI-MS ( $[M+H]^+$   $m/z = 531$ ). An adjacent bis-THF compound, desacetylurvaricin (**17**), was identified solely in the hexane extract, with a molecular mass determined by ESI-MS ( $[M+H]^+$   $m/z = 606$ ), leading to the molecular formula  $C_{37}H_{62}O_6$ . The MSMS analysis revealed intense fragments at  $m/z$  347, 275, 205, and 177, suggesting the localisation of a bis-THF ring between C-16 and C-23. This compound was previously reported in the seeds of *A. atemoya* [20]. Interestingly, nine compounds were common to the three extracts according to the MS and MSMS analyses. These compounds were identified as diepomuricanin A (**2**) and B (**3**), annotemoyin-1 (**4**) and -2 (**5**), squamocin G (**6**), squamocin C (**7**), bullatanocin (**10**), atemoyacin-E (**11**), and atemoyacin-A (**18**). The identification of compounds **2**, **3**, **4**, **5**, **6**, **7**, **10**, and **11**, was reported above.

The molecular weight of compound **18** was determined as  $m/z$  595  $[MH]^+$ , corresponding to the molecular formula  $C_{35}H_{62}O_7$ , and MSMS revealed a series of fragments at  $m/z$  489, 347, 275, 205, and 177, confirming the presence of atemoyacin-A (**18**) in all three extracts. Atemoyacin-A (**18**) was previously reported in the seeds of *A. atemoya* Hort [16]. Additional compounds that were only identified in the ethyl acetate extract of the seeds were atemoyin (**19**) and robustocin (**20**). Compound **19** was determined to have a product ion of  $m/z = 601$   $[M + Na]^+$ , and MSMS revealed intense fragments at  $m/z$  347, 275, 205, and 177. The fragmentation pattern observed in the MSMS spectrum indicated that the two THF rings of (**19**) were located between C-13 and C-22. This compound was previously reported in the methanolic and ethanolic extracts of *A. atemoya* seeds [20]. Finally, compound (**20**) is reported for the first time in *A. atemoya*, with the MS showing a compound ion at  $m/z = 585$   $[M + Na]^+$ . The MSMS revealed a fragmentation pattern with two intense fragments at  $m/z$  261 and 233, confirming the occurrence of two THF rings between C-9 and C-18. This compound was previously reported only in the seeds of *A. muricata* [43]. The fragmentation patterns of each compound reported are shown in Figure 1. The NMR and MS spectra are shown in the Supplementary Materials (Figures S5–S26).

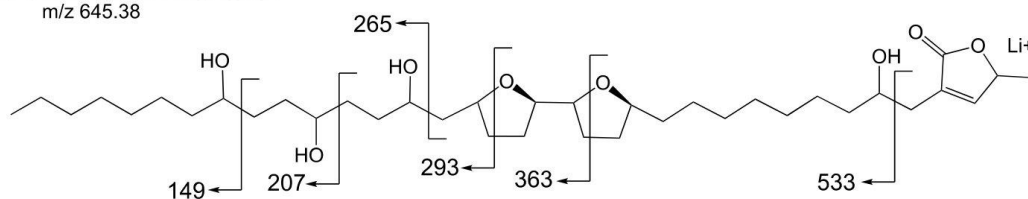
(9) **Annonisin**  $C_{35}H_{62}O_8Li$   
m/z 617.19



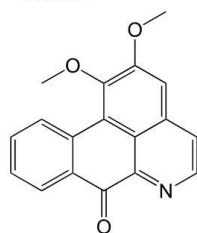
(10) **Bullatanocin**  $C_{37}H_{66}O_8Li$   
m/z 645.38



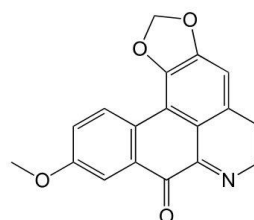
(11) **Atemoyacin E**  $C_{37}H_{66}O_8Li$   
m/z 645.38



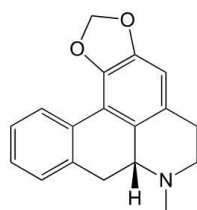
(12) **Lysicamine**  $C_{17}H_{13}NO_3$   
m/z 291



(13) **Lanuginosine**  $C_{18}H_{11}NO_4$   
m/z 304



(14) **Roemerine**  $C_{18}H_{17}NO_2$   
m/z 279



(15) **Corydine**  $C_{20}H_{23}NO_4$   
m/z 341

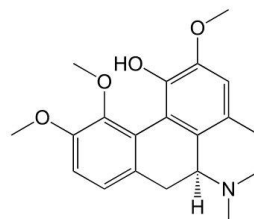
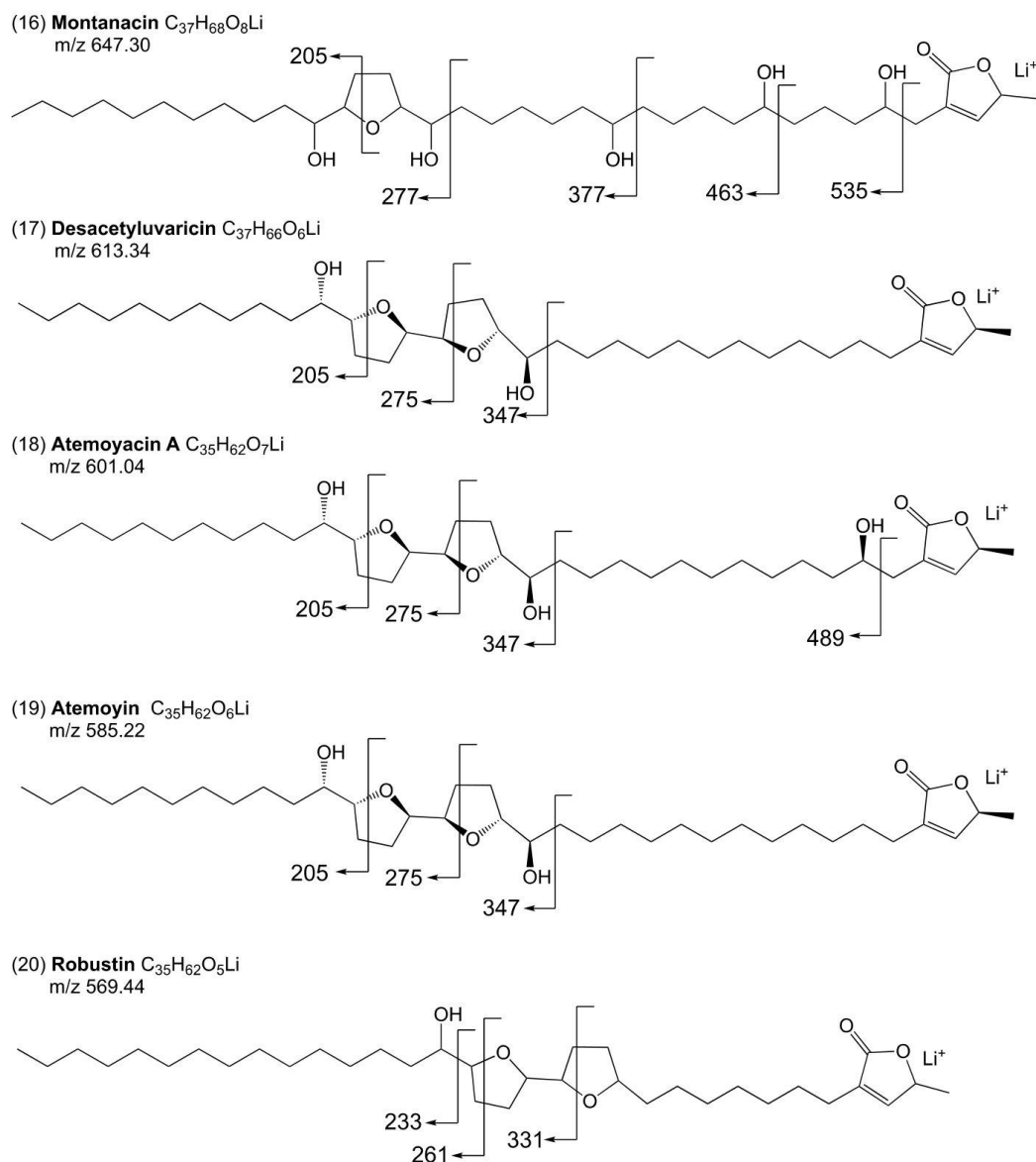


Figure 1. Cont.



**Figure 1.** Structures and fragmentation patterns for the  $[M + Li]^+$  adducts of acetogenins and alkaloids identified in the leaves, pulp, and seeds of *A. atemoya*.

#### 2.3.4. KJ Cultivar

The second cultivar (KJ) was only studied by MS analysis, with some similarities noted between the cultivars. Compounds **1** and **8** were identified in the crude leaf extracts of both the AP and KJ cultivars. Compound **16**, however, was identified in the leaf extract of the KJ cultivar but was not detected in the leaves of the AP cultivar. Compound **1** was identified only in the ethanolic extract of the KJ cultivar fruit pulp, with a molecular mass of 530



determined by ESI-MS ( $[M+H]^+$   $m/z = 531$ ), leading to the molecular formula  $C_{35}H_{62}O_3$ . Interestingly, although the extraction methods were identical for both cultivars, compound **1** was identified in all three solvent extracts of the AP cultivar pulp. The ESI-MS of the ethyl acetate extract of the KJ cultivar pulp indicated the presence of compounds **2** and **3**, with a molecular mass 564  $m/z$ , corresponding to the molecular formula  $C_{35}H_{62}O_4$ . Compound **8** was also found in both the hexane and ethanolic extracts of KJ fruit pulp, with a molecular weight determined by ESI-MS ( $[M+H]^+$   $m/z = 596$ ). However, compounds **2**, **3**, and **8** were not detected in the AP fruit pulp. Finally, compound **16** was found in both the hexane and ethyl acetate extracts of KJ pulp, with a molecular mass determined by ESI-MS ( $[M+H]^+$   $m/z = 640$ ). However, **16** was only detected in the ethyl acetate extract of the AP pulp.

The seeds of the KJ cultivar displayed similar ACs to those of the AP cultivar; however, ACs (**1**, **4**, **5**, and **17–20**) were not detected in any KJ seed extract. Two compounds, **8** and **16**, were found in all solvent extracts of the seeds from the KJ cultivar, with a molecular mass determined by ESI-MS ( $[M+H]^+$   $m/z = 640$  and 596, respectively) but were not detected in any extract of AP seeds. Additionally, compounds **2** and **3** found reported in both the hexane and ethyl acetate KJ seed extracts, with a molecular weight determined by ESI-MS ( $[M+H]^+$   $m/z = 564$ ), corresponding to the molecular formula  $C_{35}H_{62}O_4$ . This is with AP, where they were detected in all seed extracts. Other compounds identified in the seeds of both cultivars were **6**, **7**, **10**, and **11**. However, these four ACs were identified only in the KJ ethyl acetate extract of the seeds, compared with the AP cultivar, which contained these compounds in all three solvent extracts of the seeds.

### 3. Materials and Methods

#### 3.1. Plant Material Collection and Preparation

The fully matured fresh leaves, fruit, and seeds of *A. atemoya* cultivars (African Pride (AP) and KJ Pinks Mammoth (KJ)) were collected in July 2020, from a local farm in southeast Queensland, Australia. The leaves and fruits were washed with running tap water to remove dirt and debris. The leaves were spread out on a bench for air drying at room temperature in a well-ventilated area for one week. The seeds were removed from the fruit, washed, and air-dried. The fruit pulp was sliced and kept on the bench for air drying at room temperature for one month. The dehydrated leaves, pulp, and seeds were ground to a fine powder using a multifunctional grinder. The final weight of the leaf, pulp, and seed powders was 222.4 g, 380 g, and 50.6 g for the AP cultivar, and 180.9 g, 210 g, and 34.1 g for the KJ cultivar, respectively.

#### 3.2. Preparation of Plant Extracts

The dried and powdered leaves, pulp, and seeds of both cultivars were initially macerated with hexane (2.5 L) and filtered. The residues were then extracted with ethyl acetate (2.5 L) and filtered. Finally, the residue was extracted with ethanol (2.5 L). All extractions were carried out once at room temperature with stirring for 72 h and filtrated using vacuum filtration. After each extraction, the solvents were evaporated under reduced pressure, affording the yields shown in Table 2.

**Table 2.** The final weight of crude extracts of *A. atemoya* cultivars.

| Extraction Solvent | AP Cultivar |          |         | KJ Cultivar |          |          |
|--------------------|-------------|----------|---------|-------------|----------|----------|
|                    | Leaves (g)  | Pulp (g) | Seed(g) | Leaves (g)  | Pulp (g) | Seed (g) |
| Hexane             | 12.3        | 2.63     | 10.93   | 10          | 1.787    | 6.32     |
| Ethyl acetate      | 3.9         | 0.783    | 1.72    | 3.2         | 0.884    | 1.3      |
| Ethanol            | 24.7        | 25.52    | 1.5     | 22.48       | 30.2     | 1.09     |

### 3.3. Chemical and Reagents

Laboratory-grade hexane, dichloromethane (DCM), ethyl acetate (EtOAc), ethanol (EtOH), and methanol (MeOH) were obtained from the School of Chemistry, University of Sydney. HPLC-grade methanol, acetonitrile, LCMS-grade methanol, and deuterated solvents ( $\text{CD}_3\text{OD}$  (99.8% D) and  $\text{CDCl}_3$  (99.8% D) were purchased from Sigma-Aldrich (Sydney, Australia).

### 3.4. Phytochemical Screening

Each of the crude hexane, ethyl acetate, and ethanol extracts of the three plant parts was analysed by TLC in order to investigate their phytochemical constituents. A total of 2 mg of each extract was dissolved in 1 mL of solvent and spotted on a TLC plate, which was subsequently developed in a CAMAG  $20 \times 10$  cm twin-through glass developing chambers using MeOH:DCM (10:90 *v/v*) as the mobile phase. The TLC plate was then examined under UV light at 254 nm and 365 nm; then, the TLC plate was further visualised using Kedde's reagent to identify ACs. Firstly, the TLC plate was dipped in a 2% solution of 3,5-dinitrobenzoic acid in 100 mL of ethanol and left to dry for five minutes and then sprayed with 5% KOH in 100 mL of ethanol. For alkaloid identification, TLC plates were visualised with Dragendorff's reagent.

### 3.5. TLC and CC

Analytical plates for TLC were silica gel 60 F<sub>254</sub>, aluminium sheets, 200  $\mu\text{m}$ , ( $20 \times 20$  cm), and Merck silica gel 60 (particle size 0.040–0.063 mm) (Merck KGaA, Darmstadt, Germany). A few milligrams of each crude extract of the leaves, pulp, and seeds dissolved in methanol was spotted on the TLC plate and eluted using methanol:dichloromethane (10:90) as a mobile phase. Plates were visualised under ultraviolet light (UV) at 254 nm and 365 nm, and in order to visualise the compounds, Kedde's (acetogenins) and Dragendorff's (alkaloids) reagents were used. Kedde's reagent was prepared as solutions of 2% (2 g) of 3,5-dinitrobenzoic acid in 100 mL of ethanol and 5% (5 g) of KOH in 100 mL of ethanol [44]. Dragendorff's reagent was prepared as follows: solution A: 1.7 g of bismuth nitrate was dissolved in 100 mL of water:acetic acid (4:1). Solution B: 40 g of potassium iodide was dissolved in 100 mL of water. Solutions A and B were combined as follows: 5 mL A + 5 mL B + 20 mL acetic acid + 70 mL water.

### 3.6. Flash Chromatography

All purifications by flash chromatography were performed on a Reveleris iES system (W. R. Grace & Co.-Conn., Columbia, MD, USA) using the Reveleris® Navigator™ software. This instrument consists of a binary pump with four solvent selection, fraction collector and evaporating light scattering detector (ELSD). Reveleris® flash cartridges packed silica.

### 3.7. NMR

All the fractions obtained and the isolated compounds were characterised by  $^1\text{H}$ -NMR and  $^{13}\text{C}$ -NMR spectroscopy.  $^1\text{H}$  and  $^{13}\text{C}$  spectra were recorded at 400 MHz and 100 MHz, respectively, using a Varian Gemini 400-MR automated spectrometer (Agilent Technologies, Santa Clara, CA, USA) at room temperature, using  $\text{CDCl}_3$  as solvent referenced to the residual signal set at 7.26 ppm for  $^1\text{H}$  and 77.0 ppm for  $^{13}\text{C}$ .

### 3.8. MS and MS/MS

Mass spectra were carried out on a Thermo Scientific TSQ series Quantum Access Max liquid chromatography mass spectrometry (LC-MS/MS) system (Thermo Fisher Scientific, Waltham, MA, USA), using heated electrospray ionisation in positive ionisation mode. High-purity nitrogen (BOC, Sydney, Australia) at 60 psi was used as the sheer gas, and ultra-high purity argon (BOC) at 2 mTorr was used as the collision gas. The mass spectroscopy was conducted at a voltage of 4500 V. The heated capillary and vaporiser were heated at 380 °C and 350 °C, respectively. The mass spectra were acquired at a scan

range of 100–1000 m/z. Data were analysed with XCalibur 4.1.50 (Thermo Fisher Scientific, Waltham, MA, USA) software. For the MS/MS experiment, a solution of crude extracts was prepared at a concentration of 1 mg/mL in MeOH, and the injection volume was 5 µL. The optimal collision energy was 70 eV, and lithium iodide (2 mM in MeOH) was added to each sample before analysis.

### 3.9. Isolated Compounds

#### 3.9.1. Squamocin G (6)

White powder  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ) 0.87 (3H, t,  $J = 6.9$  Hz, H-34), 1.44 (3H, d,  $J = 6.7$  Hz, H-37), 2.40 (1H, dd,  $J = 15.0, 8.2$  Hz, H-3a), 2.50 (1H, br d,  $J = 15.0$  Hz, H-3b), 3.39 (1H, m, H-15), 3.84–3.93 (3H, m, H-16, -23, -24), 3.85 (1H, m, H-4), 3.84–3.90 (2H, m, H-19, 20), 5.06 (1H, qq,  $J = 6.8, 1.4$  Hz, H-36), 7.18 (1H, br s, H-35),  $^{13}\text{C-NMR}$  (400 MHz,  $\text{CDCl}_3$ ) 14.10 (C-34), 19.10 (C-37), 22.67 (C-33), 24.49 (C-22), 25.54 (C-6 or C-13), 25.61 (C-13 or C-6), 26.03 (C-26), 28.34 (C-17), 28.88–28.93 (C-18, C-21), 29.10–29.67 (C-7, C-8, C-9, C-10, C-11, C-12, C-27, C-28, C-29, C-30, C-31), 31.89 (C-32), 32.42 (C-25), 33.2 (C-3), 33.32 (C-14), 37.39 (C-5), 69.99 (C-4), 71.33 (C-24), 74.04 (C-15), 77.97 (C-36), 82.26 (C-19 or C-20), 82.51 (C-20 or C-19), 82.77 (C-23), 83.2 (C-16), 131.18 (C-2), 151.77 (C-35), 174.33 (C-1).

#### 3.9.2. Squamocin C (7)

White powder  $^1\text{H-NMR}$  (400 MHz,  $\text{CDCl}_3$ ) 0.87 (3H, t,  $J = 6.7$  Hz, H-34), 1.40 (3H, d,  $J = 7.1$  Hz, H-37), 2.25 (2H, t,  $J = 7.7$  Hz, H-3), 3.40 (1H, m, H-15), 3.59 (1H, m, H-29), 3.82–3.96 (5H, m, H-16, -19, -20, -23, -24), 4.99 (1H, q,  $J = 6.9$  Hz, H-36), 6.98 (1H, s, H-35),  $^{13}\text{C-NMR}$  (400 MHz,  $\text{CDCl}_3$ ) 14.1 (C-34), 19.2 (C-37), 22.0 (C-26), 22.6 (C-33), 24.80 (C-22), 25.15 (C-3), 25.31 (C-31), 25.64 (C-13, C-27), 27.37 (C-4), 28.36 (C-17), 28.9 (C-18, -21), 29.16 (C-5), 29.30 (C-6, C-7, C-8, C-9, C-10, C-11, C-12), 31.9 (C-32), 32.45 (C-25), 33.28 (C-14), 37.23 (C-30), 37.48 (C-28), 71.39 (C-24), 71.77 (C-29), 74.12 (C-15), 77.4 (C-36), 82.21 (C-19), 82.52 (C-20), 82.78 (C-23), 83.3 (C-16), 134.3 (C-2), 148.87 (C-35), 173.9 (C-1).

## 4. Conclusions

Several *Annona* species have been used as traditional medicines, particularly across South America and Asia, and have been phytochemically well characterised. Many of the ACs present in *Annona* species have also been shown to have anticancer activities. *A. atemoya* is a hybrid of two *Annona* species: *A. squamosa* (sugar apple) and *A. cherimola* (Chirimoya), first reported in 1907, which is gaining importance as a commercially important crop due to its creamy and sweet fruit. However, only few studies have investigated the phytochemistry of *A. atemoya*. Some previous studies have reported the presence of ACs in the seeds of *A. atemoya* [16–18,20–22]; our data are the first to show that ACs are also present in the leaves and pulp of *A. atemoya*.

In total, twenty compounds were identified: sixteen ACs (1–11 and 16–20) and four alkaloids (12–15). The seeds of the AP cultivar were the richest in ACs, with fourteen compounds identified (1–7, 10, 11, 17–20) in all three extracts, compared with the pulp, which only contained two ACs (1 and 16). In addition, the ethyl acetate crude extracts of both the leaves and seeds were richer in ACs in comparison with the other crude extracts. Compounds 6 and 7 were isolated, and their structures and absolute configuration were determined through the interpretation of the spectroscopic data and comparison with previously reported data. Montanacin A (16) is reported for the first time in the pulp of the AP cultivar and the leaves of the KJ cultivar of *A. atemoya*.

Our results demonstrated that *A. atemoya* contains a range of acetogenins and alkaloids, similar to those found in other *Annona* species. To the best of our knowledge, this is the first comprehensive study of the ACs in the leaves and pulp of *A. atemoya*. Although the two cultivars contained similar ACs, the KJ cultivar contained only 9 ACs compared with the 16 detected in the AP cultivar, indicating that further study of other cultivars should be undertaken. Given the increasing consumption of custard apples, their potential health



benefits, and the toxicity of some of the constituents found in the fruit and leaves, a study to quantify some of the relevant constituents is also warranted.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ijms24032294/s1>.

**Author Contributions:** Conceptualisation, J.R.H.; methodology, B.S.M.A.K.; validation, J.R.H. and J.E.H. formal analysis, B.S.M.A.K.; investigation, B.S.M.A.K.; writing—original draft preparation, B.S.M.A.K.; writing—review and editing, J.R.H. and J.E.H.; supervision, J.R.H. and J.E.H. All authors have read and agreed to the published version of the manuscript.

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‘And their call will close with these words: All praise is due to God (Allah), the Sustainer  
of all the worlds’

- The Qur'an, Surah Yunus