EXTRACTION OF TRITERPENOIDS SAPONINS FROM AUSTRALIAN PLANT USING SUPERCRITICAL FLUIDS

A thesis submitted in fulfillment of the requirements for the degree of Master of Philosophy

By

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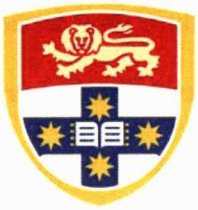

THE UNIVERSITY OF SYDNEY

School of Chemical and Biomolecular Engineering

August 2010

Abstract

It has been identified that triterpenoids saponins extracted from plants can have cardiovascular, antitumor, and anti-inflammatory activities. The Australian Acacia plant has a broad range of triterpenoids saponins. Current methods for separation and isolation of triterpenoids saponins involve using a large amount of organic solvents, which can be a drawback of using the extract as a functional food and nutraceutical.

The objective of this study was to investigate the feasibility of using supercritical fluids for the selective extraction of triterpenoids saponins from Australian Acacia plant. The process parameters, such as temperature, pressure, and solvent modifier, were optimized for the extraction of triterpeonid saponin with high cytotoxic effect on cancer cells. The results were compared with multistage organic solvent extraction. NMR was used to characterize the group of compounds extracted at each condition. *In vitro* MTT assay was used to investigate the biological activity of extracts. The results acquired from conventional extraction process demonstrated that crude extraction by methanol included both triterpeonids saponins and sugar compounds with high anticancer activity. Further fractionation steps and the use of other organic solvents decreased the anticancer activity of extracts from Acacia plants.

Neat supercritical CO_2 was efficient in extraction of fat from Acacia plants. However, saponins and other polar compounds were not extracted by neat CO_2 at 50 °C and 180 bar. Addition of small amount of methanol substantially enhanced the solvation power of CO_2 for the extraction of saponins in Acacia plant. Different groups of saponins

and sugar were extracted by varying the methanol mole fraction, temperature and pressure of SFE process. A higher selectivity and anticancer activity of the active compounds were obtained in comparison with conventional extraction methods and in a shorter period of time. The results of NMR analysis demonstrated that the extract from SFE contained a selective number of triterpenoids saponins and negligible amount of sugar group, when using low concentration of methanol in CO_2. However, the extract from methanol included various groups of triterpenoid saponins and different types of sugar compounds. Methanol concentration and temperature had significant impact on SFE; however, the effect of pressure was negligible. The optimum conditions for the extraction of anticancer active compounds within the conditions examined, was 31 °C, 90 bar and using 5 mol% methanol. The cytotoxic activity of the extract acquired at this condition was significant (IC_{50}= 0.05±0.03 μg/ml) that was nearly 400 fold higher than using neat methanol for the extraction (IC_{50}= 19.24±0.32 μg/ml).

Comparison of extracts in terms of growth inhibition on normal human skin fibroblast cell was conducted. Extract from SFE was less toxic (IC_{50}: 371.28 μg/ml) to human cell than extract from methanol (IC_{50}: 42.77 μg/ml). From the NMR analysis, it confirmed that the extract from SFE contained high quantity of triterpenoids saponins and contributed to biological activity. On the contrary, the extract from methanol was more complicated and had larger sugar content than the one from SFE. The results suggest that supercritical fluid extraction was effective for the selective extraction of saponins from *A. victoriae* with high anticancer activity.

Acknowledgements

I would like to express my gratitude to my supervisor, A. Prof. Fariba Dehghani for her kind supervision, direction and inspiration, which help me to develop research abilities and complete this study in due time. I am also indebted to Fariba for the painstaking task of reviewing this thesis and for the valuable suggestions offered, without which I would not have been able to finish this work. I would like to acknowledge my Associate Supervisor, Dr. Colin Duke for his support and guidance. His valuable discussions and supervision had a key role in completion of this project.

I am grateful for financial support of Faculty of Engineering that covers my tuition fees, and Mr. Vic Cherikoff's financial support and intellectual contribution for this project.

The valuable discussion and advice provided by Mr. Peter Valtchev at various stage of this study are greatly appreciated. My appreciation and gratitude is also extended to Dr Van Tran from faculty of Pharmacy and my colleagues at School of Chemical and Biomolecular Engineering at University of Sydney. I wish to thank Mr. Chengdong Ji for his help on statistical analysis. I also wish to thank Mrs. Elizabeth Dobrinsky who always efficiently took care of providing material and equipment needed for my research.

Finally, I thank all my family and friends for their love and encouragement including my husband Mr. Xiao Liang. Thanks for their love, support and kindness during my study.

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1. Introduction

The extracts from plants have been used as medicines, food additives, and natural pesticides. Certain plants have active compounds, which are effective for the treatment of challenging diseases. The functional ingredients are obtained by enriching traditional foods to promote a beneficial action for human health, and these ingredients from natural sources are more attractive by customers. For example, triterpenoid saponins, isolated from Australian desert tree *Acacia victoriae*, showed inhibitory effects for tumor cell growth and induce apoptosis, in part, by perturbing mitochondrial function [1]. Due to the low concentration of pharmacologically active compounds in natural plants, it is highly desirable to develop extraction methods with superior selectivity and efficiency for recovery of these compounds from raw materials [2].

Triterpenoids saponins are naturally occurring sugar conjugates of triterpenes possessing the property of forming stable froth when shaken with water [3]. They have been found as constituents of many folk medicine remedies and some recently developed drugs for wide bioactivities, such as cardiovascular, antitumor, antifungal, antiviral and anti-inflammatory agents [4]. Saponins are widespread in nature and represent a family of highly bioactive compounds. Madl et al. found that crude total saponins from *C. quinoa* act as immunological and absorption adjuvants to enhance immune responses and mucosal absorption to a substance co-administered therewith [5].

Acacia originated from Australia has been introduced as a horticultural variety throughout the world and is commonly known as prickly wattle or elegant wattle [6]. *Acacia victoriae* has a number of agricultural uses including wind breaks, shelter belts, food, critical area stabilization and as a low water-use ornamental. Different Acacia species seeds have been used as a source of food material by the indigenous Australian [7]. Among the Acacia's, *Acacia victoriae* is the most common and widespread species, present all over Australia. The ground Acacia seeds are used as a flavoring agent in pastries, breads, and desserts, especially ice-cream. They are also used in high quality coffee-like beverages [8]. Gutterman and collaborators demonstrate that the desert legume plant *Acacia victoriae* contains highly active saponins, which have selective mechanisms of action, and inhibition of tumor cell proliferation via induction of apoptosis and prevention of nuclear transcription factor-*k*B (NF-*k*B) [9-12].

The early determination of saponins in plant material was based predominantly on gravimetry or on methods taking advantage of some of their chemical and biological features [13]. The foaming property, which is a well-known feature of most saponins, was used to search for plant saponin content. Froth formation after shaking in water solution is specific to most saponins, but some of them, especially those with two or three branched sugar chains do not form stable froth, and conversely some plant extracts not containing saponins may produce froth, providing misleading information [14].

General procedures for obtaining crude saponin mixtures typically include extraction with aqueous alcoholic solution using methanol, ethanol, etc. A defatting step,

generally with petroleum ether is performed before the extraction step to remove fat impurities in saponins extract [15]. Conventional chemical protocols for extraction of saponins are difficult to be implemented and even may result in losing active ingredients during the processing steps, because of their inherent features, such as high polarity, non-volatility and low content in plants [16]. Consequently, it is desirable to develop extraction methods with better selectivity and efficiency for recovery of these compounds from raw materials.

The use of supercritical fluids, especially carbon dioxide, in the extraction of active components from plants has increased recently due to the advantages of the supercritical extraction process. Supercritical fluid extraction (SFE) usually performed with pure or modified carbon dioxide, is a rapid and selective technique for recovery of active compounds from plant matrices. In SFE, less hazardous chemical is used and the process has a minimal chemical damage to the extract [7]. Several large scale SFE plants have been in operation in Europe and Asian Countries for more than two decades [17]. Large scale processes are related to the food industry like the decaffeination of coffee beans [18, 19], black tea leaves [20] and the extraction of bitter flavours from hops [21, 22]. The maximum throughput of a single plant for extraction is well above ten thousand tons solid process per year [2]. SFE from solids is carried out by continuously contacting the solid substrate with the supercritical solvent. The solid substrate in most cases forms a fixed bed. The SCF flows through the fixed bed and extracts the desired compounds until the substrate is depleted [2]. SFE products are superior in quality and yields. For example, Johnson et al [31] developed methods for determination of fat content in fish feed. Extracts from SFE method contained a significantly greater amount of phospholipids (15-85%) than

extracts from the Soxhlet method. The unique properties of SCF and restricted regulation by environmental protection agency for minimising organic solvent in food products are key factors that promote to use SFE as an alternative technique in food processes. SFE has been used to extract triterpenoid saponins from Bamboo [32], vitamins from food samples [25], carotenoids and chlorophyll from microalgae [33], cardamom oil from cardamom seeds [34], chlorophyll from dendrocalamopsis oldhami foliage [35], and ginsenosides from North American ginseng [36].

In previously published studies, active compounds that have anticancer activity in Acacia plant have been identified. These compounds were extracted by 1:1 volume ratio of dichloromethane and methanol. It was shown that certain triterpene saponins (called avicins) from *Acacia victoriae* are selectively toxic to tumor cells at very low doses (IC_{50}: 0.2 μg/mL for Jurkat cells) [12]. However, the anticancer activity of crude saponins extract was not reported. Keung found that crude extract from *Radix puerarize* is apparently 10 times more active than daidzin as a pure compound [37]. The presence of another agent(s) may have synergistic effect on the activity of daidzin and improve its bioavailability for antidipsotropic effect.

The aim of this study was to investigate the feasibility of extracting triterpenoids saponins by SCF technique. Prior to SFE and process optimization *in vitro* study was conducted to investigate anticancer activity of extracts from *A. victoriae* plant.

2. Supercritical Fluid Extraction (SFE)

2.1 Introduction

A supercritical fluid (SCF) is a fluid at a temperature and pressure above its critical point. It can diffuse into a solid phase like a gas, and dissolve materials like a liquid. In recent decades SCFs have gained their significance in science and industrial applications because of further safety and environmental concerns for reducing volatile organic compounds (VOC), particularly in food and pharmaceutical industry [38]. Carbon dioxide is the most commonly used SCF, because it is nonflammable, inert with a critical point of 31.1 °C and 73.8 bar that is excellent for processing thermally-labile and oxygen-sensitive compounds [39]. Supercritical Fluid Extraction (SFE) is known as an environmentally-friendly process and has been used for the extraction of biological active compounds from different plants. SFE has been used in environmental, pharmaceutical, polymer and food processing. SFE efficiency can be manipulated by variation of pressure and/or temperature, and it may achieve a high selectivity [10]. SFE from solid materials is the most developed application, mainly for the food products (coffee, tea) [26, 27, 40-42], food ingredients (hops and aromas, colorants, vitamin-rich extracts, specific lipids) [29, 30, 43, 44], and nutraceuticals [45-48]. The advantages of using SFE is elimination of organic solvents from process, selective extraction of active ingredient and potential to produce pesticides free product that are attractive in food processing [49, 50].

2.2 Physical Principles of SCFs

A supercritical fluid is a fluid at above its critical temperature and pressure as shown in Figure 2-1. At the critical point, the gas and liquid phases merge to form a single homogeneous fluid phase. Under these conditions, the fluid acquires a liquid like density and its viscosity and diffusivity are close to gases as shown in Table 2-1 [51]. A commonly accepted opinion is that the density of SCF and the physicochemical affinity of the solute for the solvent, is the basis of its solvent power [52]. The feasibility to tune these properties with pressure and temperature renders SCFs promising solvents for chemical reactions and extractions.

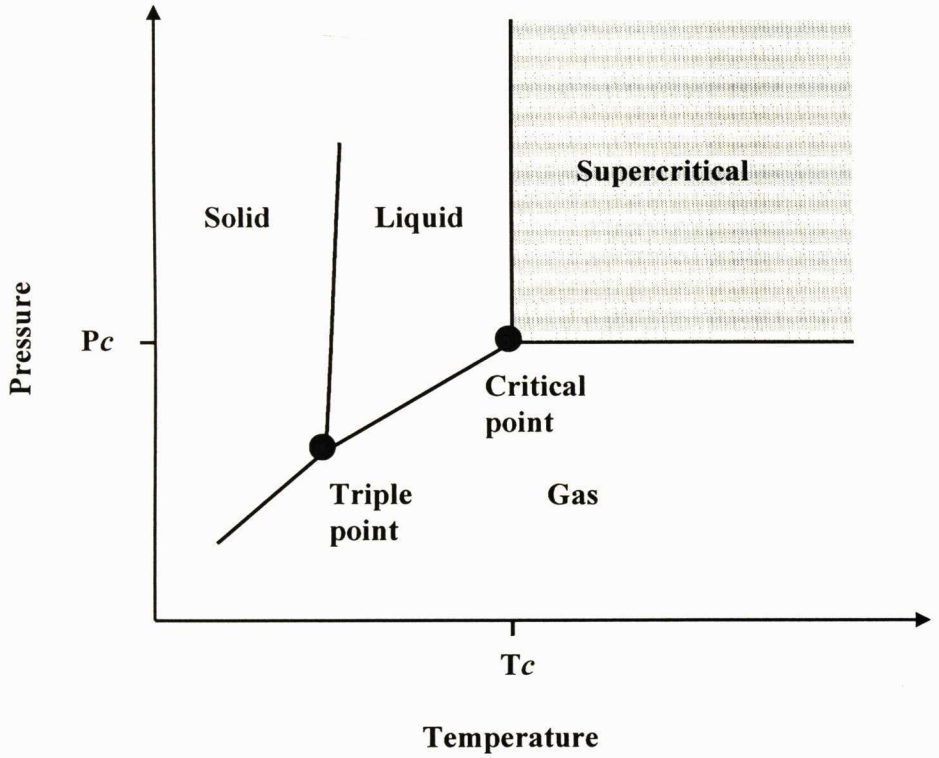


Figure 2-1 Typical phase diagram for a pure compound [2]

Table 2-1 Characteristic values for physical properties of gases, liquids and supercritical fluids [2]

State of the fluid	Density (g/cm^3)	Diffusivity (cm^2/s)	Viscosity (g/cm/s)
Gas			
P=1 bar, T=15-30 °C	$(0.6\text{-}2.0)\times10^{-3}$	0.1-0.4	$(0.6\text{-}2.0)\times10^{-4}$
Liquid			
P=1 bar, T=15-30 °C	0.6-1.6	$(0.2\text{-}2.0)\times10^{-5}$	$(0.2\text{-}3.0)\times10^{-2}$
Supercritical fluid			
$P=P_c$, $T\approx T_C$	0.2-0.5	0.7×10^{-3}	$(1\text{-}3)\times10^{-4}$
$P=4P_c$, $T\approx T_C$	0.4-0.9	0.2×10^{-3}	$(3\text{-}9)\times10^{-4}$

Dissolved compounds can be recovered from supercritical fluid simply by decreasing the pressure and/or increasing the temperature, both of which reduce fluid density [49]. Generally, the solubility of a given solute increases with both increasing density of the solvent and increasing vapor pressure of the solute. Consequently, the solubility of a solute in a SCF increases with increasing pressure, whereas it decreases, remains constant or increases with increasing temperature, depending on the predominant factor. However, solubility in liquids usually surpass those in SCFs and that the dissolving power of a SCF approaches that of a liquid solvent at high enough densities [38].

The dynamic viscosity of a SCF is comparable to a gas which is an advantage for mass transfer, since natural convection effects are inversely proportional to the square

of the kinematic viscosity [53]. It can penetrate into porous solid materials more effectively, which results in rapid extraction fluxes and a substantial reduction in extraction time through rendering greater mass transport properties [3]. Diffusion coefficient of solutes in SCFs is close to gases; this is beneficial for processes that diffusion of a component is a limiting step. Viscosity and diffusivity of a SCF are dependent on temperature and pressure, as is the case for density. For a SCF, viscosity increases and diffusivity decreases as pressure is increased, approaching values of a liquid. An increase in temperature leads to a decrease in viscosity of the SCF, whereas diffusivity will increase with an increase in temperature [54].

A fluid at the vicinity of critical point exhibits special properties. The difference between liquid and vapor suddenly disappears at the critical point, and a number of physical properties approaches zero (e.g. enthalpy of vaporization and surface tension), whereas others diverge theoretically (e.g. isothermal compressibility). Last but not least, the fluid shows the phenomenon of critical opalescence, a characteristic of the phase transition at the critical point [38].

When a liquid is heated in a sealed vessel, boiling does not occur at SC point. The density of liquid decreases as a result of its expansion and approaches to the density of vapor and the surface between two phases disappears. The temperature at which the interface disappears is the critical temperature T_C and the corresponding vapor pressure is the critical pressure P_c. Above the critical temperature the liquid phase of substance does not exist.

For a reduced temperature ($T_R=T/T_C$) in the range of 0.9-1.2 the reduced solvent

density ($\rho_R=\rho/\rho_C$) can increase from gas-like values of 0.1 to liquid-like values of 2.5 as the reduced pressure ($P_R=P/P_C$) is increased. But as T_R increased to 1.55, the supercritical fluid expanded and reduced pressures greater than ten are required to obtain liquid-like densities. By operating in the critical region, the pressure and temperature can be used to tune density, which in turn adjusts the solvent power of a supercritical fluid. The density of a pure solvent changes in the region of its critical point dramatically, as shown in Figure 2-2 [55].

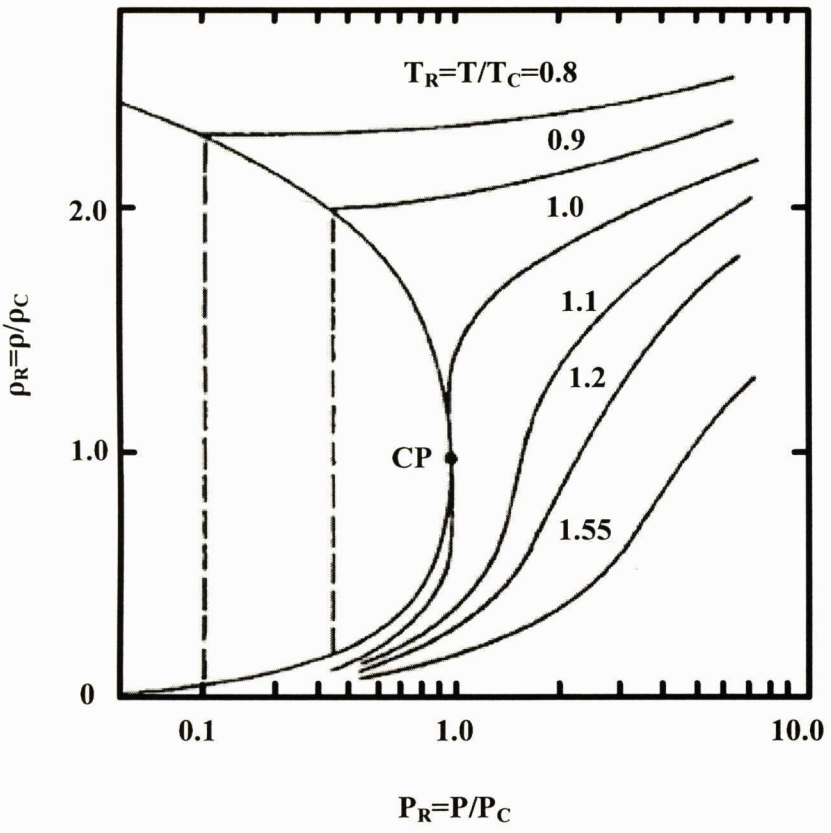


Figure 2-2 Variation of the reduced density (ρ_R) of a pure component in the vicinity of its critical point

In many supercritical fluid systems, a retrograde phenomenon occurs, close to critical region, where the increase in solute vapor pressure is sufficient to fully compensate the loss of solvent power caused by the reduction of solvent density [49]. When temperature is increased, the solute vapor pressure enhances and this effect may be dominant to the effect of temperature on solvent density, resulting in an overall higher solubility. The effect of temperature on the solvent power of a SCF can be elaborated by the solubility profile of solid naphthalene (T_m=80.2 °C) in supercritical ethylene near its critical point (T_C=9.3 °C, Pc=50.5 bar). As illustrated in Figure 2-3, at pressures above 90 bar, the solubility of naphthalene in supercritical ethylene was increased with increasing temperature [55]. Below this pressure, by increasing temperature the solubility of naphthalene was decreased. Although this example suggests that there is correlation between solvent density and strength. It is the interactions between solvent and solute molecules that determine how much solute dissolves in a SCF solvent. By compressing the solvent and increasing the density, the probability that solvent and solute molecule interacts. The type of interactions, such as hydrogen bonding, depends on the physical characteristics of each species in the mixture. Some food processing systems have succeeded in controlling and exploiting this complex thermodynamic behavior, to affect simultaneous extraction and fractionation at relatively low pressure [56].

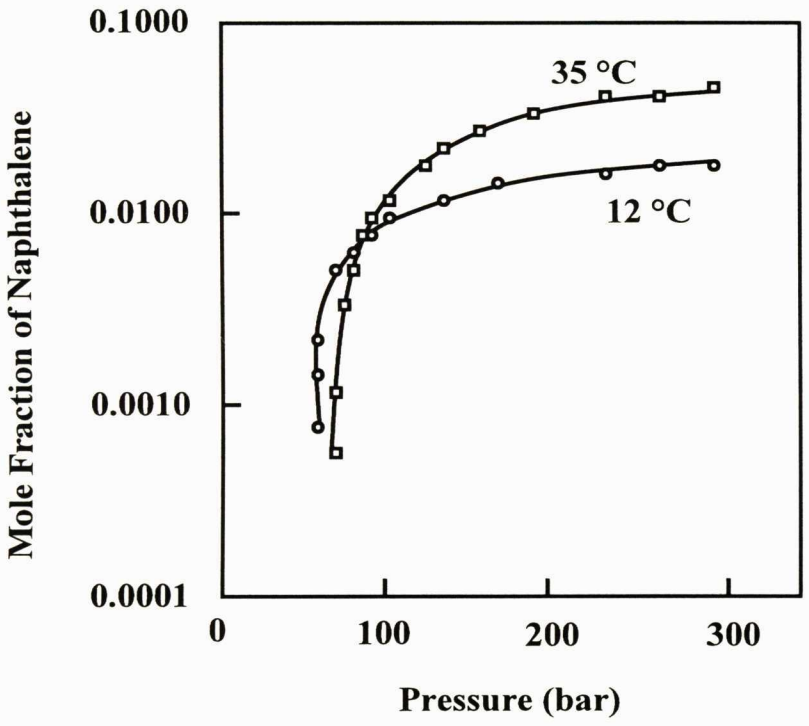


Figure 2-3 Solubility behavior of solid naphthalene in supercritical ethylene [55]

2.3 Solvents Used as Supercritical Fluids

The critical properties of SCF solvents that are commonly used at commercial scale are listed in Table 2. Many hydrocarbons have a critical pressure close to 45 bar. The critical temperature of SCFs is enhanced by raising their molecular weight, and increasing their polarity or intermolecular hydrogen bonding. The critical temperature of water is high due to the strong hydrogen bond; a large amount of thermal energy is required to break the hydrogen bonds and evaporate the water [55]. Additionally, if oxygen was not carefully purged, water at high temperature can be corrosive and might cause damage to extraction vessels [10]. Supercritical water is nontoxic, nonflammable, and ecologically benign; however, it is not suitable for the extraction

of thermally labile compounds. On the other hand, it is being tested for hazardous-waste detoxification [57] and hydrocarbon reforming [58-61]. A process for extracting active compounds or polymers from a plant within a short period of time by supercritical water was reported [62, 63].

As can be seen in Table 2-2, the critical temperature of N_2 and CO_2 is significantly lower than alcohols and heavy hydrocarbons, which suggests the use of specific supercritical fluids for different applications. For example, carbon dioxide, ethane and ethylene are attractive solvents for the extraction of heat-sensitive flavors, pharmaceuticals, labile lipids, reactive monomers and critical point drying and cleaning of microelectronics because the critical temperatures of these solvents are near ambient. Higher molecular weight hydrocarbons with high critical temperatures are used for processing nonvolatile substances such as processing of heavy hydrocarbons from petroleum fractions, recovery and purification of lubricant oils, coal liquefaction and polymer processing [55]. Recent years, subcritical water was used for the development of high-rate hydrolysis saccharification, the extraction of lignocelluloses and manufacturing of glucose, oligosaccharides, and other monosaccharide [64-68]. Subcritical water was also used for the production of microparticles and the extraction of some food products [69].

Table 2-2 Critical properties of various solvents [51, 55, 70-73]

Solvent	**Critical Temperature (°C)**	**Critical Pressure (bar)**	**Critical Density (g/ml)**
Carbon dioxide, (CO_2)	31.1	74.8	0.27
Ethane, (C_2H_6)	32.4	49.5	0.29
Ethanol, (CH_3-CH_2-OH)	243.2	62.2	0.24
Fluoroform, (CHF_3)	26.3	49.2	0.26
n-Heptane, (C_7H_{16})	267.3	27.8	–
n-Hexane, (C_6H_{14})	234.4	30.5	0.26
Methane, (CH_4)	-82.4	46.6	0.29
Methanol, (CH_3-OH)	240.1	82.0	0.22
Nitrous oxide, (N_2O)	36.6	73.4	0.27
Water, (H_2O)	374.4	224.1	0.23

The most commonly used supercritical fluid in food processing is CO_2, because it has a moderately low critical pressure and quite a low critical temperature. Carbon dioxide is removed from the extract upon depressurization and it can be vented to the atmosphere or recycled without harm. Furthermore, high purity CO_2 is available with comparably low cost [38]. Early systematic studies on the solubility of a range of lipophilic natural substances in CO_2, show that, generally, solubility decreases with increasing molecular weight within a homologous series, and increases with the addition of polar groups such as hydroxyl, carboxyl or nitrogen [49]. Although SC-CO_2 has been used to extract a variety of non-polar compounds from both biological and non-biological sources, its current commercial applications involve the extraction

of natural products where there is a requirement for low temperature processing, high mass-transfer rates and negligible amount of solvent carried into the final product. The extraction may be facilitated by a preliminary size reduction from solid [74].

2.4 Effect of Modifier

The solubility of high molecular weight and polar compounds in CO_2 is limited. Polar or non-polar modifiers have been added to CO_2 to increase the solubility of such compounds. A modifier or a co-solvent, is commonly a volatile compound that is miscible with the primary supercritical fluid, and usually constitutes only a small percentage of the overall fluid composition [49]. The modifier interacts strongly with the solute and significantly increases the solubility of the original SCF solvent [75]. The modifiers are used to selectively extract and enrich the more polar target molecules from the raw material [76]. In addition, modifiers often enhance extractions from solid materials by disrupting the bonding between solutes and the solid matrix [36].

The most acceptable and efficient modifiers in industrial practice include water and short-chain alcohols [70]. Polar compounds such as triterpenoids saponins, for example, show very limited solubility in pure CO_2 and in such cases either a more polar supercritical fluid should be used or a modifier such as those listed in Table 2-3 can be added to enhance the solvent strength [77]. Among all the modifiers, methanol is the most commonly used because it can disrupt the bonding between the solutes and plant matrices [78]. To gain a better understanding of the solvation power of SCF, researchers have studied bulk properties such as dielectric constant, which describe

the effect of solvents on reaction rate in terms of pure electrostatic interaction between ions or dipolar molecules and solvent molecules in their initial, final, or transition state [79, 80]. Ethanol, though not as polar as methanol, may be a better choice in SFE of natural products because of its lower toxicity. Several reports have successfully employed ethanol as a modifier in SFE for the extraction of organic compounds from plants [81]. Depending on the properties of the samples and the desired compounds, the best modifier usually can be determined based on preliminary experimental results. Although moisture in raw plants materials usually is undesirable because it may cause clogging problems, in some cases it could be an advantage because water may function as a modifier for SFE of certain compounds [82-85].

Table 2-3 Additives commonly used to modify CO_2 for SFE [3]

Modifier	**T_c (°C)**	**P_c (atm)**	**Molecular mass**	**Dielectric constant at 20°C**	**Polarity index**
Methanol	239.4	79.9	32.04	32.70	5.1
Ethanol	243.0	63.0	46.07	24.3	4.3
Propan-1-ol	263.5	51.0	60.10	20.33	4.0
Propan-2-ol	235.1	47.0	60.10	19.3	3.9
Hexan-1-ol	336.8	40.0	102.18	13.3	3.5
2-Methoxyethanol	302	52.2	76.10	16.93	5.5
Tetrahydrofuran	267.0	51.2	72.11	7.58	4.0
1,4-Dioxane	314	51.4	88.11	2.25	4.8
Acetonitrile	275	47.7	41.05	37.5	5.8
Dichloromethane	237	60.0	84.93	8.93	3.1
Chloroform	263.2	54.2	119.38	4.81	4.1

Modifier	T_c (°C)	P_c (atm)	Molecular mass	Dielectric constant at 20°C	Polarity index
Propylene carbonate	352.0	-	102.09	69.0	6.1
N,N-Dimethylacetamide	384	-	87.12	37.78	6.5
Dimethyl sulphoxide	465.0	-	78.13	46.68	7.2
Formic acid	307	-	46.02	58.5	-
Water	374.1	217.6	18.01	80.1	10.2
Carbon disulphide	279	78.0	76.13	2.64	-

The solubility range, selectivity and versatility of supercritical carbon dioxide systems can be extended and modified dramatically by the use of modifiers. Among the relatively few theoretical studies of supercritical fluid modifiers that have been published, the observations of Dobbs and Johnston (1987) provide an interesting example of both qualitative and quantitative effects of a modifier (3.5 mol% methanol) on the solubility of two structurally similar solutes (benzoic acid and hexamethyl benzene) in supercritical CO_2. Their data, shown in Figure 2-4, indicates that, the solubility of both solutes was increased by addition of 3.5 mol% methanol in CO_2. This effect was more pronounced for benzoic acid, underlining that modified CO_2 can be used for the separation of these two compounds. Modifiers such as methanol enhance solute solubility because of physiochemical between these compounds. The increase of solvent density and the presence of hydrogen bonding between the benzoic acid and methanol were accounted for the enhancement of the solubility and selectivity [86]. When adding a modifier, care must be taken to ensure the fluid mixture is in the supercritical state, resulting in homogeneous phase. The effect of mole fraction of methanol on the critical properties of CO_2: methanol mixture is

presented in Figure 2-5. The critical temperature of 20 mol% methanol and CO_2 mixture increased to 80 °C and 150 bar, therefore, the SCF extraction can be conducted at above these conditions to utilize its unique properties [1].

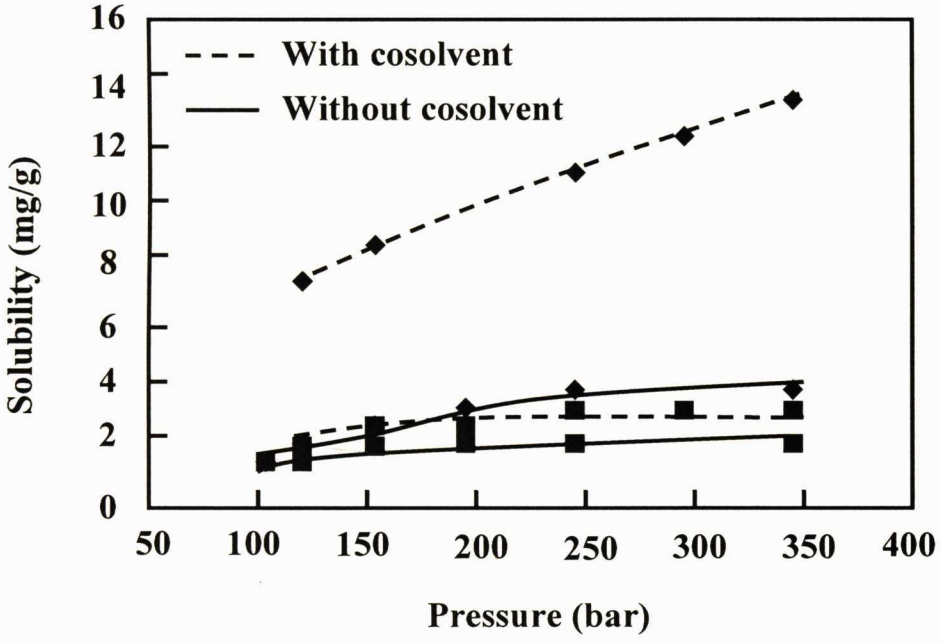


Figure 2-4 The effect of methanol on the solubility of both benzoic acid (◆) and hexamethyl benzene (■) in supercritical CO_2 system [86].

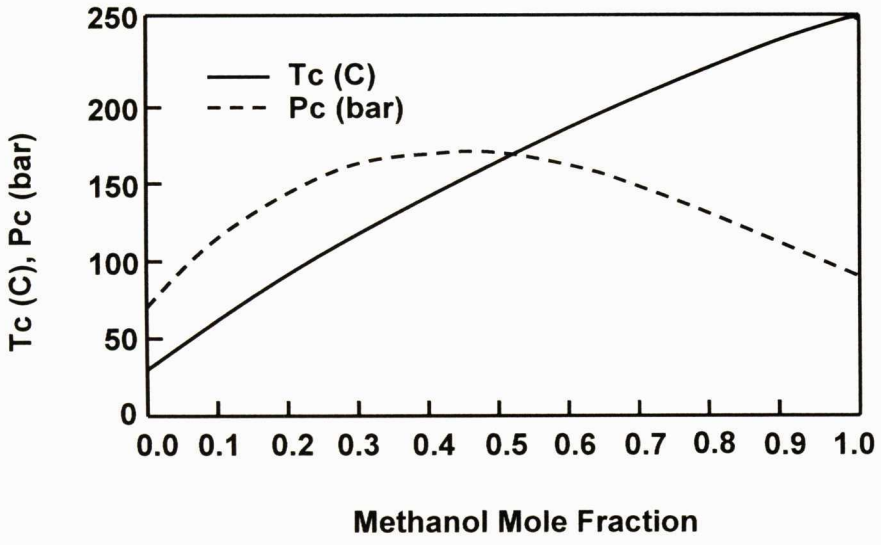


Figure 2-5 Relationship between the critical temperature, pressure and mole fraction of a CO_2-methanol mixture [1]

Despite of significant improvement in solvation power of modified SCF, there are some disadvantages in using co-solvents. The use of modifiers to improve recoveries sometimes may suffer from the disadvantages that it is necessary to strip the modifier from the product, particularly when solid traps are used, after extraction. In these cases, the trap temperature may be set above the boiling point of the modifier to avoid the formation of a liquid phase in the solid trap, which affects its retention capability. The generation of a homogeneous CO_2-modifier mixture (only one supercritical phase of constant composition during the extraction) complicates instrumentation as two synchronized high pressure pumps are required. Alternatives such as premixed fluids and the addition of modifier to the sample are unviable because modifier content changes during cylinder depletion and extraction processes, respectively. In the latter alternative, two-phase formation can also occur [87].

2.5 Supercritical Fluid Extraction Design

SFE is conceptually simple to perform and does not require sophisticated equipment. Extracted food material is often solid, and therefore semi-batch extraction is frequently used [70]. A schematic diagram of a semi-batch supercritical fluid extraction system is presented in Figure 2-6. The extraction process consists of a high-pressure pump and pump for delivery of modifier in case it is required, an extraction vessel and one or more separators in which the solvent is depressurized and the extract is collected. A heat exchanger is used to adjust the temperature of process materials, and they are commonly equipped with independent control of temperature and pressure, in such way that fraction of the extracted compounds can be carried out by a stepwise depressurization. Therefore, different compounds can be obtained within each separator, depending on their solubility in the supercritical fluid [51]. Additional equipment includes pipes, valves, measuring and controlling devices [2]. In many cases, it is essential to install a refrigerated system to trap volatile compounds and also to recycle the SCF. So far used in SFE systems, vessels between 10 L to 1000 L have been used in commercial scale SFE process [39].

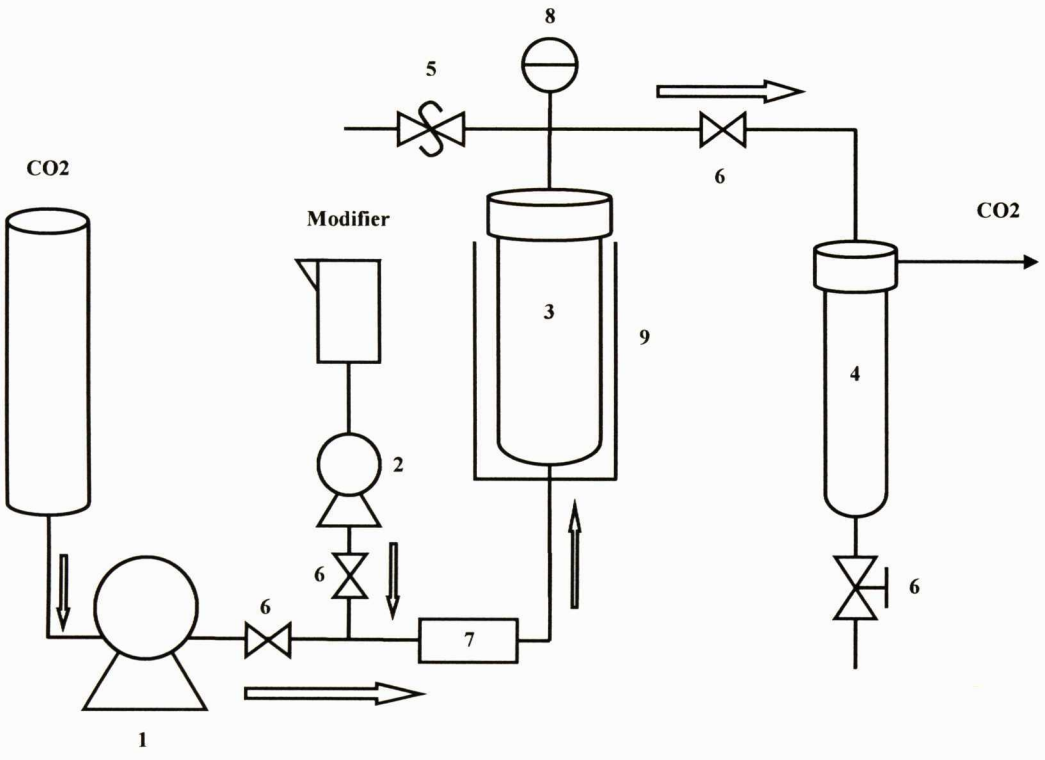


Figure 2-6 Schematic diagram of a supercritical fluid extraction (1) CO_2 pump, (2) modifier pump, (3) solid samples extraction cell, (4) fractionation cell, (5) pressure relief valve, (6) ball valve, (7) mixer, (8) pressure indicator, (9) heating jacket.

The nature of the material and the desirable extract, type of SCF, and extraction conditions are factors that can have a significant impact on the extraction efficiency [10]. The major process parameters are temperature, pressure, flow rate, the modifier and extraction time. In this section, the effect of some of these factors is described briefly.

2.6.1 Effect of Pressure and Temperature

The solubility of a target compound in a SCF is a major factor determining its

extraction efficiency. Solubility of a substance in a supercritical fluid is the sum of two factors: the vapor pressure of the substance, which is a function of a temperature, and the solvating power of the supercritical fluid, which is a function of fluid density [3]. Hence solubility is controlled experimentally by selecting the extraction pressure and temperature.

The solubility of naphthalene at 45°C and various pressures is presented in Figure 2-7. This solute is slightly soluble in CO_2 at 75 bar (Threshold Pressure) and as the pressure increases the solubility rises, especially around 90 bar, up to its maximum value. The pressure plays a critical role in the extraction efficiency. An elevation of pressure at a given temperature results in an increase in the fluid density, which enhances the solubility of a desirable solute. Consequently, at higher pressures, lower volume of fluid is required for the extraction. For example, one needs to double the volume of CO_2 to extract 70% of diuron herbicide from a contaminated soil, when working at 110 bar instead of 338 bar [57]. However, high pressure is not always recommended for the extraction of natural products that comprised of a multi-component system as the selectivity may decrease and the production of complex extracts can complicate analysis.

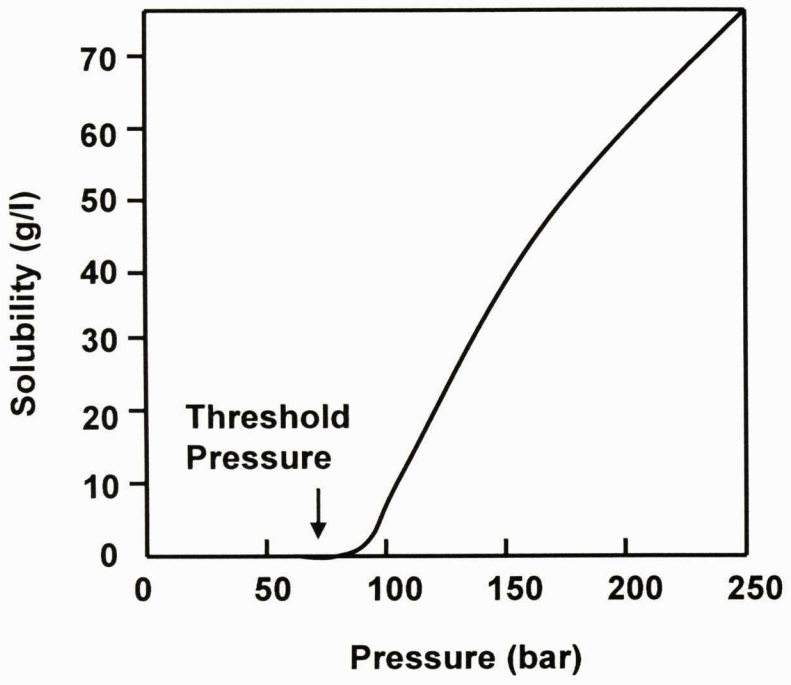


Figure 2-7 The effect of pressure on the solubility of naphthalene at 45°C [7]

The effect of temperature on the extraction efficiency cannot be predicted as several opposing factors are involved. Primarily care must be taken to ensure the operating temperature does not affect the thermal stability of the desired compound. At a constant pressure, the density of CO_2 decreases when the temperature is increased. This effect is more pronounced as the fluid compressibility increases. Higher temperatures often promote extraction recoveries owing to increased solute diffusion coefficients and vapour pressure in the fluid with increasing temperature [7]. Temperature elevation can cause swelling of the matrix, which may influence the rate of extraction [3]. Generally, for a non-volatile solute, the dominant factor in extraction is the SCF density; therefore, at higher temperatures a lower extraction recovery is achieved. However, for a volatile compound, the dominant factor can be vapour pressure at a certain range of pressure and therefore, the extraction efficiency is enhanced by increasing the temperature.

2.6.2 Effect of flow rate and extraction time

The flow rate of supercritical fluid may have an impact on the extraction efficiencies; the slower the fluid velocity, the deeper it penetrates the matrix. The flow velocity can be expressed by the linear velocity, which relies on the flow rate and the vessel geometry. For a given extraction vessel, the flow rate can be easily changed by using a new restrictor. Decreasing the flow rate resulted in decreasing SCF velocity, which enhances the yield of extraction [88]. Care must be taken during the dynamic extraction to achieve saturation point, in which the amount of solute extracted is equal to equilibrium solubility. The saturation concentration may not be achieved if high flow rate is used. It is, therefore, crucial in each extraction process to determine the range of flow rate that extraction can be conducted close to equilibrium.

Papamichail et al. [5] studied the SFE of oil from milled celery seeds using CO_2 as a solvent. They investigated the effect of CO_2 flow rate on the extraction from celery seeds. The increase of the solvent flow rate leads to the increase of the amount of oil extracted from celery seeds versus extraction time (Figure 2-8). On the other hand, the amount of the extracted oil per kilogram of CO_2 used was higher for the lower flow rate due to the intra-particle diffusion resistance. This, actually, has as a result the smaller slope of the extraction curve in Figure 2-9 for the higher flow rate.

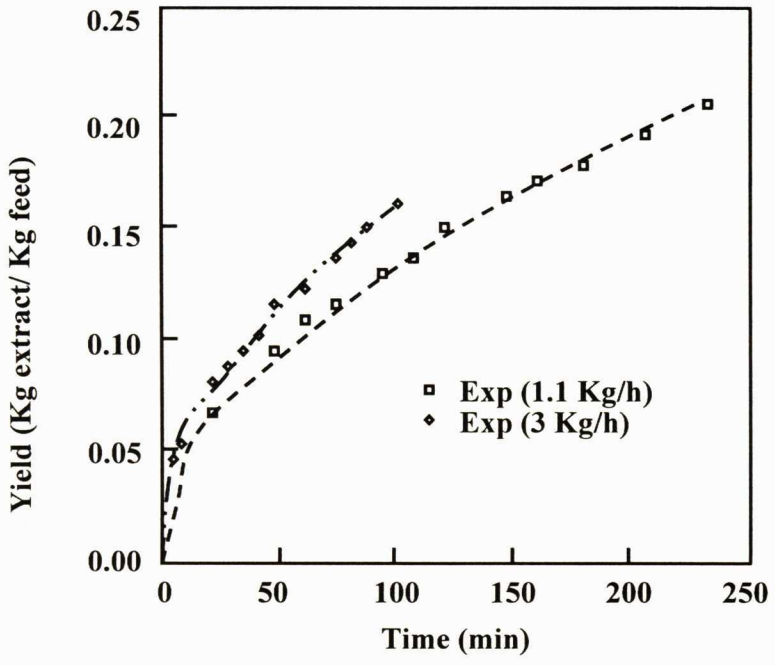


Figure 2-8 Effect of solvent flow rate on the extraction yield vs. extraction time at 150 bar, 45 °C [5].

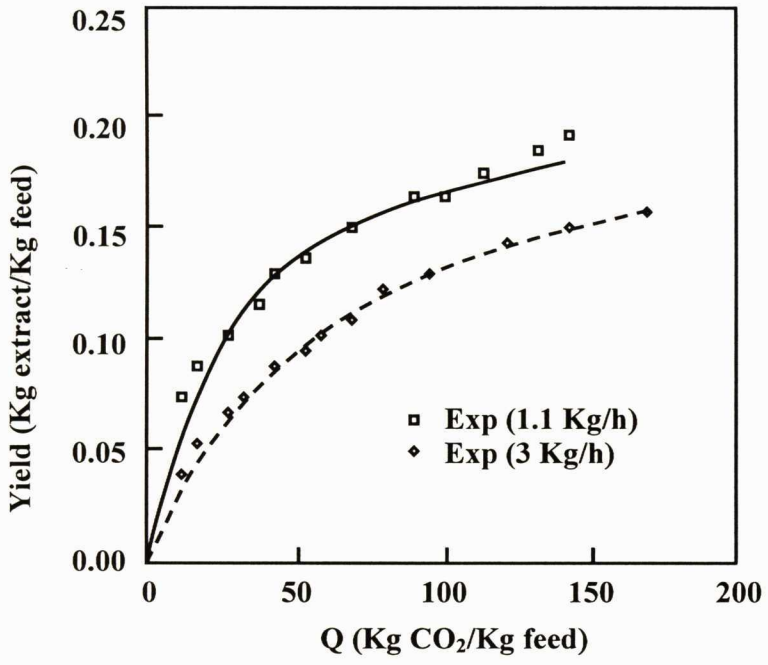


Figure 2-9 Effect of solvent flow rate on the extraction yield vs. the specific amount of solvent (Q) at 150 bar, 45 °C [5].

It is important to maximize the contact of the supercritical fluid solvent with the sample material to approach the saturation and enhance the efficiency of SFE. It was reported that, a 10-20 min static extraction prior to dynamic extraction improved the extract recoveries in SFE of aflatoxins [7]. However, extraction is never complete in a finite time, being generally initially rapid but then approaching a plateau: the time required to remove 99% of a particular analyte may be at least ten fold higher than the system that only requires extracting 50%. The relationship between extraction efficiency and time for a typical process was shown in Figure 2-10. The last part of the extraction is governed by diffusion.

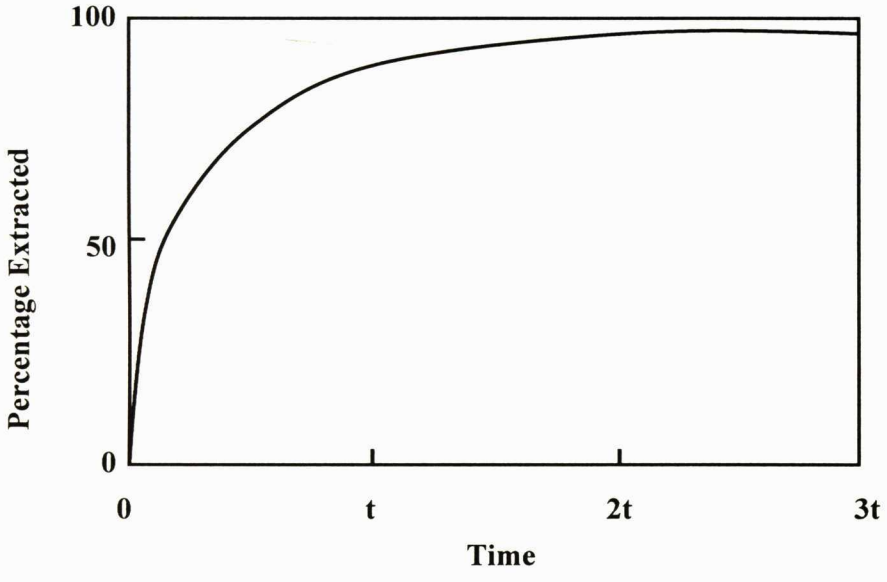


Figure 2-10 Theoretical curve of percentage extraction versus extraction time [3].

2.6.3 Effect of particle size

The particle size controls the mass transfer kinetics by increasing surface area, which

improved the access of CO_2 to the soluble components [89]. In general, higher extraction efficiency can be achieved by applying smaller size particle, which resulted in an increase in mass transfer surface, and in quantity of soluble fraction [88]. Hawthorne and co-workers discussed elution of the analytes from the matrix in the vessel to determine the rate-limiting step in SFE. A larger size of sample was extracted at a similar rate as a smaller one, and a static extraction step was nearly as effective as a dynamic extraction step performed for the same time [90].

Louli et al. [6] extracted oils from parsley seed with supercritical CO_2 at different conditions. The effect of particle size of solid matrix on the yield of extraction was shown in Figure 2-11. The extraction rate was increased by decreasing the size of the seeds. This effect is more dominant at higher pressures. This is due to the higher amount of oil released as the seed cells are destroyed by milling. Moreover, after milling the diffusion paths in the solid matrix become shorter resulting in a small intra-particle resistance to solute diffusion.

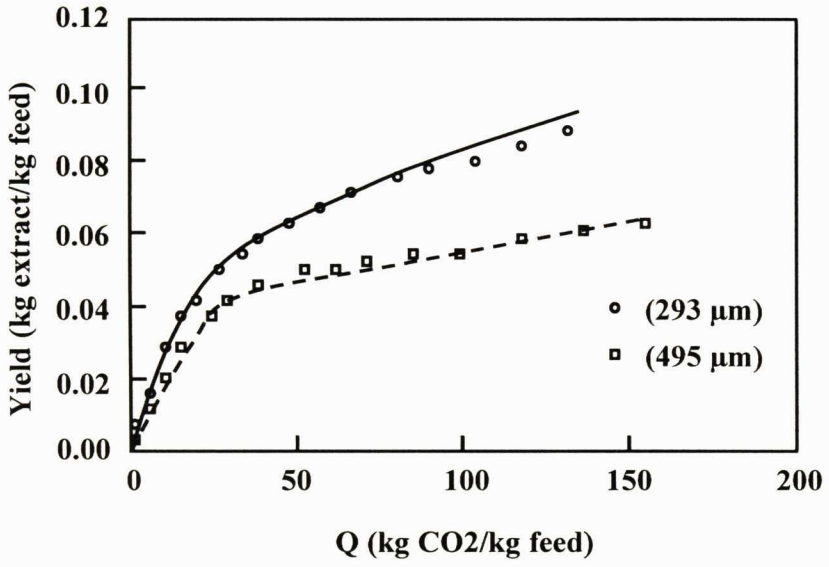


Figure 2-11 Effect of particle size of Parsley seed samples on the extraction rate (data are presented as the extraction yield vs. the specific amount of solvent (Q) at 100 bar, 45 °C, and a solvent flow rate of 1.1 kg CO_2/h) [6].

Ziemons et al. used supercritical CO_2 to extract tagitinin C from Tithonia diversifolia [91]. At 137 bar and 40 °C, the extraction yield of tagitinin C from the leaves of *T. diversifolia* was increased nearly four-fold by decreasing particle size from 125-250 µm to 0-63 µm. From Figure 2-12, it is clear that decreasing particle size increases the yield. Furthermore, the range between 0-63 µm gives a yield nearly six-fold higher than the particle ranging from 125-250 µm.

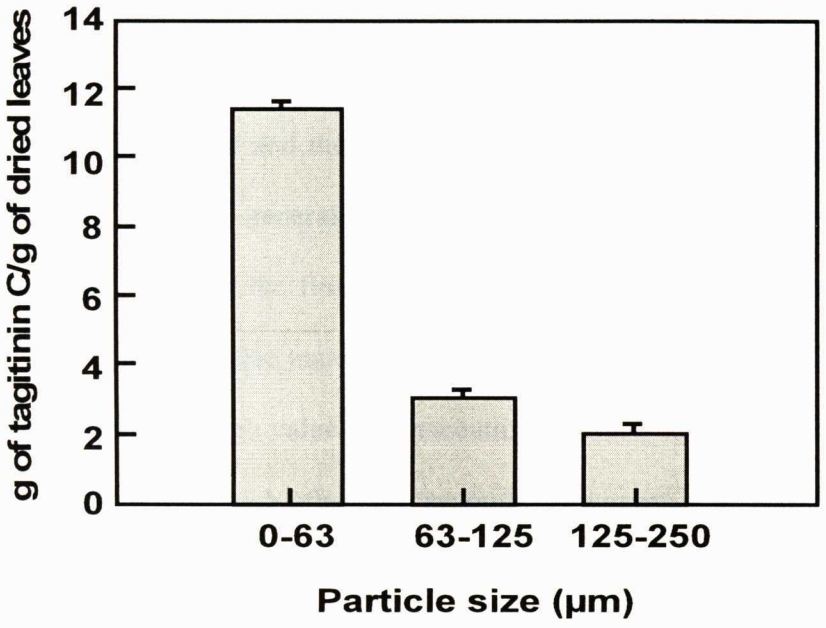


Figure 2-12 Effect of particle size on the extraction yield of tagitinin C from the dried leaves of Tithonia diversifolia (137 bar, 15 g of CO_2, flow rate 1 mL min^{-1}, 40 °C).

2.7 Applications

SCFs have been widely used in extraction and recovery of high-value compounds. Experience accumulated in recent years on the use of SCFs has been reached to a step that it is possible to envision their applications beyond the common practices of extraction [92].

2.7.1 Supercritical Fluid Chromatography (SFC)

SFC can be used for analysis, where it combines many of the advantages of high performance liquid chromatography (HPLC) and gas chromatography (GC) [93]. It can be used with non-volatile and thermally labile analytes with the universal flame ionization detector. In SFC, generally narrower peaks are produced due to rapid diffusion [94]. The purity of the final products is very high, but the cost makes it suitable only for very high-value materials such as pharmaceuticals [95]. SFC can be used for the production of high value pharmaceutical products that are difficult to be purified and separated from each other. For example, Thienpont et al. compared SFC with conventional HPLC on separation of the four stereoisomers of the antifungal drug itraconazole [96]. The result showed SFC achieved a reduction of more than 40% in processing time without loss of selectivity and a 60% reduction in organic solvent requirements.

2.7.2 Supercritical Fluid Reaction (SFR)

SFR is operated in supercritical media, mainly in large scale petrochemical plants and in fine chemistry [45]. Changing the conditions of the reaction solvent can allow separation of phases for product removal, or single phase for reaction [97]. Temperature and pressure can tune the reaction down preferred pathways, e.g., to improve yield of a particular chiral isomer [98]. There are also significant environmental benefits over conventional organic solvents [99]. A good example of catalysis reaction in SCFs is the synthesis of formic acid derivatives (e.g. *N,N*-dimethylformamide (DMF), *N,N*-diethylformamide (DEF), and Methyl formate (MF)) in $SCCO_2$. In this case $SCCO_2$ serves as both reactant and a solvent [100]. Noyori's group demonstrated that the process efficiency of synthesis of DMF and MF was

substantially enhanced with the application of $SCCO_2$ compared with condition that organic solvent was used as a media for reaction [100]. The high efficiency of the $SCCO_2$ –based process was mainly attributed to the favourable mass transfer effect, high solubility of H_2 and the weak solvation of homogeneous catalyst in $SCCO_2$ [100]. In 1999, Baiker *et al.* reported a continuous amination process of propanediols in supercritical ammonia ($SCNH_3$) that was used both as a solvent and reactant [101]. In this process, the selectivity for producing the corresponding primary diamine was significantly improved [101].

2.7.3 Other Applications

Other SCF applications that are now under commercial development includes polymer processing, ceramics and carbons manufacture, fabrication of foams and aerogels, particle design, impregnation, dyeing, cleaning, and pollution abatement [42, 45, 102, 103].

The widest application of supercritical fluids is in extraction as shown in Figure 2-13 [102]. The extraction of valuable materials from solid substrates by means of SCFs has been carried out on a commercial scale for more than two decades. Chemical reactions represent the second largest group with longer tradition and steady behaviour. A cross analysis of the supercritical water curve has been steeper in slope since 1995. Particle generation processes seem to keep a strong position at present. The field of surface treatment application techniques, starting in the 1980s with varnish application techniques has now expanded to sophisticated wafer treatment methods and application of dielectric layers. Impregnation is a comparably small field

but has gained considerable interest since 1995. An important innovation was textile dyeing with supercritical carbon dioxide.

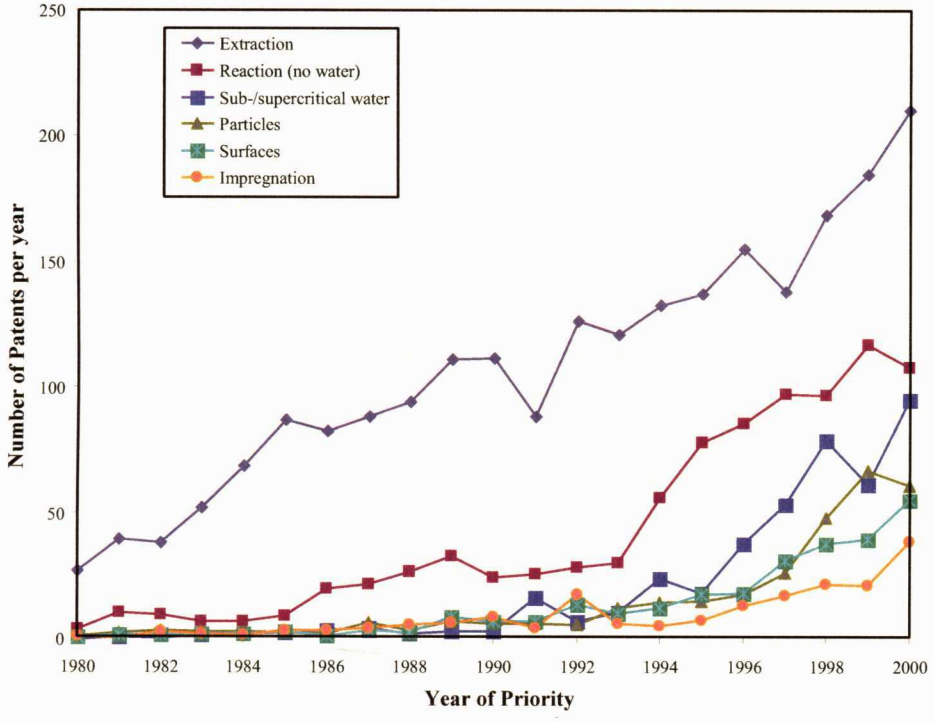


Figure 2-13 Historical development of process principles of supercritical patent applications [102]

SFE is mostly distributed in Europe, the USA, Japan, and in the South East Asian Countries, as can be seen in Figure 2-14. Commercially successful hop extraction plants have been established in several countries, including Australia, UK, Germany and USA [2]. There are several commercial plants for the decaffeination of coffee and/or tea, notably in Germany, UK, Japan, USA and Austria. The third area of significant commercial development in this field is in the flavour and fragrance industry, with commercial plants operating in several countries, including UK, France, Germany, USA and Japan [2]. These industrial plants are commonly batch or semi-

batch systems, and vary in capacity according to the nature of the product and the operating requirements of the process. Some relatively small extraction plants (100-500 Litre extraction volume), have been used successfully by the Japanese food industry to process a range of high-value food ingredients and medicinal compounds [49]. Several commercial flavour extraction plants, such as those used by Universal Flavors Ltd (UK) and CUB (Australia) are designed to extract approximately 500 kg at a time, with three to five extraction vessels in series, in a semi-batch process [104]. In the CUB process, this allows up to 4 tons of hops to be processed, yielding up to 800 kg of hop extract per day. Larger extraction vessel, such as 4000 Litre has been used for the decaffeination of tea and hop extraction in Germany [49].

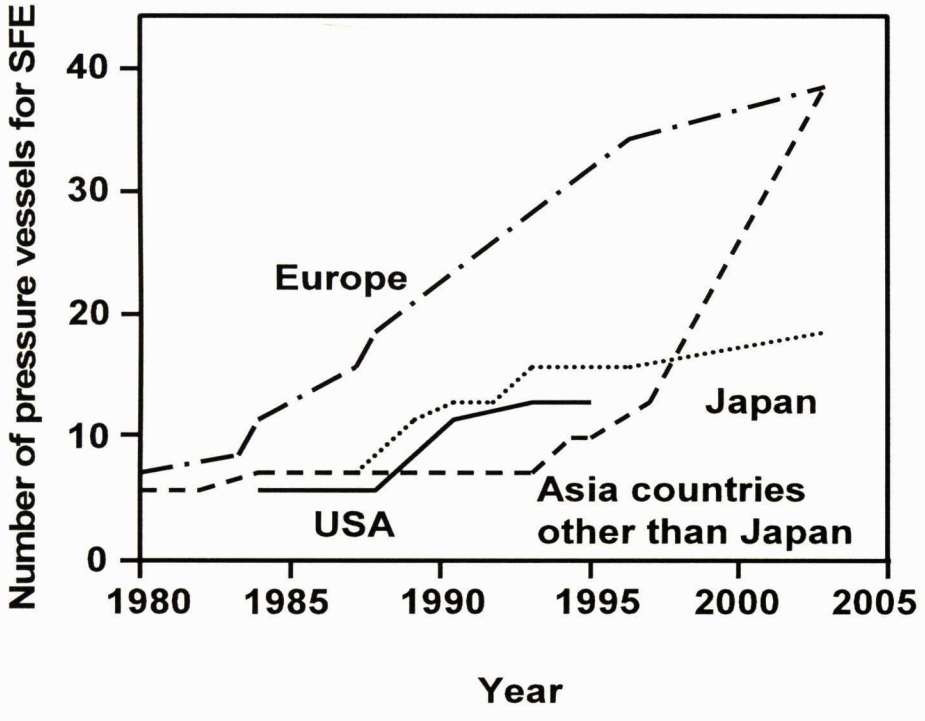


Figure 2-14 Total number of pressure vessels >0.1 m^3 for supercritical extraction processes [2].

The general distribution of the development with supercritical fluids in different fields from 1999 to 2007 is shown in Figure 2-15. As can be seen in Figure 2-15a, more than 30% of SFE methods are devoted to food and agriculture, followed by 24% concerning fuel industry. The applications in food and agriculture flavours and natural products (37%) were highest, followed by oils and essential oils (26%), fats determination, metabolic residues and pesticides, as shown in Figure 2-15b.

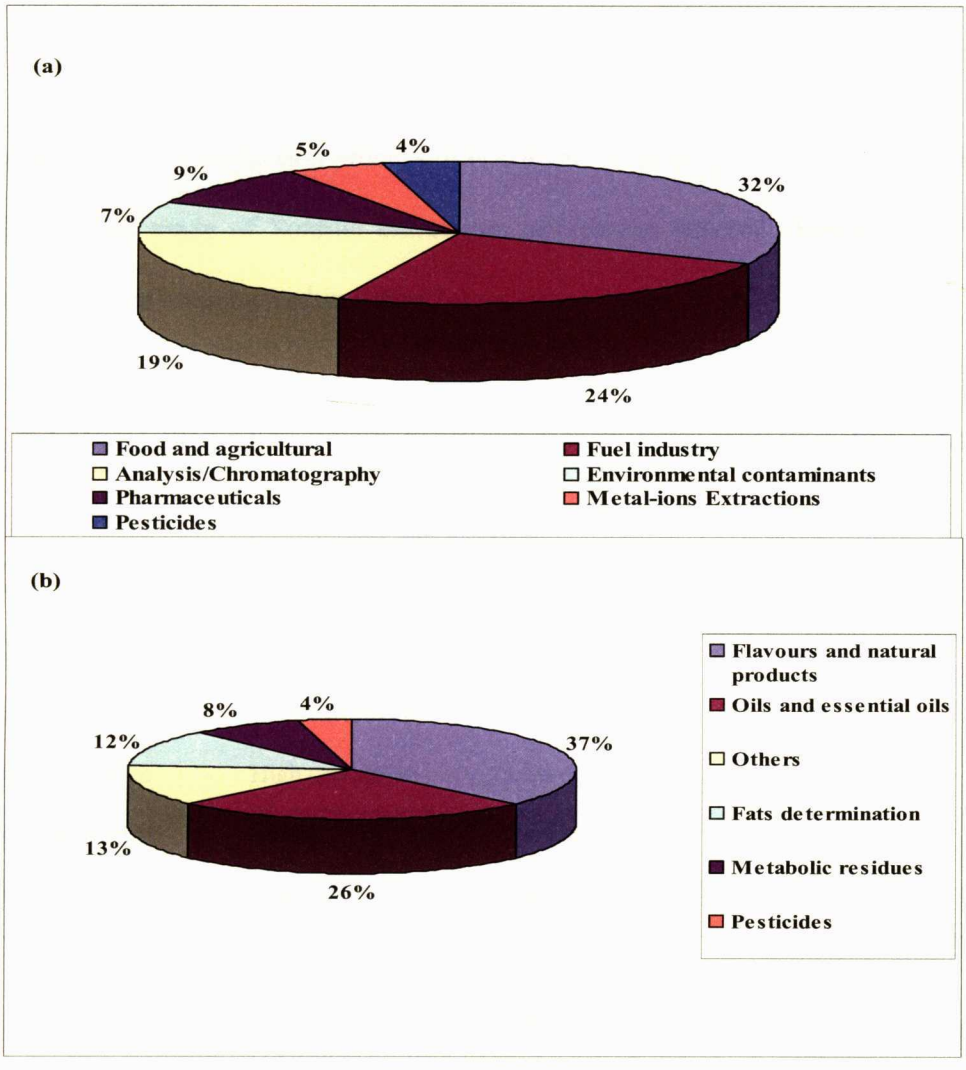


Figure 2-15 (a) Graphical representation of the results obtained of a literature search between 1999 and 2007. (b) Graphical representation of SFE application in food science and technology between 2001 and 2005 [51, 105-107].

SFE of pharmacologically active substances found in medicinal plants has become one of the more promising applications. Most research applications extract the useful ingredients at pressures greater than 200 bar and temperature between 40 °C and 80 °C [74].

2.7 Cost Comparison

In commercial scale production process, it is critical to optimize the cost of process. A comparison was made of Soxhlet extraction, microwave-assisted extraction (MAE) and with SFE that summarized in Table 2-4.

Table 2-4 Comparison of Soxhlet extraction, microwave-assisted extraction (MAE), and supercritical fluid extraction (SFE) [50]

Factor	**Soxhlet**	**MAE**	**SFE**
Investment	Small	Medium	Large
Process time	Long (up to 48 h)	Short (<30 min)	Short (< 60 min)
Solvent consumption	High (200-500 ml)	Low (<40 ml)	Minimal (< 5 ml)
Method development	Simple	Simple	Complicated
Sample treatment	Required	Required	Not required

Soxhlet extraction is by far the most cost effective technique in terms of investment costs; however, it requires large quantity of solvent thorough sample treatment is time consuming. MAE is an interesting alternative because of its medium investment costs and the possibility of performing multiple fast extractions with little solvent consumption. However, it invariably requires clean-up and involves long cool-down

times for the extraction cells. The choice of solvent may be limited as it should preferably be able to absorb microwaves, such as pure non-polar solvents [108].

SFE has the advantage that it constitutes automated systems for unattended performance. SFE is rapid, tenable solvent power can be used for extraction and fractionation of various compounds without changing solvent and elimination use of organic solvent. Instead of expensive, high purity organic solvents, SFE can also have a cost savings. In SFE efficiency can be manipulated by variation of pressure and/or temperature, and it may achieve a outstandingly high selectivity [10]. The main shortcoming is its very high investment cost and the need for a continuous service agreement with the instrument supplier as the instrument have several disposable parts that must be replaced on a routine basis. Carefully structured research showing the advantages of SFE over conventional extraction techniques and a critical comparison between them would probably foster usage of SFE to the extent that one would expect from its potential. A number of official methods are bound to be replaced with SFE alternatives in the future because of the outstanding advantages of SFE. Reported costs for production rates around 1000 ton/year of solid feed are in the range of 3 USD/kg feed. Economy of scale may bring costs down to less than 0.5 USD/kg for batch operation [2]. Continuous operation would further reduce costs.

3. Triterpenoids Saponins

3.1 Introduction

Saponins are glycosylated compounds generated by the terpenoid pathway, classified as triterpenoids, steroids, or steroidal glycoalkaloids [12]. They are widely distributed in a large number of plants that are important in human and animal nutrition [109, 110]. Pharmacological and biological properties of triterpene saponins from different plant species have been studied, including fungicidal, antiviral, spermicidal or contraceptive, cardiovascular, anti-inflammatory and antitumor activities [111]. While previous reports have identified triterpene compounds that have any of a number of applications, there still a strong need for identification of additional, novel, biologically active terpenoid metabolites. Many saponins are toxic to normal mammalian cells, and careful screening of novel compounds is required to discover saponins that are specifically cytotoxic to cancer cells but nontoxic to normal cells. There is a great diversity in the structure and activity of these compounds, which poses challenges in their characterization, elucidation of biological activities, and application against particular ailments. Apart from discovering highly effective and specific triterpene saponins, stable production and efficient application methods for homogeneous therapeutic compounds are needed. Achieving the difficult goals of identifying novel bioactive triterpenoids saponins, determining the feasibility of production using SFE of these compounds, and developing application technology could provide entirely new treatment for a diverse set of human cancers, in which therapeutic options are currently limited.

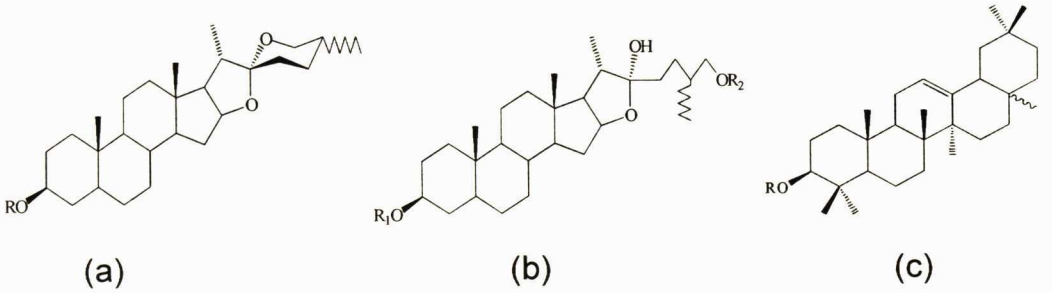
3.2 Properties of Triterpenoids Saponins

3.2.1 Chemistry of Triterpenoids Saponins

Several thousand terpenes and terpenoids occur in many plants [112-114]. The terpenes are biosynthetically constructed from isoprene units [113]. The C_5H_8 isoprenes polymerise and subsequently fix the number and position of the double bonds. The basic molecular formulae of terpenes are thus multiples of C_5H_8. Most terpenes have cyclic structures and are classified by the number of C_5 isoprene units that they contain. Given the many ways the basic C_5 units can be combined, it is not surprising to observe the amount and diversity of the structures [115].

The triterpene group of compounds includes sterols and triterpenes, which can accumulate as glycosides (saponins) in extensive amount in plant [4]. Saponins are classified according to their aglycone skeleton. The first group consists of non-steroidal saponins, which are the most common and occur mainly in the dicotyledonous angiosperms. The second group consists of the steroidal saponins which are derived from the tetracyclic triterpenoids and isoprene units and are almost exclusively present in monocotyledonous angiosperms. A third class called steroidal amines, which are also referred to as steroidal alkaloids [116]. Steroidal saponins consist of a steroidal aglycone, a C_{27} spirosatne skeleton which generally consists of a six-ring structure (Figure 3-1a). The hydroxyl-group may be engaged in a glycosidic linkage so that the aglycone structure remains pentacyclic (Figure 3-1b). This is referred to as a furostane skeleton. Triterpenoids saponins consist of a triterpene

aglycone, which consists of a C_{30} skeleton, compromising a pentacyclic structure (Figure 3-1c) [117].



(a) (b) (c)

Figure 3-1 Aglycone skeletons of pentacyclic (a) steroidal *spirostane*, (b) steroidal *furostane* and (c) triterpenoid saponins. The R-group is a sugar moity [4]. Tetracylic terpenes such as lanosterol, sitosterol and cycloartenol synthase (CAS_1) [8].

3.2.2 Biological Activities of Triterpenoids Saponins

Avicin inhibition of tumor cell proliferation via induction of apoptosis

Jayatilake et al. assessed the biological activity of *A. victoriae* triterpene glycosides against tumor cells using partially purified avicin fractions and two purified triterpene glycosides called "avicin D" and "avicin G" (Figure 3-2) [118]. These mixtures and purified compounds markedly inhibited the growth of several tumor cell lines. Minimum growth inhibition was observed in nonmalignant cells (human fibroblasts, mouse fibroblasts, and immortalized breast epithelial cells).

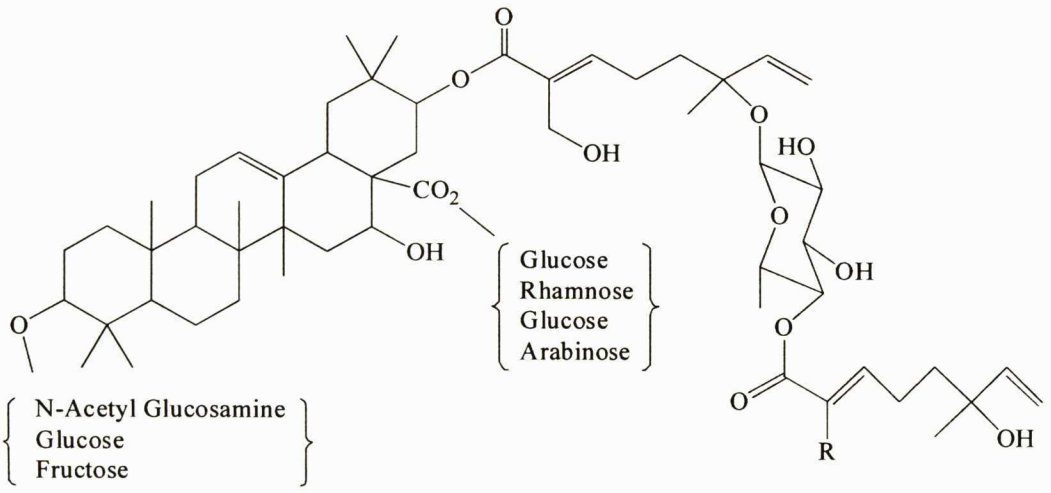


Figure 3-2 Chemical structure of avicins D (R: OH) and G (R: H).

Triterpenoids saponins may provide a method for preventing or treating cancer. It was shown that avicins D and G from *A. victoriae* are selectively toxic to tumor cells at very low doses (IC_{50}: 0.2 μg/mL for Jurkat cells) [12]. Resistance to programmed cell death (apoptosis) is an integral part of cancer cell development, and reestablishment of control of apoptosis is a known target mechanism for anticancer drugs. The release of cytochrome *c* through the outer mitochondrial membrane into the cytosol is one confirmed pathway to induce the initiation of apoptosis. Haridas et al. studied the effects of added avicins, a several fold increase in the cytosolic levels of cytochrome *c* was observed within 30-120 min in whole Jurkat cells and within a minute in the cell-free system [19]. There was no effect observed on mitochondrial membrane, which indicated that avicins influenced the outer membrane of the organelle without modifying the bioenergetics of the internal membrane. It is concluded that avicins have a direct effect on mammalian cell mitochondria, and this effect could be directly related to their ability to trigger the onset of apoptosis in cancer cells [19].

In vivo studies of avicin activity

Hanausek et al. extended the studies of isolated avicins to preclinical analysis of mouse skin cancer using two different protocols [20]. One involved varying doses of avincins were applied of 100 nmol of 7,12-dimethylbenz[a]anthracene (DMBA) twice a week for 4 weeks, and the second approach involved a low-dose of 10 nmol DMBA to initiate carcinogenesis, followed by repeated applications of 12-*O*-tetradecanoyl-phorphol-13-acetate (TPA), which is known to be a tumor promoter. In both approaches, avicins were applied at 12 weeks to selected mice before the DMBA or TPA. In the animals receiving avicin pretreatment, avicins produced a 70% decrease in the number of mice with papillomas and a greater than 90% reduction in the number of papillomas per mouse in both protocols.

Inhibition of nuclear transcription factor-kB (NF-kB)

Haridas et al. examined the effect of triterpenoids saponins mixture on tumor necrosis factor (TNF)-induced activation of nuclear transcription factor-*k*B (NF-*k*B) in human Jurkat cells [9]. They recognized that NF-*k*B regulates the transcription of a number of genes involved in immune and inflammatory pathways. Triterpenoids saponins treatment resulted in decreasing expression of NF-*k*B-regulated proteins such as inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX-2). The authors concluded that avicins reduced both oxidative and nitrosative cellular stress and thereby suppress the development of malignancies and related diseases.

3.3 Extraction

Although numerous variations are possible, current general procedures for obtaining crude saponin mixtures typically include extraction with methanol, ethanol, water or aqueous alcohol. Wattle seeds have a high content of fat, which will affect the purity of active compounds and need to be removed before extraction. A defatting step, generally with petroleum ether, performed before the extraction step [119]. Then dissolution or suspension of the extract in water and shaking or washing the solution or suspension with n-butanol saturated with water. A dialysis step is also included to remove small water soluble molecules such as sugars [14]. Table 3-1 lists selected previous studies on organic extraction of triterpenoids saponins.

Table 3-1 Extraction of triterpenoids saponins from natural plants using different solvents

Solvents	Plant	Yield (%)	Applications	Reference
MeOH	Ilex oblonga	0.039	Hypocholesterolemic, antioxidant	[120]
70% MeOH	Achyranthes bientata Bl.	0.0025	Antilipemic, cardiotonic	[121]
20% MeOH	Acacia victoriae	0.058	Purgative, emetic, expectorant	[118]
95% EtOH	Gleditsia sinensis Lam.	2.24	Expectorant, pesticide	[122]
80% EtOH	Lysimachia	1.5	Choleresis, diuresis,	[123]

Solvents	Plant	Yield (%)	Applications	Reference
	christinae		anti-inflammation	
80% EtOH	Zizyphus jujube Mill.	0.015	Treat virtual trouble, insomnia and hyperhidrosis	[124]
70% EtOH	Aesculus chinensis Bunge var Chekiangensis	0.032	Anti-tumor	[125]
$CHCl_3$-MeOH (9:1)	Cyclanthera pedata	0.019	Anti-inflammatory, hypocholesterolaemic hypoglycemic	[126]
$CHCl_3$-MeOH (9:1)	Amaranthus caudatus	0.018	Anti-nutrients	[127]

The most efficient extraction from Acacia plant was achieved using methanol or aqueous methanol [128]. Although water is typically a less efficient extraction solvent for saponins (unless specifically water-soluble glycosides are desirable) it has advantages of being easily lyophilized and giving a cleaner extract. Depending on the proportion of water used for extraction, either monodesmosidic or bidesmosidic saponins may be extracted [129, 130]. As we can see in Table 3-1, the yields were not high which indicates triterpenoids saponins have a relatively low content in natural sources, and the complete recovery of these compounds may be complicated. Since triterpenoids saponins have relatively large molecular weights; their isolation can be challenging because of complex mixtures of closely related compounds, differing

subtly in the nature of the aglycone. Especially in plants, saponins are accompanied by very polar substances such as saccharides and coloring agents. It is desirable to develop a method and solvent that minimise number of steps in isolation of saponins.

3.4 Application of Triterpenoids Saponins Extract

The triterpene compounds from *Acacia victoriae* are contemplated to be useful for a wide variety of applications in addition to anti-inflammatory and anti-tumor [131], for example, anti-fungal and anti-viral agents, piscicides or molluscicides, contraceptives, antihelmintics, UV-protectants, expectorants, diuretics, regulators of cholesterol metabolism, cardiovascular effectors, anti-ulcer agents, analgesics, sedatives, immunomodulators, antipyretics, angiogenesis regulators, agents for decreasing capillary fragility, agents to combat the effects of aging, and agents for improving cognition and memory [11, 127, 132].

These compounds have a role in the regulation of angiogenesis. Angiogenesis or neovascularization is defined as the growth of new blood vessels [133, 134]. Tumors and cancers induce angiogenesis to provide a life-line for oxygen and nutrients for the tumor to thrive. The development of new blood vessels also provides exits for malignant cancer cells to spread to other parts of the body [135]. Angiogenesis inhibition therefore benefits cancer patients. On the other hand, angiogenesis is required at times such as wound healing. These wounds can be external wounds or internal organ wounds that result from accidents, burns, injury and surgery [128]. Thus, agents that promote angiogenesis have a great potential for use in therapy for wound healing.

A particular application of triterpenoid saponins is as an active ingredient in topical compositions, including cosmetics, designed for treating skin aging [32]. Specific changes to the skin that may result from aging and that may be prevented or treated in accordance herewith, whether chronologically or externally induced [135-138].

4. Separation and Characterization of Triterpenoids saponins in *Acacia victoriae*

4.1 Introduction

The aim of this study was to determine whether there is an anticancer compound or group of compounds in extract from *Acacia victoriae*. In this chapter, it was attempted to use different solvent to fractionate the extract into different polarity groups. Analytical methods for identifying the groups and also *in vitro* technique to assess biological activity of extract were developed.

Over the past 20 years, Acacias have been subjected to extensive study because of their increasing popularity as a health supplement. *Acacia victoriae* is a widespread species that grows in Western Australia, east through central Australia to Queensland, south to Adelaide area in the South Australia. This species is most commonly found on depositional landforms such as floodplains or alluvial flats, but also grows on rocky hillsides and ridges [23].

With the development of modern chromatographic techniques, much progress has been made in identifying bioactive components in Acacias, such as triterpenes and polysaccharides [12, 19, 22, 118, 128, 139, 140]. For the triterpenes alone in *Acacia victoriae*, avicin D and G have already been isolated, which markedly inhibited the growth of several tumor cell lines [118]. Different methods used for processing and extraction of chemical components were to focus on the isolation and purification of

major triterpene components in *Acacia victoriae* for utilization in subsequent studies. Traditional separation methods were applied in order to obtain crude triterpenes.

4.2 Materials and Methods

4.2.1 Materials

The seedpods harvested from *Acacia Victoriae* and Acacia gum were kindly supplied by Vic Cherikoff Pty Ltd. The sample was oven-dried, ground to 3-mm particle size in a Wiley mill, and sieved by 1.0 mm laboratory test sieve (Endecotts Ltd. London, England). Millipore ultrafiltration device was purchased from Millipore. All the chemicals, including methanol (MeOH), ethanol (EtOH), petroleum ether, glycerol, dimethyl sulfoxide (DMSO), chloroform ($CHCl_3$), *n*-butanol (*n*-BuOH), acetic acid (HOAc) and ethyl acetate (EtOAc), were purchased from Merck Chemical Co. MeOH-d4 and Deuterium oxide were purchased from sigma for NMR analysis. The NMR tubes (8 inch length, 5 mm diameter) were purchased from NovaChem Co. The TLC plate (Silica gel) was purchased from Merck Chemical Co. CO_2 gas (Food Grade of 99.9% purity) was supplied by BOC Co. Ltd. All chemicals were used without further purification.

All the cell culture tools flasks, well-plates and centrifuge tubes were purchased from BD Falcon Biosciences. Advanced Dulbecco's modified eagle medium (Advanced DMEM), Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), Antibiotic-Antimycotic (AA), and GlutaMAX were

purchased from GIBCO, Invitrogen Australia Pty Limited. 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and pen-strep were purchased from Sigma. A 2.5 mg/mL MTT solution was prepared in MilliQ water.

4.2.2 Defatting

The Acacia plant was defatted prior to the extraction of triterpenoids saponins to eliminate the interference of this group of compounds during the extraction by other solvents. Soxhlet technique was used for defatting and determining fat content of Acacias, as shown in Figure 4-1. Petroleum ether, common solvent for defatting, was used in Soxhlet procedure [20, 31, 141]. In all cases, 10 g of *A. victoriae* sample was extracted with 250 mL petroleum ether at 60 °C for a period of at least 24 hrs to ensure whole fat was extracted from the plant. After removal from the apparatus; the raw material was dried in the oven at 40 °C until a constant weight was achieved. Fat content was determined by gravimetric method and expressed as percent by weight. The measurement was repeated at least three times to verify the repeatability.

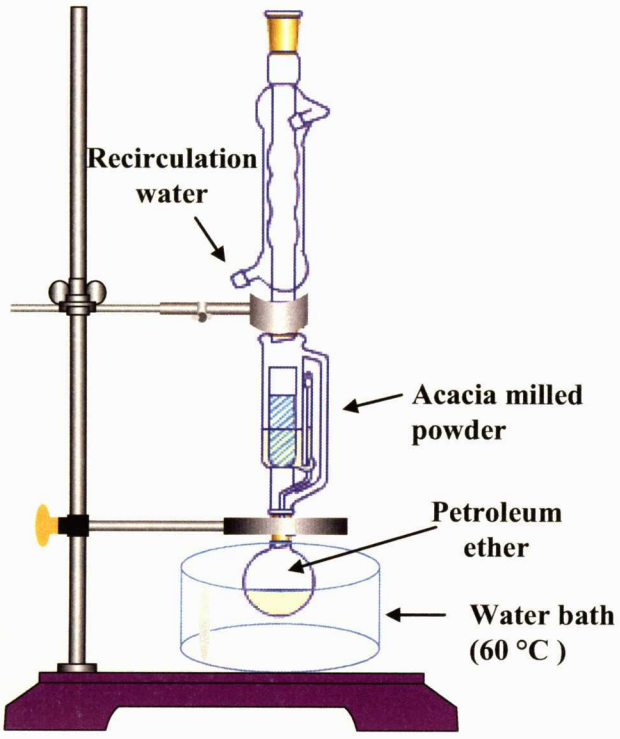


Figure 4-1 Soxhlet extraction apparatus used for defatting and extraction

4.2.3 Extraction of Triterpenoids saponins by Organic Solvent Extraction

The effect of solvent on the extraction of compounds from Acacia plant was determined. In each experiment, 5 g fine powder of defatted *Acacia Victoriae* was suspended in 25 mL solvent using a schott bottle. The extraction was conducted at ambient temperature for a period of 16 hrs using a shaker at 200 rpm. After which the supernatant was separated from solid residue using a filter paper. Millipore ultrafiltration device with 5 kD cut off was used to separate any residue of proteins that may be extracted by the selected solvent. The extract was then collected by evaporating the solvent using rotary evaporator at room temperature.

After reviewing large number of literatures, solvents with different degree of polarity were selected for the extraction of triterpenoids saponins from *Acacia victoriae*[18, 32, 112, 120, 121, 126, 142]. The solvents include water, 1:1 volume ratio of water: methanol, methanol, 1:1 volume ratio of ethanol:glycerol and ethyl acetate.

4.2.4 Multistage Liquid-liquid Extraction for the Fractionation of Saponins

The aim of this study was to fractionate extracted compounds from Acacia plants, to separate them by degree of polarity and also to investigate the effect of polarity of triterpenoids saponins on their anticancer activity. This effect was compared with total extracted triterpenoids saponins from *A. victoriae*. A protocol elaborated in Figure 4-2 was used for the fractionation of different polarity triterpenoids saponins. For this purpose, 10 g defatted powder of the seedpods of *Acacia Victoriae* was extracted with methanol overnight at room temperature to obtain crude extract. The solid residues were then extracted with 1:1 volume ratio of water and methanol, solvent separated under reduced pressure using rotary evaporator at 60 °C, and a dark green residue (0.98 g) was obtained (Fraction A). Fraction A was obtained by increasing the polarity of solvent and extracting any polar active compound that may maintain in the residue, and the feasibility of extracting any polar active compounds in solid residues.

Methanol was evaporated from first stage extraction solution and the solid was washed with ethyl acetate to remove less polar compounds. Triterpenoids saponins that were not soluble in ethyl acetate were precipitated and total solid was obtained in Fraction B was 1.28 g. Fraction B was then subjected to liquid-liquid partitioning by

adding the same volume of *n*-butanol and water successively, yielding two fractions: less polar fraction dissolved in *n*-butanol (Fraction C, 0.37 g) and polar one in water.

For further fractionation, 2.85 g sodium chloride was added to the water fraction (25.65 mL) to prepare a 10 wt% NaCl solution by means of salting out based on the principle that triterpenoids saponins are less soluble at high salt concentration [143]. The extract was then partitioned by addition of equal volume of *n*-butanol to aqueous phase. Fraction D (0.14 g) and E (2.86 g) were obtained from *n*-butanol phase and water phase, respectively. Fraction D was expected to be polar active compounds left in the water phase after previous separation and major compound in Fraction E was sodium chloride after evaporating the solvent by rotary evaporator. The order of these fractions in terms of degree of polarity was A > E > B > D > C. All the fractions were then analysed by Thin Layer Chromatography (TLC), NMR and MTT assays.

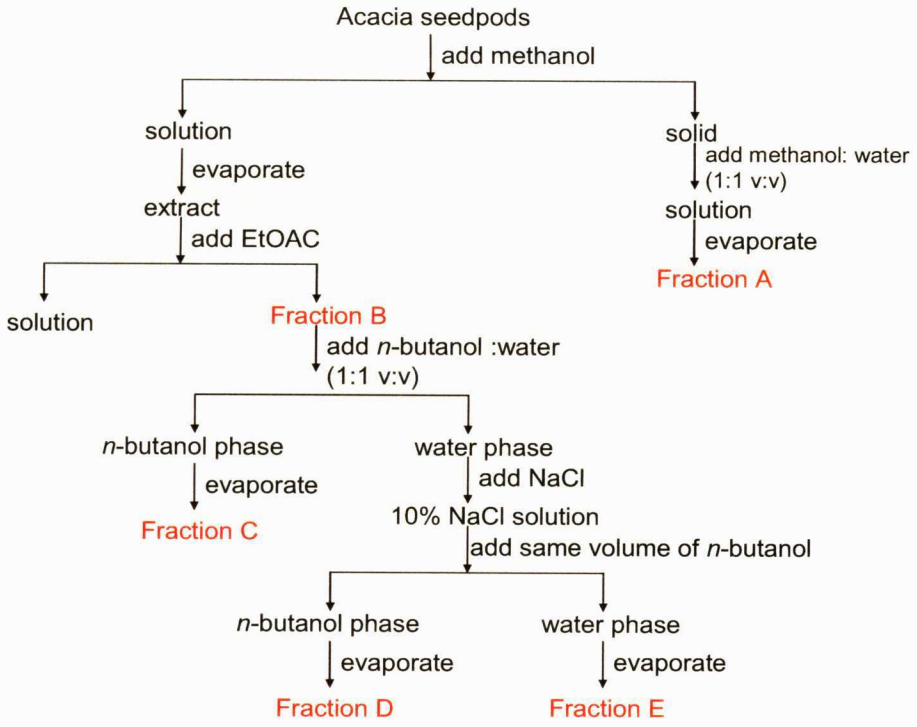


Figure 4-2 Fractionation procedure of Acacia plant

4.2.5 Thin Layer Chromatography (TLC)

Extract from each solvent system was first dried and then dissolved in methanol to prepare a 10 mg/mL solution. Gynosaponins TN-1 isolated from *G. pentaphyllum Makino*, total EP saponins isolated from *Chenopodium quinoa* (kindly supported by Dr. Colin Duck) and sucrose were chosen as standards. TN-1 and Total EP and sucrose were chosen as polarity indicator, and the information of sugar group could be obtained from TLC spectrum. Two mobile phase systems with different polarities were used including $CHCl_3$-EtOAc-MeOH-H_2O (2:5:4:1) and *n*-BuOH-EtOH-HOAc-H_2O (8:2:1:3).

A small amount of solution containing the sample was applied to a plate at one cm from the edge of the plate. The plate was then dipped into the solvents and placed in a sealed container. The mobile phase was diffused into the plate by capillary action and carried the components in the sample at different rate because of the variation in their interaction between mobile and stationary phases. The plate was removed from the mobile phase after the solvents was approached to the top of the plate, then placed under UV light using both short and long wavelengths to observe separate spots. As the chemicals being separated may be colourless and may not captured under UV, the plate was then soaked into colour developing agent (10 vol% sulphuric acid in ethanol) that allowed the visualization of spots under heating and the images of the plate were taken for further characterisation.

4.2.6 Nuclear magnetic resonance (NMR)

The proton NMR is an important and well-established technique for determining the organic compounds. Prior to analysis, less polar extracts were dissolved in MeOH-d4 (Fraction A-D), and more polar compounds were dissolved in Deuterium oxide (Fraction E). The solution was placed in a uniform NMR tube, and oriented between the poles of a powerful magnet, and is spun to any magnetic field variations, as well as tube imperfections. The experiments were carried out on Varian 400MR NMR facility.

4.2.7 Cell Culturing

A2780 cells are an ovarian cancer cell line (kindly provided by Royal North Shore Hospital, Australia). The cells were cultured in flasks containing 5 mL Advanced DMEM (Dulbecco's modified Eagle's medium) supplemented with 2% FBS (Fetal Bovine Serum) or DMEM supplemented with 10% FBS and 1.25% Glutamine and Antibiotic-Antimycotic at 37 °C in 5% CO_2 incubator. A2780 cells were split on 3 or 4 days basis throughout the experiment.

GM3348 cells are a human skin fibroblast cell line (obtained from the Coriell Cell Repository). The cells were cultured in flasks containing 5 mL DMEM (Dulbecco's modified Eagle's medium) supplied with 10% FBS (Fetal Bovine Serum) and 1% pen-strep at 37 °C in 5% CO_2 incubator. GM3348 cells were split on 5 or 6 days basis throughout the experiment.

4.2.8 MTT Assay

The selection of desired products relies on the outcome of the *in vitro* bioassay for determining the efficacy and activity of each extracted fraction. The extract was tested by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] assay, which offers a quantitative and convenient method to determine cytotoxicity of potential anti-tumor agents [14, 128]. The fractions with desirable activity achieved by MTT assay were selected for further characterisation.

The MTT assay is a well-documented cell viability assay and has been further improved as a high-throughput screening-capable assay since it was first developed by Mosmann (1983) [143, 144]. It is an established colorimetric method for evaluating a cell population's response to external factors based on the reduction of tetrazolium salt by mitochondrial succinic dehydrogenases in viable cells yielding insoluble purple formazan crystals [145, 146], which are later solubilised in DMSO (dimethyl sulfoxide) producing a purple-colored solution. The absorbance of this colour intensity solution can be quantified by measuring at a certain wavelength (575 nm) by micro-plate reader [147]. Anti-tumor activity is evaluated using IC_{50} determined by non-linear regression analysis. MTT assay is a suitable and reliable tool for lager scale studies as it is performed in 96-well plates and the results are automatically read in short term, and no radioisotopes are used [146].

Cell lines undergoing exponential growth were suspended in fresh medium at a concentration of 1×10^5 cells /mL and inoculated in a 96-well flat bottomed plate in a volume of 100 µl per well and kept under 5% CO_2 incubator at 37°C overnight. The

seeding density depends on the growth characteristics of the cells and was selected to avoid a 100% confluence of untreated cells. After overnight incubation, cells were attached as controlled by microscopy and treated with extracts at different concentrations, as shown in Fig. 4-3. Two blanks were designed for removing the effect of cells and using as a reference. The plates were incubated at 37°C for 72 hrs and 20 µl of MTT (2.5 mg/mL) were added to each well and the plate was incubated for 4 hrs at 37 °C. The supernatant was then removed and the formazan crystals were dissolved with 200 µl of DMSO. Finally, the absorbance at 570 nm was recorded using a Bio-Rad 680 microplate reader. Antitumor activity was evaluated using IC_{50} determined by non-linear regression analysis.

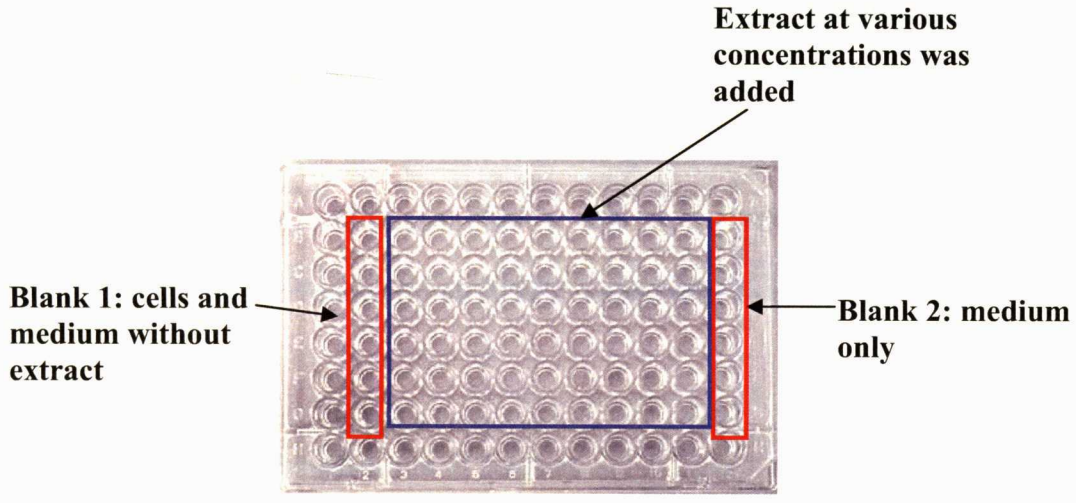


Figure 4-3 Schematic diagram of 96-well plate used for MTT assay

MTT was conducted to test the cytotoxic effect of organic solvents on A2780 first, as it was crucial to obtain the growth inhibition information of solvents. The extract dissolved in both DMSO and Acacia gum (a 5 wt% solution) was tested to obtain the best solvent system to extract active compounds from *A. victoriae*. The outcomes of

the above stages of study were used to develop the optimum conditions for the extraction of desired fractions from plants using SCF process.

4.3 Results and Discussion

4.3.1 Fat Content of *Acacia Victoriae*

Fat was removed from plant to minimize its residue as a source of contamination in saponins extracts from different solvents. The results of the experiment show that the amount of fat in *Acacia Victoriae* sample was 5±0.1 wt%. Fat is slightly soluble in solvents such as MeOH, EtOH and DMSO. If the sample was not defatted, extraction by dissolving the solid residue in a solvent or acacia gum solution, a cloudy solution was formed that can interface in characterisation with TLC, NMR, and MTT assays. Therefore, defatting was performed for all Acacia sample prior to extraction.

4.3.2 TLC Analysis for Identification of Extract

Thin layer chromatography (TLC) is widely used for the analysis of natural products [148-151]. TLC plays an important role in the quality control of food and drugs to identify the ingredients and impurities; it has been used for assessing the purity and the stability of product [152, 153]. In all studies, silica gel and RP-18 were commonly used as solid stationary phase; however, the mobile phase was selected according to the polarity of desirable saponins presented in the sample [22, 154].

In this project, TLC was used to analyse extract from *Acacia* plant. The TLC images acquired for the analysis of extracts from different organic solvents is shown in Figure 4-4. Extracts from water, methanol: water (1:1 v:v), methanol and ethyl acetate were labelled E1, E2, E3 and E4, respectively. Gynosaponins TN-1 and total EP saponins were labelled TN-1 and EP in the spectrum. The extracts from methanol and methanol: water (1:1 v:v) had saponins as some bands detected under UV light. The extract in ethyl acetate had no saponins as no spot was observed under UV light. In the system that $CHCl_3$-EtOAc-MeOH-H_2O (2:5:4:1) used as mobile phase, most compounds were detected at the bottom of plate. It can be concluded that they were polar or have molecular structures close to sugar. However, in the solvent system that *n*-BuOH-EtOH-HOAc-H_2O (8:2:1:3) used as mobile phase, the colour and position of the standard and sample were different, which indicates compounds were not sucrose, and they were polar (at least 3 glycosyl groups) or other type of sugar. Therefore, the active compounds in *A. victoriae* were polar, and further analysis by NMR was required for their identification.

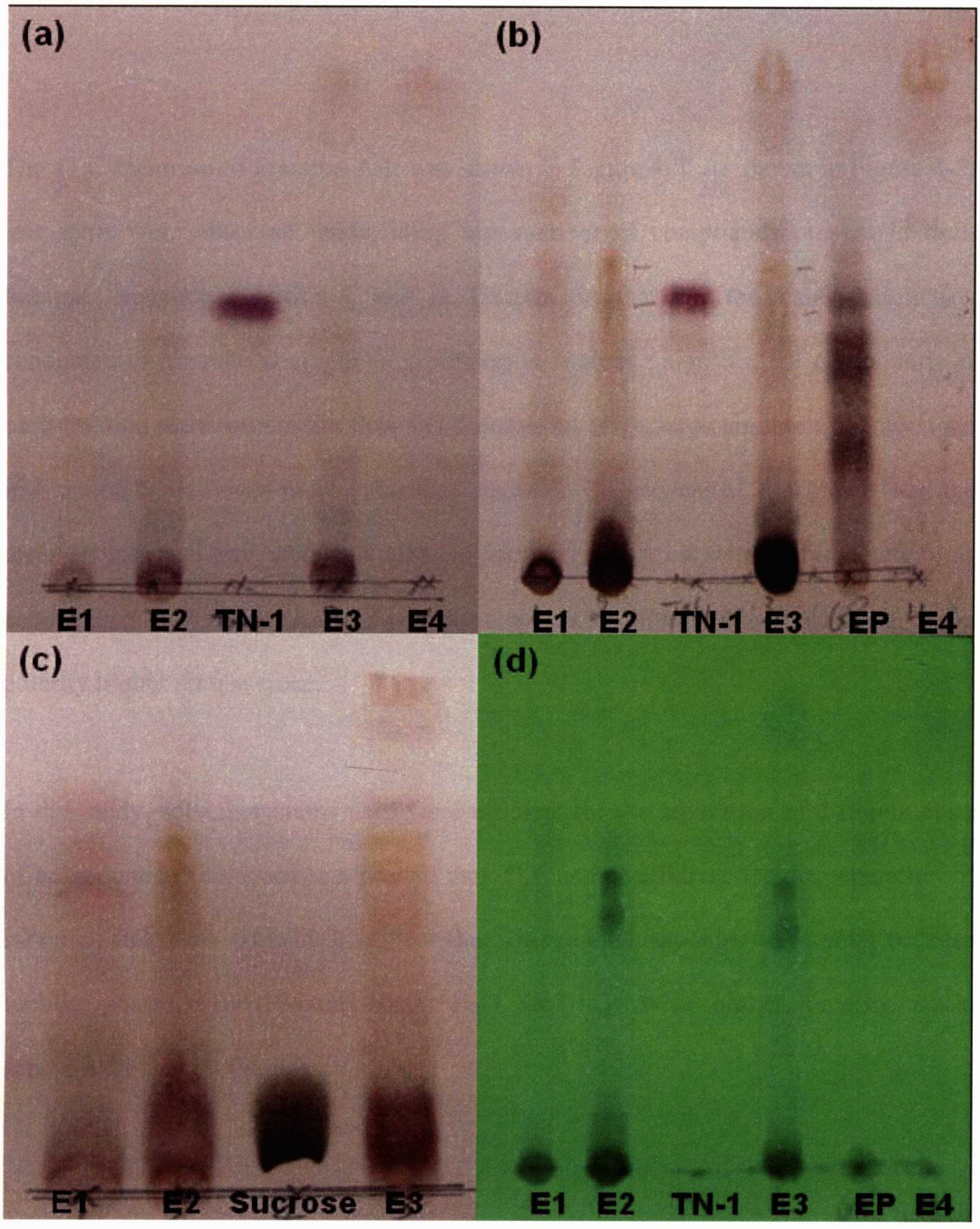


Figure 4-4 TLC analysis for samples obtained from extraction of *Acacia* using E1: water, E2: methanol: water (1:1 volume ratio), E3: pure methanol and E4: ethyl acetate. (a) and (b) for system that $CHCl_3$-EtOAc-MeOH-H_2O (2:5:4:1) as mobile phase, (c) for *n*-BuOH-EtOH-HOAc-H_2O (8:2:1:3) as mobile phase, (d) under UV light at long wavelength (320 nm-400 nm) for system that $CHCl_3$-EtOAc-MeOH-H_2O (2:5:4:1) as mobile phase.

The TLC spectrum of Fraction A-E was shown in Figure 4-5. As shown in Figure 4-5, less spots were observed, underlining less number of compounds present in each fraction, especially Fraction C and D. Despite the fact that the fractionation was conducted to separate compounds according to various polarities, the compounds in each fraction were more polar than TN-1 saponins, and a large amount of compounds in Fraction B was even more polar than sucrose. The content of Fraction B was the most complicated one because it was obtained from the first step of fractionation, as can be seen in the spectrum, the polarity range of Fraction B was from TN-1 to the polarity higher than sucrose.

In this study, solvent systems used were efficient for the separation of different class of compounds. The spectrums proved that TLC is not efficient for the separation of saponins and other extracts. It is clear that triterpenoids saponins were polar because mobile phase *n*-BuOH-EtOH-HOAc-H_2O (8:2:1:3) with higher polarity could separate the extract roughly.

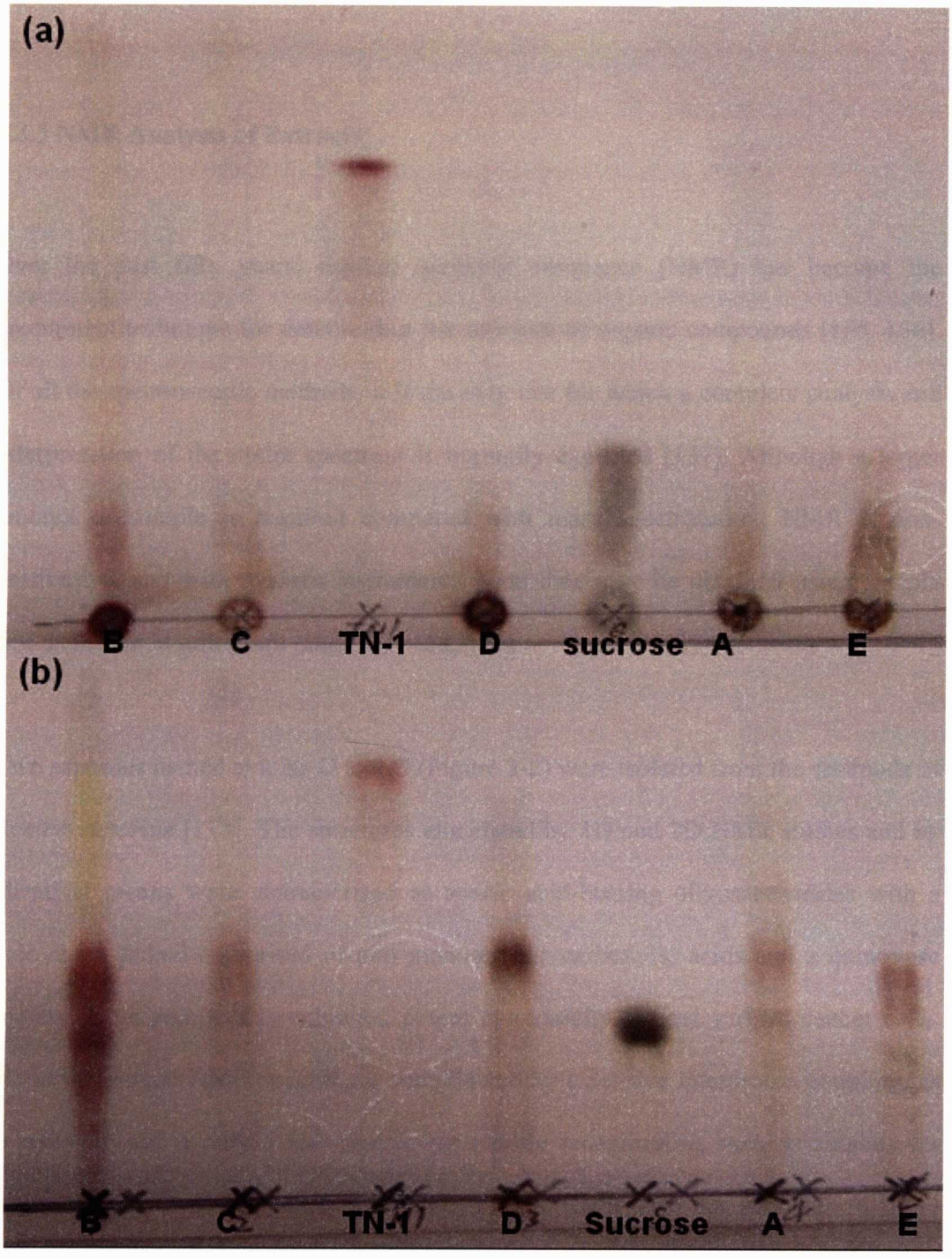


Figure 4-5 Comparison of TLC of samples obtained by multistage liquid-liquid extraction with sucrose and TN-1 standard. A: Fraction A, B: Fraction B, C: Fraction C, D: Fraction D and E: Fraction E. (a) $CHCl_3$-EtOAc-MeOH-H_2O (2:5:4:1), (b) *n*-ButOH-EtOH-HOAc-H_2O (8:2:1:3)

4.3.3 NMR Analysis of Extracts

Over the past fifty years, nuclear magnetic resonance (NMR) has become the preeminent technique for determining the structure of organic compounds [155, 156]. Of all the spectroscopic methods, it is the only one for which a complete analysis and interpretation of the entire spectrum is normally expected [157]. Although a larger amount of sample is required compared with mass spectroscopy, NMR is non-destructive, and with modern instruments good data may be obtained using sample size as low as in milligram range [21, 158, 159].

Two saponins named avicins D and G (Figure 3-2) were isolated from the seedpods of *Acaica victoriae* [118]. The structures elucidated by 1D and 2D NMR studies and by chemical means were characterized as acacic acid-bearing oligosaccharides with a side chain linked comprised of two monoterpene carboxylic acids and a quinovose moiety. Both compounds exhibited potent cytotoxicity against various cancer cells. However, proton NMR spectra are complicated by extensive interproton coupling, as a result of which only a few signals are readily recognizable, such as singlets for angular methyl groups [130]. Therefore, proton NMR was not efficient for pure saponin chemical structure identification. Proton NMR, however, provides a promising option to analysis of crude triterpene group. The full assignments of proton NMR resonances for triterpenoids saponins have been investigated because typically more than 20 protons will have chemical shifts crowded in the region 0.5-2 ppm [160], while signals between 3-5 ppm present sugars [157, 161].

In this study, almost all the major compound in Fraction E was sodium chloride. All other fractions may have triterpenoids saponins at various polarity range. Triterpenoids saponins may be concentrated in Fractions B, C and D, while Fraction A may have a few as it was the fraction obtained from solid residue. The aim of NMR analysis was performed to confirm, whether the extracted compounds in each fraction contained any saponin compounds.

The NMR spectrums of active fractions were shown in Figure 4-6. Fraction A has more polar compounds compared with Fraction B; this is in agreement with the polarity of solvents used. Fraction C and D has strong signals between 0.5 and 2 ppm, underlining presence of large quantity of triterpenes in these fractions. Fraction C was more complicated than Fraction D, because the latter was purified by liquid-liquid partitioning. The main constitute of Fraction E was sodium chloride, therefore, the spectrum of Fraction E was simple and had signals for sugar rather than triterpenes. The NMR results demonstrated that the multistage fractionation slightly separated groups of saponins, however, further stage is required to isolate each saponin compound.

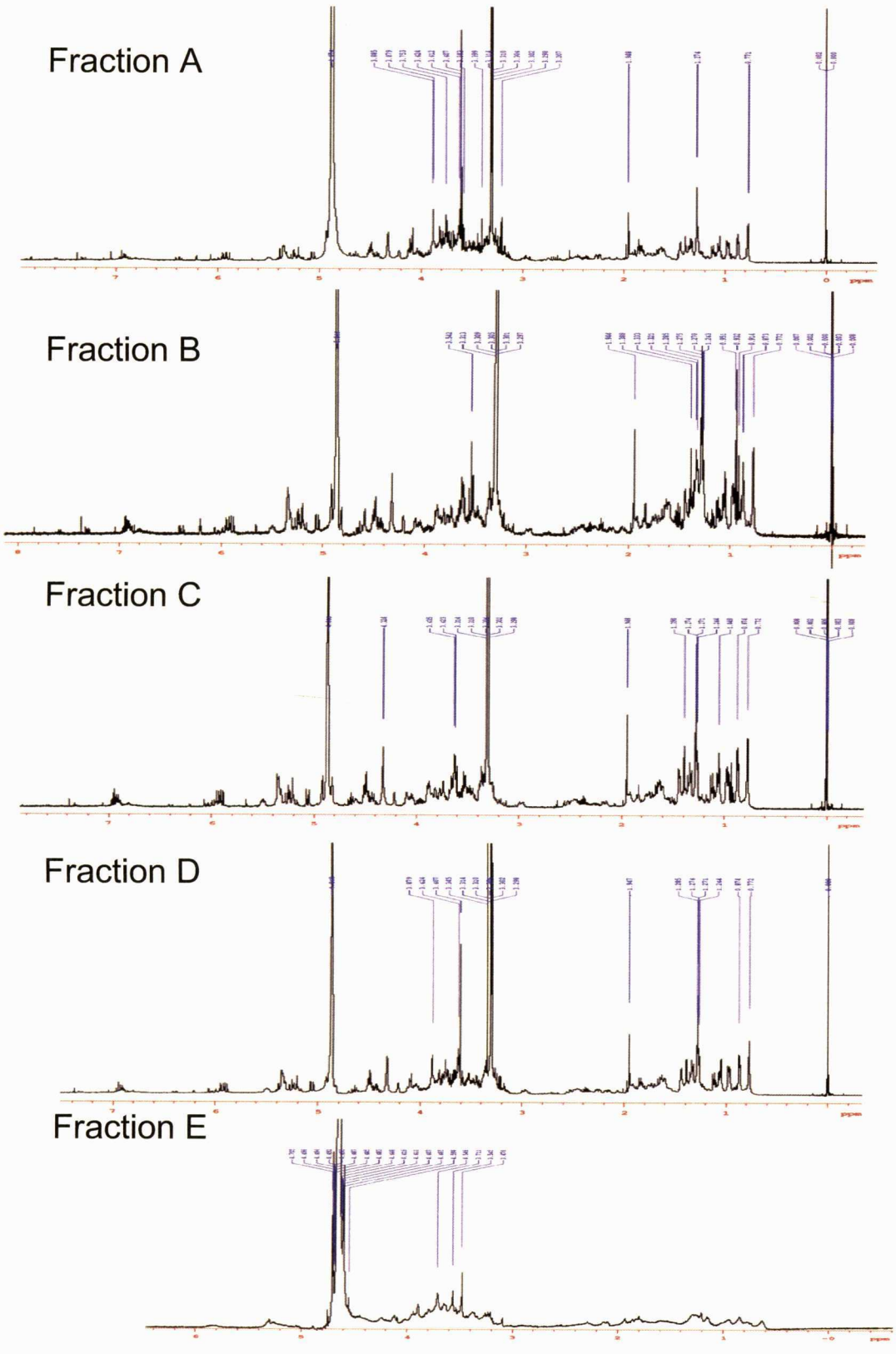


Figure 4-6 NMR spectrums of active fractions

4.3.4 Cytotoxic test of solvents on A2780 cell line

The growth inhibitory effects of various solvents against A2780 are shown in Figure 4-8. This data was used to calculate the mean IC_{50} values for each solvent. These organic solvents have been chosen to extract triterpenoids saponins and it is necessary to test their anticancer activity. As depicted in Figure 4-8, the absorption decreased by increasing the concentration of each solvent. The mean IC_{50} values are 24.1±0.3 vol% for Acacia gum, a 5 wt% solution, 6.0±0.09 vol% for Methanol: Dichloromethane (1:1), 3.1±0.17 vol% for Ethyl acetate, 2.9±0.25 vol% for DMSO, 2.3±0.11 vol% for Ethanol: Glycerol (1:1) and 1.8±0.24 vol% for Methanol. Methanol has the lowest value, therefore, more toxic to the cell compared with other solvents. From above study, we can conclude that all organic solvents examined resulted in cell growth inhibition and their residue in the extract may result in anti-cancer effect for long term exposure. The growth inhibition activity of DMSO and 5 wt% Acacia gum were compared with other organic solvents. Among all solvents system, Acacia gum solution was the best as it had less cytotoxic effect on the cells.

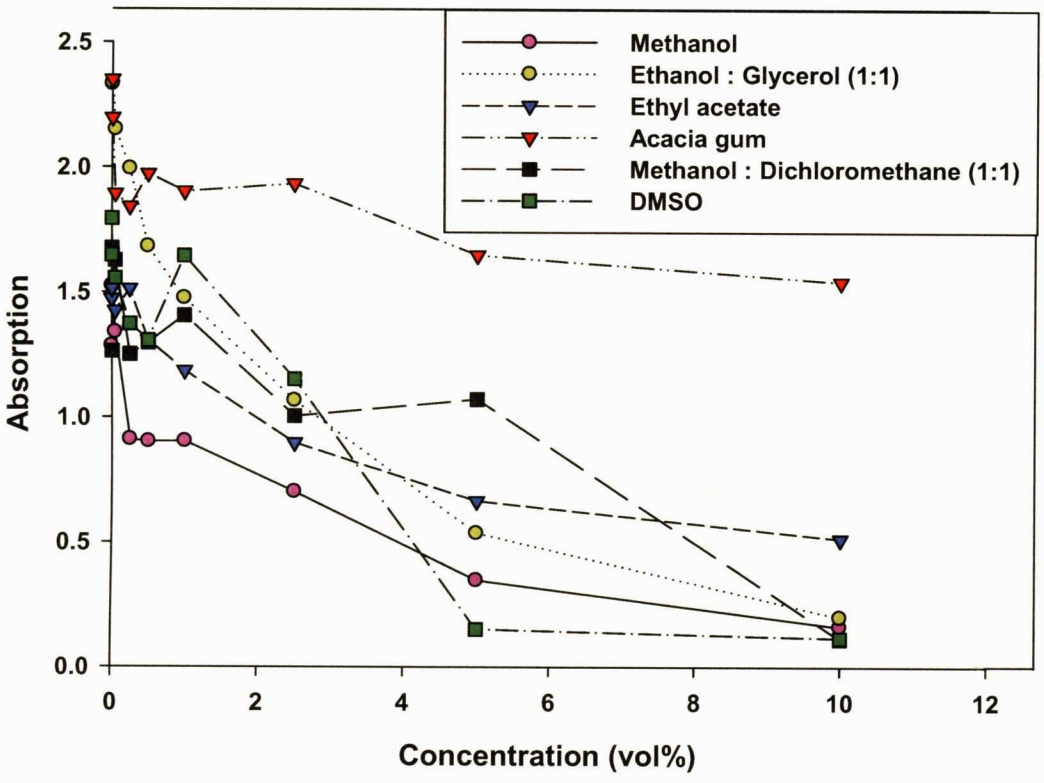


Figure 4-7 Growth inhibitory activity of solvents on A2780

4.3.5 Fractionation of saponins and their cytotoxic activity

The anticancer activity of fractions A-E obtained from liquid-liquid and liquid-solid partitioning were measured using MTT assay. Each fraction was dissolved in known amount of aqueous solution containing 5 wt% acacia gum. The growth inhibitory effects of all the fractions are shown in Figure 4-7 and the mean IC_{50} values were calculated. The IC_{50} values for fraction A-E were shown in Table 4-1. Fraction B exhibited the highest activity, which was in agreement with the previous study by Keung et al that crude plant extract was more potent than the pure compounds in terms of biological activity[37]. The IC_{50} values for fractions C and D acquired from *n*-butanol phase by liquid-liquid partition displayed high activities, implying high

solubility of active compounds in *n*-butanol phase. The differences of IC_{50} values among Fraction B, Fraction C and Fraction D were inconspicuous which demonstrated that all these three fractions contained considerable amount of triterpenoids saponins.

Fraction A had the lowest activity, corroborating that all active ingredients were already extracted by methanol. Methanol: water mixture extracted polar compounds with lower anti-cancer activity. The main constitute of Fraction E was sodium chloride after evaporation, which resulted in permeation pressure between both sides of the cell membrane and lethal effects on cells. This study confirmed previous hypothesis, and made consistent with previous NMR analysis that fractions with strong signals in 0.5-2 ppm, presented triterpenoids saponins, demonstrated higher anticancer activity on A2780 cell line.

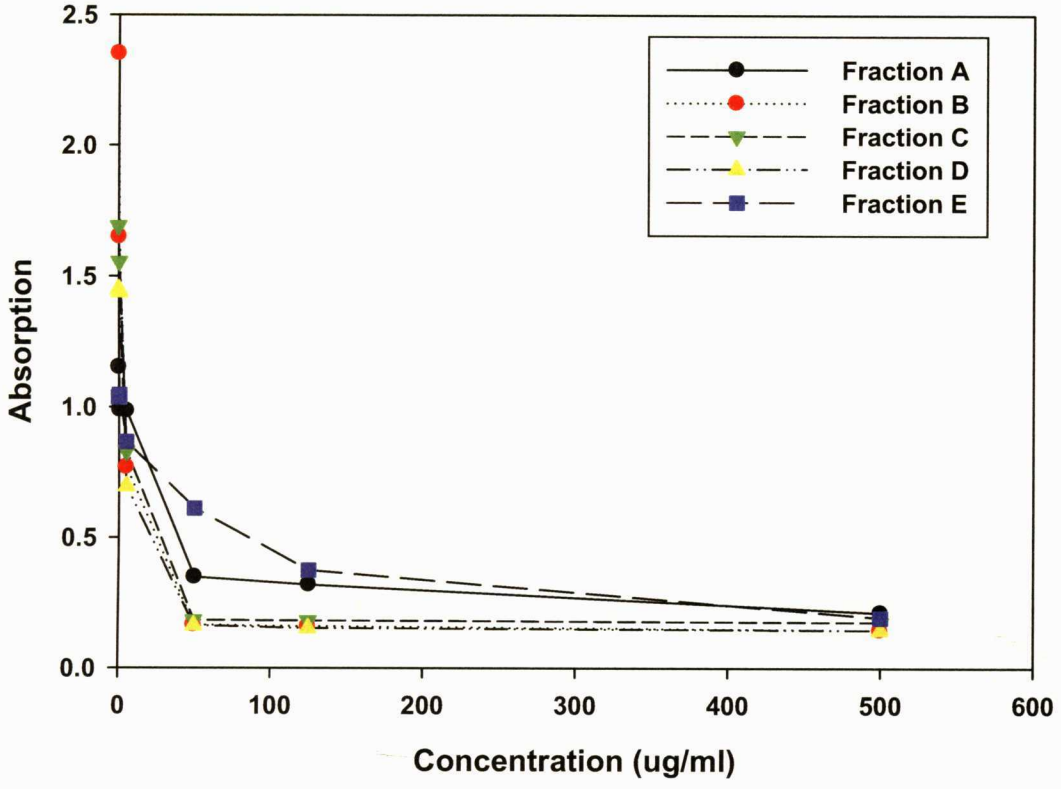


Figure 4-8 Anti-cancer activity of fractions extracted by various solvents after partitioning and dissolving in 5 wt% Acacia gum in A2780 cell line

Table 4-1 Values of IC_{50} for each fraction.

Fractions	A	B	C	D	E
IC_{50} (µg/ml)	27.311±0.21	10.764±0.34	11.975±0.06	12.632±0.11	14.458±0.17

4.3.6 Cytotoxic Effect of Extract on A2780

The dried saponins extracted by different solvents from *Acacia victoriae* were dissolved in 5% wt Acacia gum and their anti-cancer effect on A2780 cell line was determined. Acacia gum is a natural gum made of hardened sap taken from two

species of acacia tree, Acacia Senegal and Acacia Seyal. Acacia gum is a mixture of saccharides and glycoproteins gives it the properties of a glue, and binder which is edible by humans. It can be easily dissolved in water and miscible with saponins as they have the same functional group. It makes sense to use 5 wt% acacia gum as a solvent; the extracts from different solvent were dissolved in 5 wt% acacia gum and a series concentration was prepared to conduct MTT assay.

The results shown in Figure 4-9, fractions obtained from methanol: water (1:1 volume ratio) and methanol presented significantly higher antitumor activity against A2780 cell line (20.38±0.51 μg/ml and 19.24±0.32 μg/ml, respectively), wherein the IC_{50} value for water and ethyl acetate extract were 146.4±0.22 μg/ml and 145.7±0.47 μg/ml, which were 7 folds higher than extract from methanol: water (1:1 volume ratio) and methanol. This result is in agreement with the results reported in the literature [14, 128] that methanol and aqueous methanol were the best solvents to extract triterpenoids saponins from natural plants. The differences in antitumor activities may be attributed to their different structure of saponins and monosaccharide distributions.

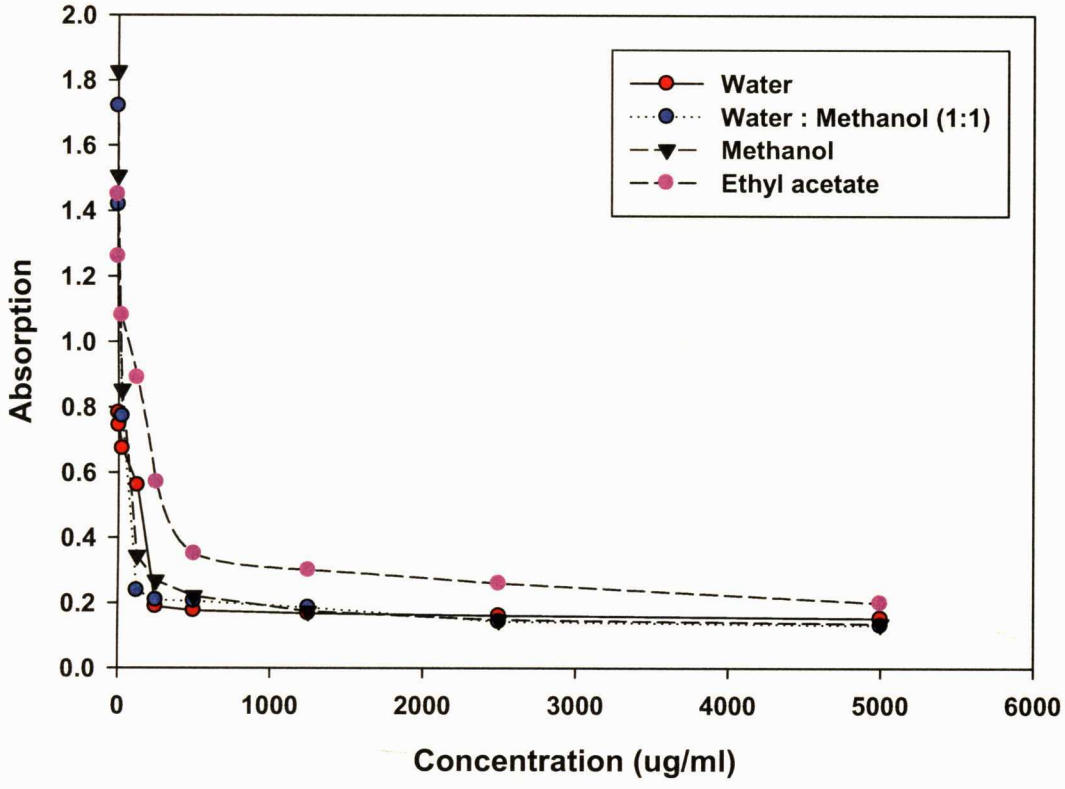


Figure 4-9 Growth inhibitory activity of extract from different selected solvents dissolved in 5 wt% acacia gum in A2780 cell line

In this study, various organic solvent systems were tested to extract active compounds from *A. victoriae*. Methanol was the best solvent to extract active compounds, which shows the best growth inhibition activity. Methanol extraction was also chosen as a reference for the following experiments to conduct further identification and compare with supercritical fluid extraction.

4.4 Summary

Triterpenes are major biologically active components in *A. victoriae*, and widely accepted as agents responsible for the anticancer activities of this plant. In this study, the amount of fat in *A. victoriae* was evaluated and the fat content was 5±0.1 wt%. Defatting is crucial to remove non-polar (fat) compounds from plant to prevent their interference for extraction and MTT assay. Crude triterpenes were isolated with different organic solvent systems and further fractionated. *In vitro* MTT assay was conducted to study anticancer activity of both extracts. Methanol was the most efficient solvent for the extraction of active compounds from *A. victoriae* that demonstrated anticancer activity.

It was attempted to use multistage fractionation for the separation of saponins and other polar compounds. The results of TLC and NMR analysis demonstrated that triterpenoids saponins extracted from *A. victoriae* were relatively polar compared with TN-1 saponins. NMR spectrum confirmed the present of various types of triterpenes in extracts from all fractions. However, proton NMR used in this study was unable to determine the molecular structure of these saponins. A complex liquid chromatography technique is required to separate each saponin for ^{C}NMR, 2D NMR and LC-MS to identify the molecular structure of each compound; this was beyond the scope of this project. The MeOH extracts demonstrated to highest cytotoxic effect on cancer cell line A2780. The addition of several stages of fractionation using liquid chromatography substantially increases the cost of process, and the result of this study demonstrated this is eventually will not enhance the cytotoxic activity of extracted fractions. It is therefore not necessary to further fractionate saponins for the

production of an anticancer formulation and the development of neutraceutical products.

From above discussion, it is confirmed crude triterpenoids saponins extracted from *A. victoriae* demonstrated higher cytotoxic activity than that of fractions obtained by liquid-liquid partitioning.

5. Extraction of Triterpenoids Saponins from *A. victoriae* by Supercritical Fluid

5.1 Introduction

The commercial Acacias products available are typically prepared by the use of organic solvents or hot water extraction [162]. Water is not efficient in extraction of triterpenoids saponins and the use of volatile organic solvent is not desirable because of restricted environmental regulations, particularly in food processing [163]. Supercritical fluid extraction (SFE) is one of promising benign technologies that can overcome this issue. SC CO_2 modified with MeOH has been used for the extraction of saponins from Bamboo [32]. The yield of process was between 0.98 wt% and 2.2 wt%. SFE has not yet been used for the extraction of saponins from *A. victoriae*.

In this chapter, the feasibility of using SCF CO_2 for the extraction of saponins ingredients from *A.victoriae* was investigated. The effect of process variables such as temperature, pressure, modifier concentration on the extraction efficiency and also activity of extract were determined.

5.2Apparatus and Experiment Procedure

A schematic diagram of the experimental apparatus for extraction using supercritical fluid is shown in Figure 5-1. The apparatus consisted primarily of an ISCO 500D syringe pump, a preheating coil, a custom made 90 mL high pressure vessel, a sample collection and a water bath. The temperature was controlled by Thermoline TU1 Unistat heater/circulator. Both sides of the vessel were filled with glass wool (Alltech Associate Australia) to avoid blockage of the lines connecting to the extraction vessel. A 0.5 μm filter was installed after the exit valve to collect the solid extract during the experiment. A cold trap was connected to the exit valve to collect any residues of extract. The cold trap was filled with 20 mL methanol (a good solvent for saponin) and placed in a Dewar jar that was filled with acetone-dry ice mixture to keep the temperature low for the collection of extract [128].

Liquid carbon dioxide was fed into the syringe pump (ISCO Model 500D) at ambient temperature. After the pump was filled with CO_2 it was connected to the system. Carbon dioxide was then passed through a preheating coil, to reach to a desired temperature, and then filled the extraction vessel that was packed with *Acacia Victoriae* powder. The extract was collected either in filter with 0.5 μm assembled at the exit for solid or cold trap for liquid.

At each temperature, after purging air from the system, the exit valve was shut down and the system was pressurised to a predetermined pressure, and it was then isolated for at least one hour to approach equilibrium. After which the dynamic extraction was commenced; the exit valve was opened slightly and the inlet flow rate was adjusted

between 5 and 10 mL/min, and the pump was run at a constant pressure mode to maintain a constant pressure in the system. The extract was collected in the collection system. A gravimetric technique was used to determine the extraction efficiency. At the end of each experimental run, the mass of extract collected was determined by weighing the collected sample in the valve, filter and dried sample from cold trap. Methanol was evaporated under the vacuum overnight. The volume of CO_2 used during the solubility measurement was recorded using the flow meter installed in the pump.

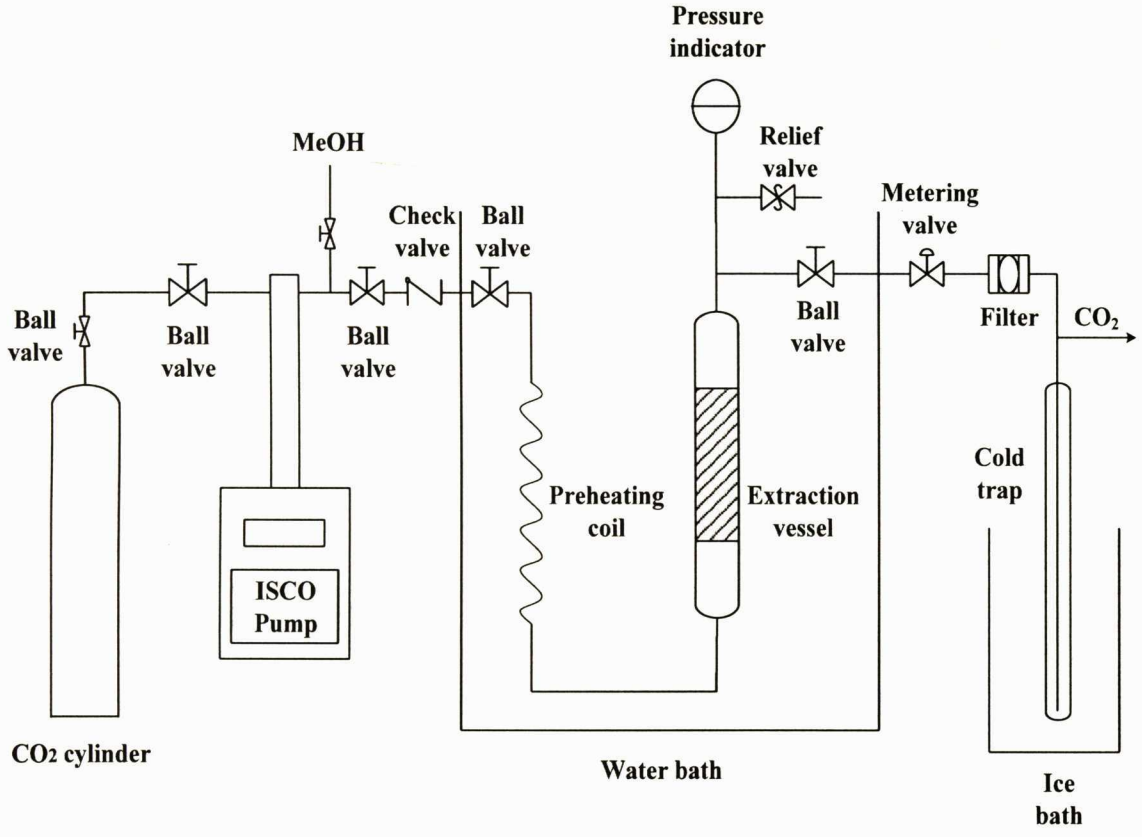


Figure 5-1 Supercritical CO_2 extraction apparatus

The validity of the experimental procedure was assessed by measuring naphthalene solubility in CO_2 at 35 °C and comparing these data with the one reported in the literature. This setup was used for the extraction of both fat and active compounds from *A. victoriae*. Every measurement and extraction was repeated at least three times to verify the repeatability.

5.3 The Preparation of CO_2-Modifier Mixture

The procedure for the preparation of modified CO_2 was described in detail in previous study [164]. In summary, in each run, the piston of the syringe pump was moved to the top and all the lines were purged with CO_2 to ensure there is no residue of other solvents in the system. The volume of modifier required for the preparation of specific mole fraction was estimated from the following equation:

$$V_{MeOH} = \frac{X_{MeOH}\rho_{CO_2}V_T MW_{MeOH}}{\rho_{MeOH}(1-X_{MeOH})MW_{CO_2} + \rho_{CO_2}X_{MeOH}MW_{MeOH}}$$

X : mole fraction

ρ : mass density (g cm^{-3})

V_T : total volume of the syringe pump with the attached fitting (cm^3)

MW : molecular weight

The CO_2 modified with MeOH was prepared by the following method. Required amount of MeOH was injected directly into the syringe pump at the stage the pump was run in refilling mode. After which the inlet valve for the pump was opened to fill

the pump with CO_2 and the outlet was left closed. During this period, cooling water was circulated around the pump heal to maintain the temperature at 5 °C. The pump was then isolated and the piston was moved up and down at pressure above 150 bar to mix the CO_2 and MeOH. The pump was then left overnight at constant pressure mode for complete mixing of modified CO_2.

5.4 Statistical Analysis

Statistical significance was determined for replicates of 3 by an independent ANOVA (analysis of variance) test to investigate the effect of each parameter in SFE using Excel software. Data are represented as mean ± standard deviation (SD).

5.5 Results and Discussion

5.5.1 Solubility of Naphthalene in Supercritical CO_2

In order to verify the reliability and efficiency of the solubility apparatus and the technique employed in this study, the solubility of naphthalene in supercritical CO_2 was determined. The solubility data for naphthalene in supercritical CO_2 at 35 °C acquired in this work was compared with the one in previous study [165]. The solubility was measured in both low (e.g. 85 bar) and high pressures (e.g. 131.1 bar). The results shown in Figure 5-2 were in good agreement with the data in literature with error less than 8%. The close proximity of these results to those acquired by

McHugh et al validates the use of the apparatus designed for extraction. The isotherm demonstrates an increase in analyte solubility with increased pressure as a result of the effect of solvent density. These results also indicate that equilibrium was established between CO_2 and naphthalene within the high pressure vessel at the flow rate used.

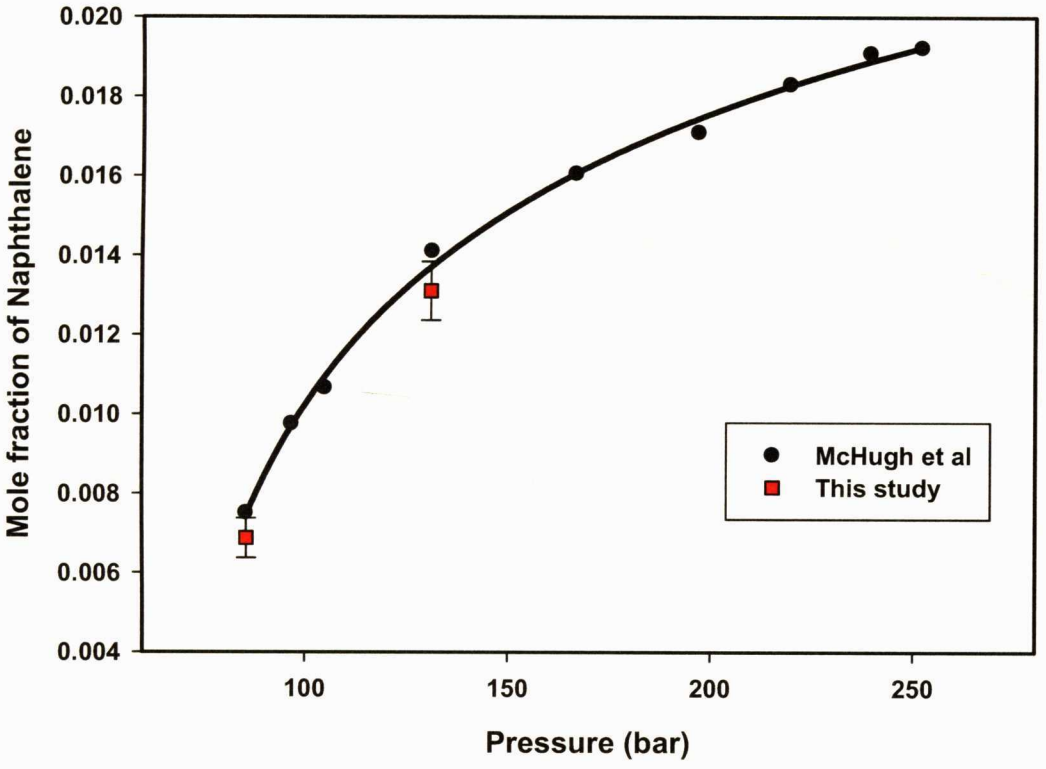


Figure 5-2 Solubility of naphthalene in supercritical CO_2

5.5.2 SFE of *A. victoriae* using neat CO_2

The data acquired in previous study demonstrated that triterpenoids saponins in *Acacia victoriae* were relatively polar. It is, therefore, anticipitated that neat CO_2 is

unlikely to extract this group of compounds. Non defatted ground seedpods were used for the SCF extraction.

Pure supercritical CO_2 was used for the extraction of compounds from Acacia plant. The effect of temperature on the extraction efficiency of CO_2 was determined at 180 bar. As shown in Figure 5-3, the amount of extract was first increased by increasing the temperature of process, and reached the highest efficiency near the critical point, then decreased that may be due to decreasing CO_2 density. The obtained maximum yield in this study (0.32 mg/g CO_2) is consistent with previous study (0.5 mg/g CO_2) [166]. The enhancement on the amount of material extracted was due to increasing the vapour pressure of the solutes by elevating the processing temperature [3, 7]. The extract was mostly fat, as it was not miscible in methanol and methanol: water (1:1 volume ratio), however, it was soluble in hexane. The results corroborate that at condition examined pure CO_2 was efficient for defatting of the Acacia and not effective for the extraction of triterpenoids saponins that are polar compounds. At the optimum condition (25 °C, 180 bar), 1.6 L CO_2 was required to completely remove the fat from 10 g *Acacia victoriae*.

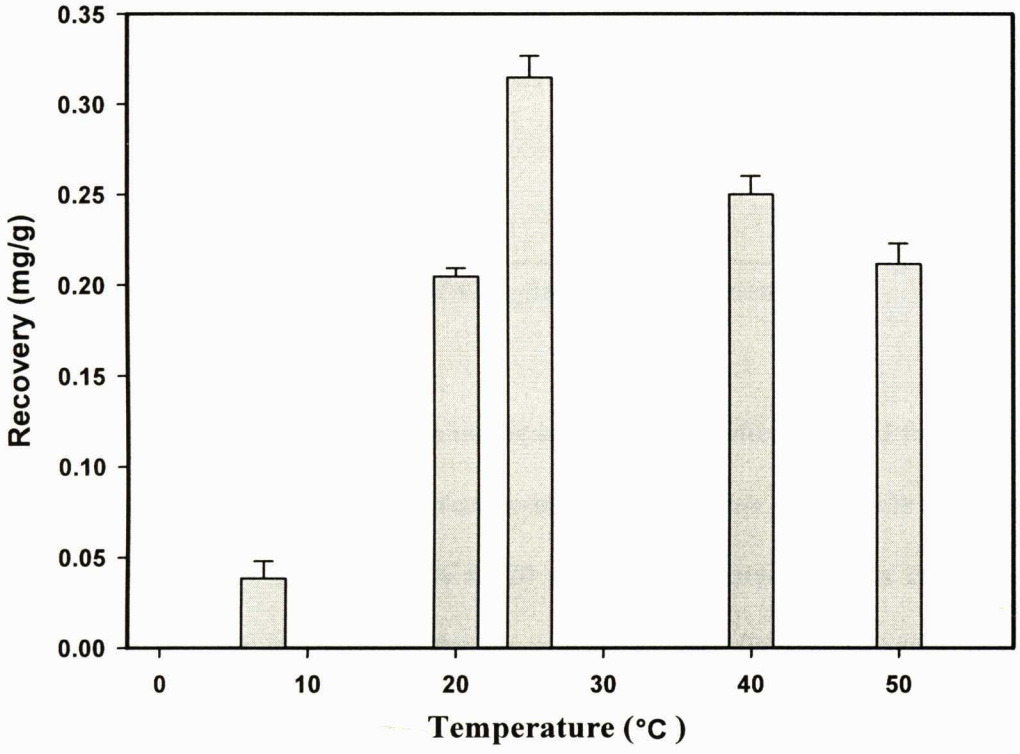


Figure 5-3 Extract obtained by pure supercritical CO_2 at 180 bar

The solubility of high molecular weight and polar compounds in CO_2 is limited. Polar or non-polar modifiers have been added to CO_2 to increase the solubility of such compounds. A modifier or a co-solvent, is commonly a volatile compound that is miscible with the primary supercritical fluid, and usually constitutes only a small percentage of the overall fluid composition [49]. The modifier interacts strongly with the solute and significantly increases the solubility of the original SCF solvent [75]. The modifiers are used to selectively extract and enrich the more polar target molecules from the raw material [76].

As it was not feasible to extract polar compounds such as saponins using neat CO_2, the effect of a modifier on the extraction of saponins was determined. Methanol was

the most efficient solvent to extract triterpenoids saponins from *A. victoriae* as reported by Arntzen [14]. The effect of using methanol as a modifier on the extraction of active compounds from Acacia plant was determined.

5.5.3 The Effect of Modifier on SC CO_2 Extraction Efficiency

The aim of this work was to determine the amount of modifier required for extracting active compounds from defatted seedpods of *A. victoriae*. The mole fraction of methanol was varied from 1 mol% to 20 mol%. The extraction was conducted at 50 °C and 180 bar to optimize the amount of MeOH for triterpenoids saponins recovery.

It was anticipated that raising MeOH concentration increases the extraction efficiency of CO_2 for active compounds such as triterpenoids saponins [167]. The results in Figure 5-4 show that the total compound extracted by modified CO_2 was increased from 0.145 mg/g to 0.8265 mg/g when the concentration of MeOH was elevated from 1 mol% to 20 mol%. However, it should be noted that the addition of large amounts of modifier considerably changed the critical point of the mixture and that the fluids were no longer supercritical [29, 168]. It is also desirable to use as possible lower amount of organic solvent during the extraction process.

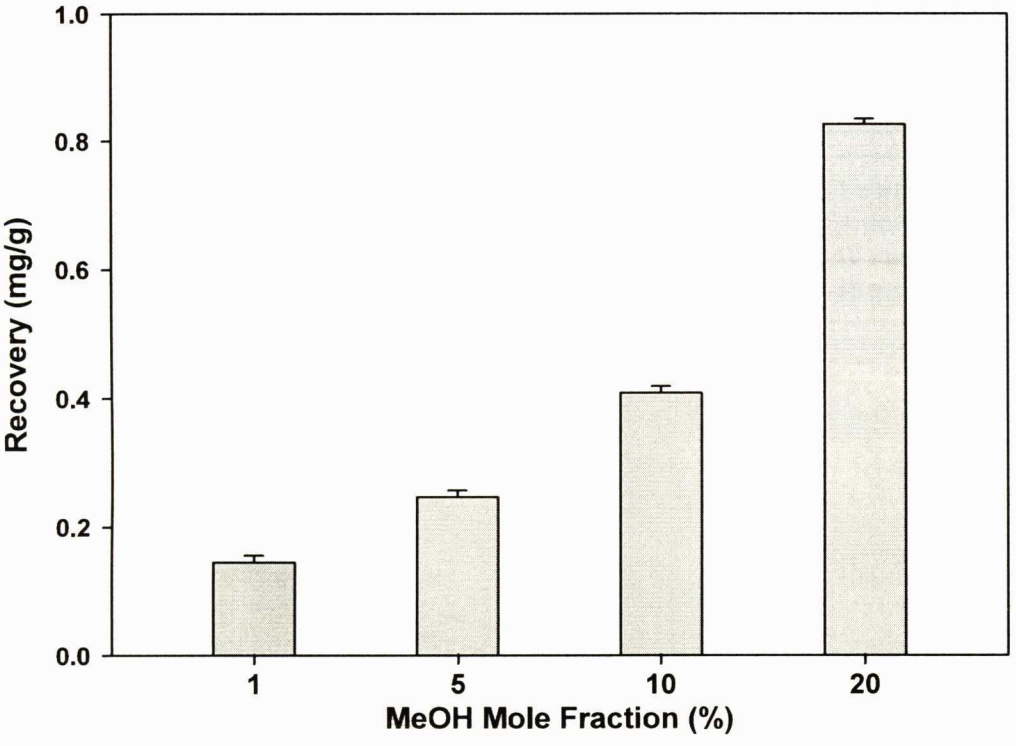


Figure 5-4 Effect of methanol on the CO_2 extraction efficiency for recovery of saponins from *Acacia* plant (P=180 bar, T=50 °C)

The results for biological activities are shown in Figure 5-5. The values of IC_{50} for 1 mol%, 5 mol%, 10 mol% and 20 mol% were 42.95±0.83 μg/ml, 32.96±0.36 μg/ml, 82.32±0.34 μg/ml and 73.57±0.82 μg/ml, respectively. The anticancer activity at 5 mol% methanol was the highest; nearly threefold of that at 10 mol%. By increasing the mole fraction of MeOH, the polarity of the solvent mixture was increased, which may result in the extraction of sugar groups in *A. victoriae*, and decreasing cytotoxic effect. However, the anticancer activity of extract obtained by using 5 mol% MeOH in CO_2 at 50 °C, 180 bar was lower than using neat MeOH at ambient condition (IC_{50} 19.24 μg/ml). The polarity of CO_2 and MeOH can be tuned by the variation of

temperature and pressure of process. These parameters may affect the extraction efficiency and the biological activity of extracted compounds.

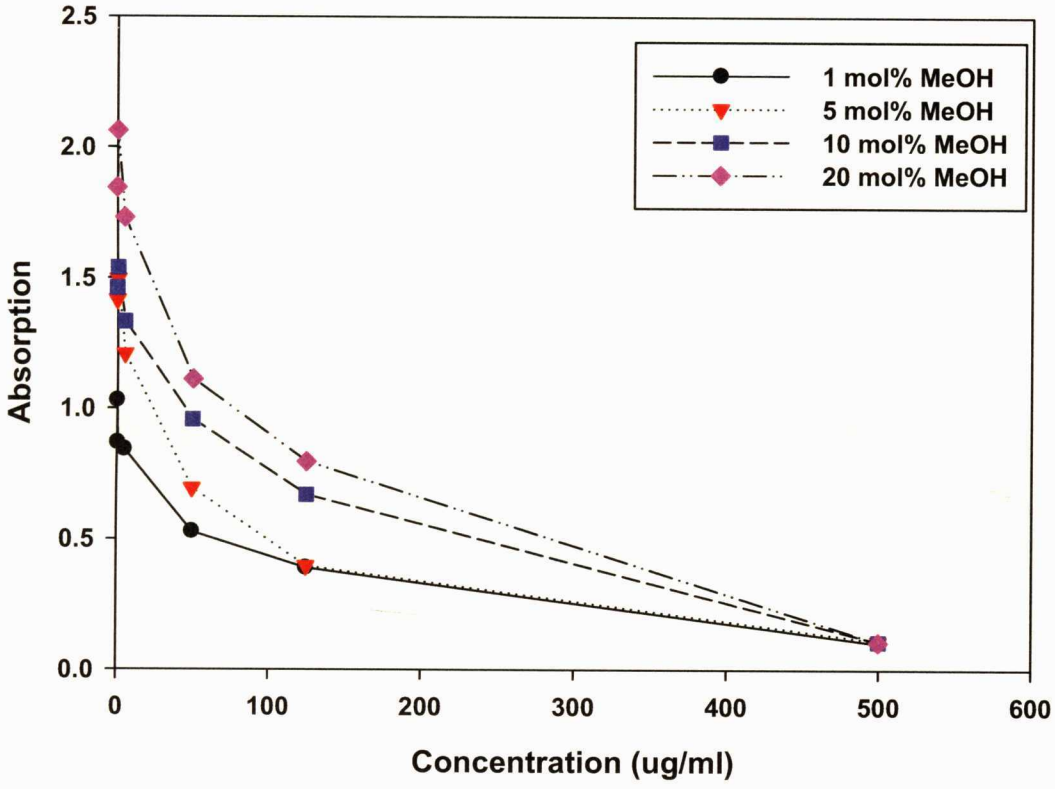


Figure 5-5 Cytotoxic effect of various SCF extracts on A2780 cell

The NMR spectrum of extract from SFE using 5 mol% and 20 mol% MeOH is shown in Figure 5-6, which demonstrated that extract using 5 mol% MeOH contained less sugar than using 20 mol% MeOH. Therefore, even the recovery of 20 mol% was higher, more sugar was extracted which was not contributed to biological activity.

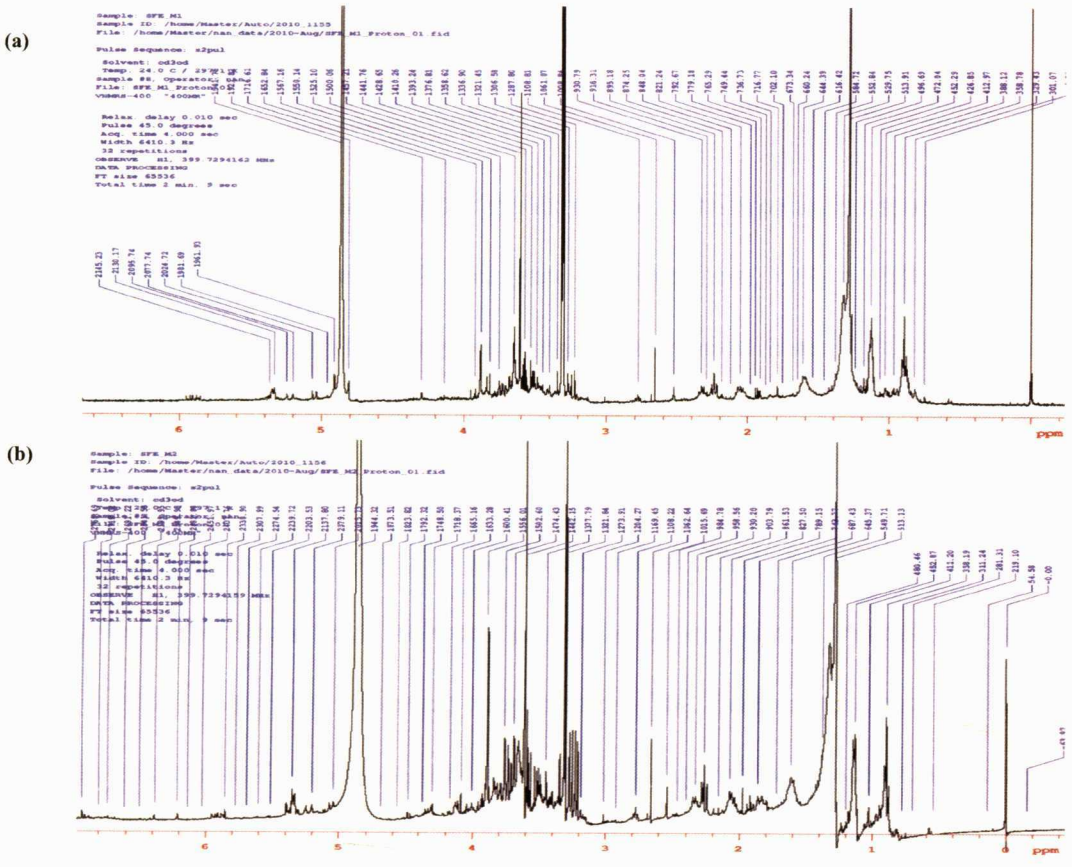


Figure 5-6 NMR spectrum of SFE extracted component from *Acacia* plant. (a) 5 mol% MeOH; (b) 20 mol% MeOH

Various amounts of methanol used exhibited different effect in changing the fluid polarity and thus had diverse effect on the solubility enhancement of active compounds. The optimal extraction condition was selected based on the biological activity rather than recovery. Therefore, the optimum amount of MeOH for the extraction of triterpenoids saponins from *A. victoriae* was 5 mol%. Using this low quantity is also desirable in terms of minimizing the use of organic solvent.

5.5.4 Effect of Operating Temperature

Operating temperature was optimized for the extraction of triterpenoids saponins from defatted seedpods of *A. victoriae*. In this part of experiment, CO_2 at 180 bar was modified with 5 mol% MeOH while the temperature varied from 7 °C to 50 °C. Temperature has two opposing effects. High temperature increases vapor pressure of solute that enhances the solute solubility; High temperature decrease CO_2 density, and enhance its salvation power [169]. The overall extraction effect of supercritical fluids usually follows the competition between increasing solute vapor pressure and the reducing SC-CO_2 density by elevating temperature [170]. Temperature also affected the polarity of the whole solvent system, which may change the compounds extracted.

The effect of operating temperature on the triterpenes recovery is shown in Figure 5-7. The yield was increased dramatically, when the temperature increased from 7 °C to 25 °C. The extraction recovery had declining trend when temperature was increased from 25 °C to 40 °C. Further increase from 40 °C to 50 °C increased the yield, and reached the highest yield with the recovery value was 0.2471 mg/g.

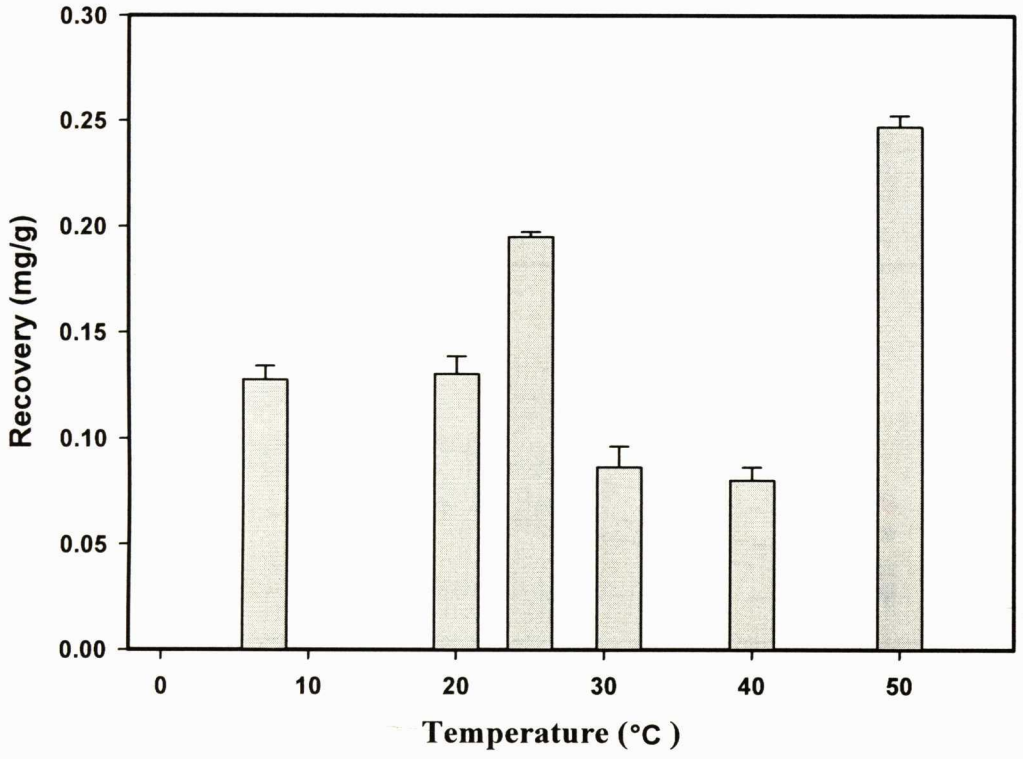


Figure 5-7 The effect of temperature on the saponins extracted from *Acacia victoriae* using CO_2 modified with 5 mol% MeOH at 180 bar

The effect of operating temperature on the biological activity of extract is shown in Figure 5-8. The values of IC_{50} for 7 °C, 20 °C, 25 °C, 31 °C, 40 °C and 50 °C were 14.43±0.32 μg/ml, 3.91±0.37 μg/ml, 23.38±0.90 μg/ml, 0.18±0.03 μg/ml, 4.12±0.27 μg/ml and 32.96±0.36 μg/ml, respectively. The anticancer activity of the extract at 31 °C was the highest, even higher than pure avicins (IC_{50}: 0.2 μg/mL for Jurkat cells) separated from *Acacia victoriae*[12].

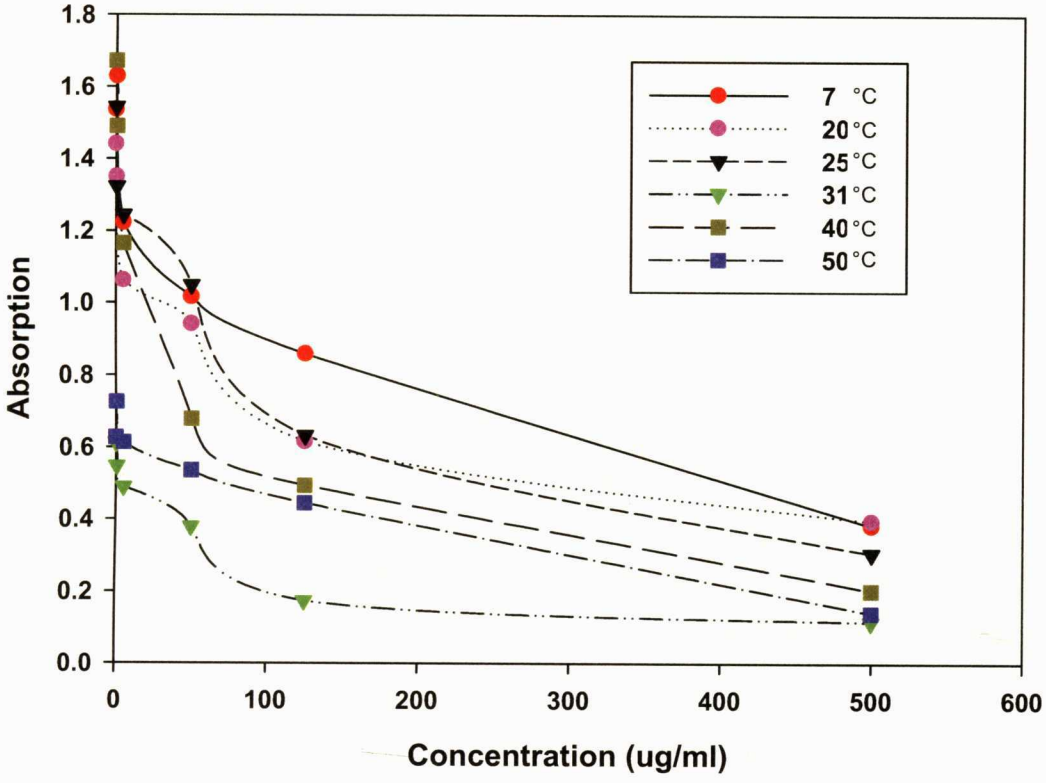


Figure 5-8 Anti cancer activity of *Acacia victoriae* extract on A2780 cell using CO_2 modified by 5 mol% MeOH at 180 bar and various temperatures for extraction

The NMR spectrum of extract from SFE using 5 mol% MeOH at 31 °C and 50 °C respectively was shown in Figure 5-9, which demonstrated that extract at 31 °C contained more triterpenes and less sugars than at 50 °C, because stronger signals were observed between 0.5 ppm and 2 ppm and weaker signals were depicted between 3 ppm and 5 ppm. The total recovery was 25 % higher at 50 °C. However, the amount of extracted trierpenoids saponins with biological activity was more at 31 °C compared with 50 °C.

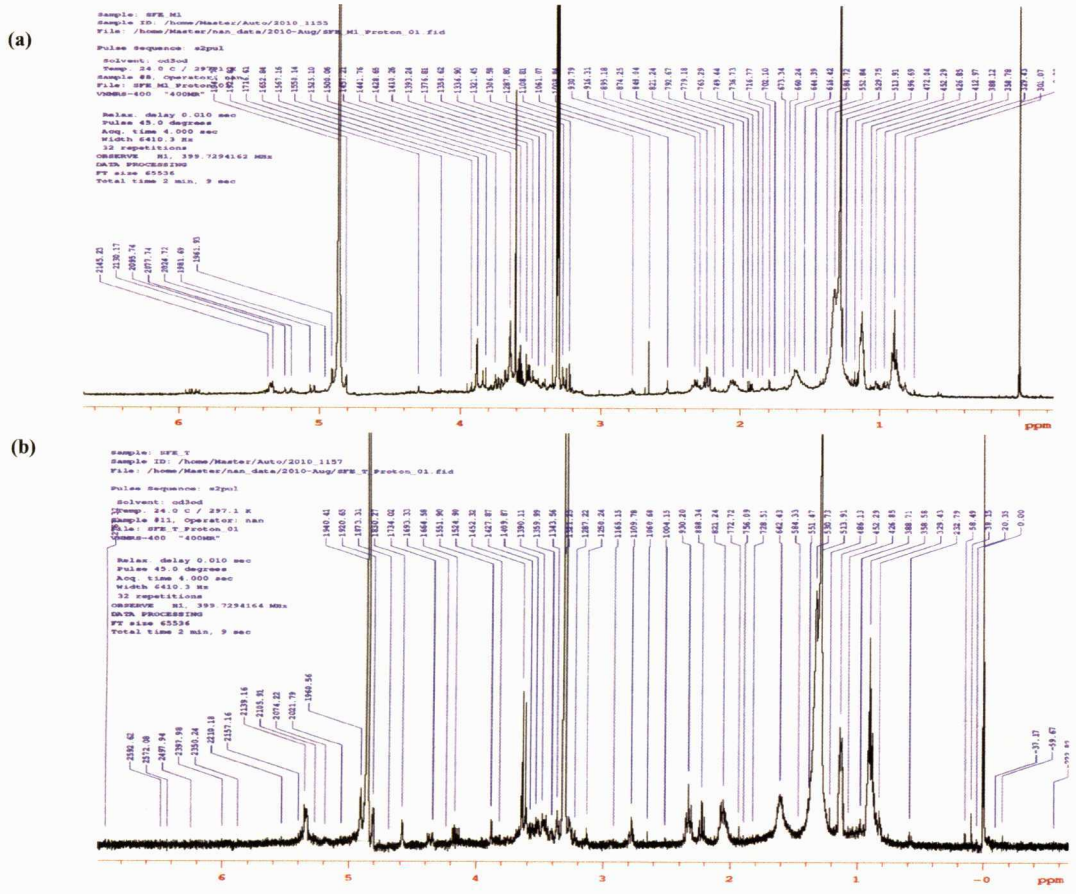


Figure 5-9 NMR spectrum of effect of temperature on extract component from *Acacia* plant. (a) 50 °C; (b) 31 °C

In this study, the extraction recovery was higher at 50 °C. However, the anticancer activity was higher at 31 °C. Therefore, the optimal operating temperature for extraction active compounds from *A. victoriae* was 31 °C.

5.5.5 Effect of Operating Pressure

The extraction of triterpenoids saponins from *A. victoriae* was conducted to optimize operating pressure. Defatted *A. victoriae* was extracted by CO_2 at 31 °C modified with

5 mol% MeOH while the pressure was varied from 60 bar to 180 bar. The effect of operating pressure on the extraction efficiency was determined. As shown in Figure 5-10, by increasing the pressure from 60 bar to 90 bar, the amount of extract was raised from 0.001 mg/g to 0.2092 mg/g. However, the extraction yield had decreasing trend when pressure was increased from 90 bar to 180 bar. Higher pressure can increase the density of the fluid mixture that would contribute to solubility enhancement. However, as pressure was increased further, the fluid was less compressible, so that the increase in density was not expected to be very significant. As can be seen in Figure 5-10, pressure at 90 bar was the best condition to extract triterpenoids saponins from A. victoriae, which is highly in agreement with the previous report by He et al. that the best condition to extract active compounds from natural plant was at the condition just a little above critical point [29].

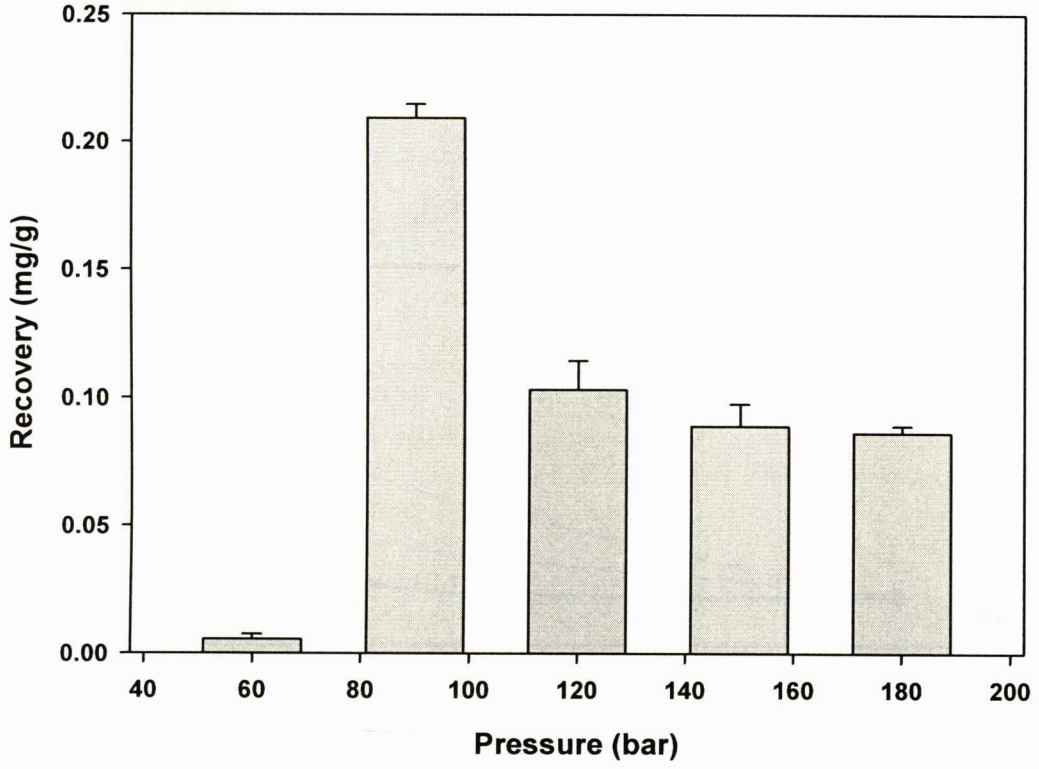


Figure 5-10 The effect of pressure on the amount of extract from *Acacia victoriae* using CO_2 modified with 5 mol% MeOH at 31 °C

The effect of operating pressure on the biological activity of extract is shown in Figure 5-11. The extract at 60 bar was very low and impossible to recover and perform anticancer activity test. The values of IC_{50} for 90 bar, 120 bar, 150 bar and 180 bar were 0.05±0.03 μg/ml, 0.67±0.04 μg/ml, 0.21±0.02 μg/ml and 0.18±0.03 μg/ml.

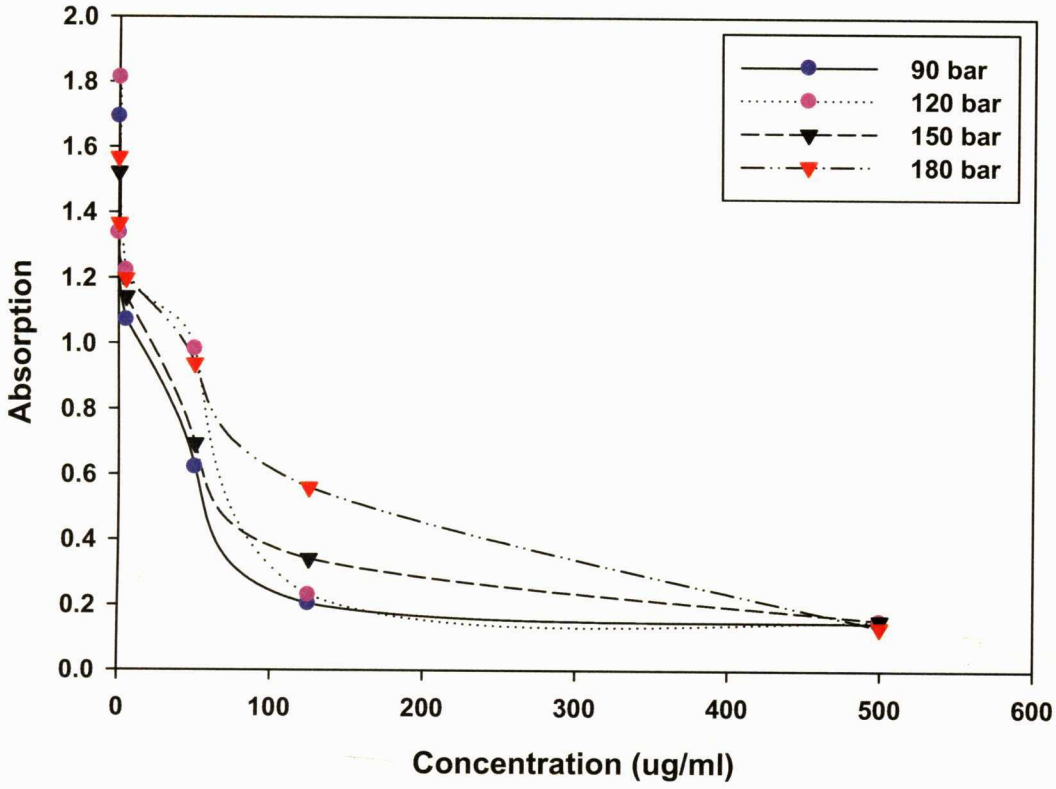


Figure 5-11 Anti cancer activity of *Acacia victoriae* extract obtained using CO_2 modified by 5 mol% MeOH at 31 °C and various pressures on A2780 cell

The NMR spectrum of extract from SFE using 5 mol% MeOH at 31 °C, 90 bar and 180 bar respectively is shown in Figure 5-11, which demonstrated that extract at 90 bar contained nearly same amount of triterpenes but less sugars than at 180 bar as weaker signals were observed between 3 ppm and 5 ppm. Therefore, based on the above findings, it is concluded that the optimal operating pressure was 90 bar.

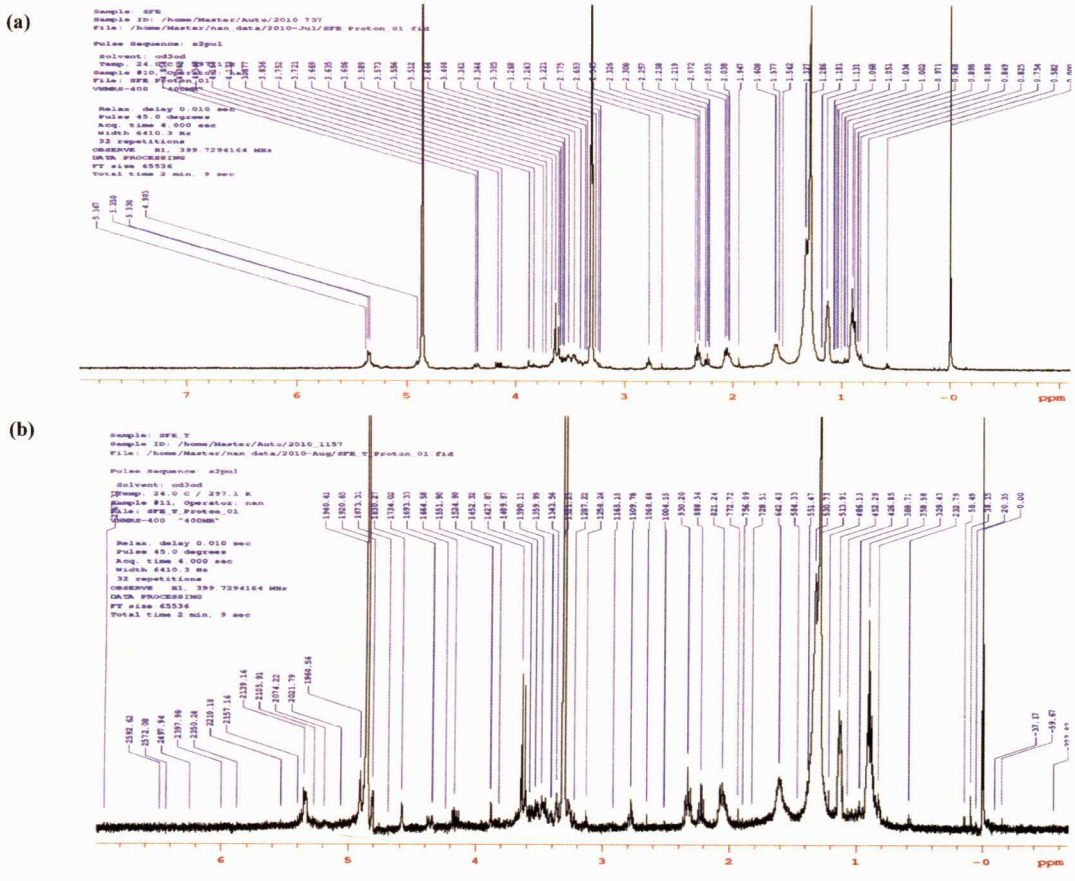


Figure 5-12 NMR spectrum of effect of pressure on extract component from *Acacia* plant. (a) 90 bar; (b) 180 bar

5.5.6 ANOVA Test

In statistics, analysis of variance (ANOVA) is a collection of statistical models, and their associated procedures, in which the observed variance is partitioned into components due to different sources of variation [29, 30]. In this study, ANOVA analysis was conducted at 95% confidence level to test the comparable factors (MeOH, T and P) in SFE process. As $P<0.0000$, MeOH had the most significant

effect on both IC_{50} and recovery, and operating temperature took the second place. However, operating pressure had the least effect because $P>0.05$.

5.5.7 Growth Inhibitory Activity of Extracted Compounds on GM3348

MTT assay was carried out to assess the growth inhibitory activity of the extract on human skin fibroblast cells GM3348 as an example of normal cell line. This study allowed determining whether extracted saponins can selectively perform cytotoxic effect on cancer cell line and with low effect on normal cells.

The growth inhibitory activity of extract from both SFE and MeOH is shown in Figure 5-13. The values of IC_{50} were 371.28 μg/ml and 42.77 μg/ml, respectively. The extract from MeOH had more than eight fold of growth inhibitory activity than that from SFE on normal human cell. It can be concluded that compounds extracted by modified CO_2 had less toxic effect on normal cell, and also had higher anticancer activity on cancer cell line. The advantages of SFE were not only at efficiency, cost, environmental consideration, but also its product had less effect on normal cell.

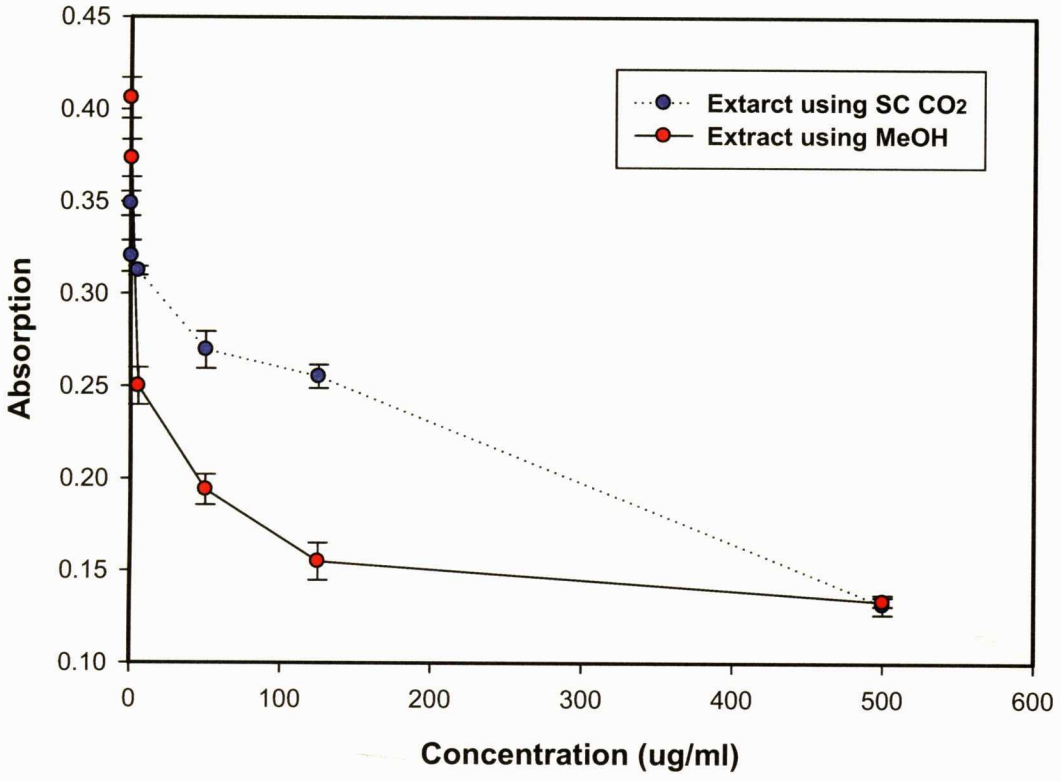


Figure 5-13 Growth inhibitory activity of extract from SC CO_2 and MeOH on GM3348

5.5.8 NMR Analysis for Comparison of Extract using MeOH and SCFs

NMR analysis was conducted to compare the extract using organic solvent extraction and supercritical fluid extraction at each optimal condition. It is desirable to obtain the information of the difference in chemical structure related to biological activity and extraction for both systems.

The NMR spectrums are shown in Figure 5-14. The signals between 0.5 and 2 ppm typically belong to triterpenes [142, 156, 171-174] and signals between 3 and 5 ppm presented sugars [175-177]. The extract from MeOH was complicated on the content of compounds, which had strong signals between both 0.5-2 ppm and 3-5 ppm. On the contrary, the extract from CO_2 modified by 5 mol% MeOH at 31 °C and 90 bar only have few peaks between 0.5 and 2 ppm and some peaks between 3 and 5 ppm. There was a strong signal at around 1.3 ppm, which means the present of large amount of triterpenoids saponins may contribute to biological activity.

From the spectra, both extracts were mixture and the one from SCFs contained selective compounds, which exhibited higher biological activity. It is concluded that supercritical fluid extraction was more efficient on isolation of active triterpenoids saponins from *A. victoriae* that demonstrated anticancer activity.

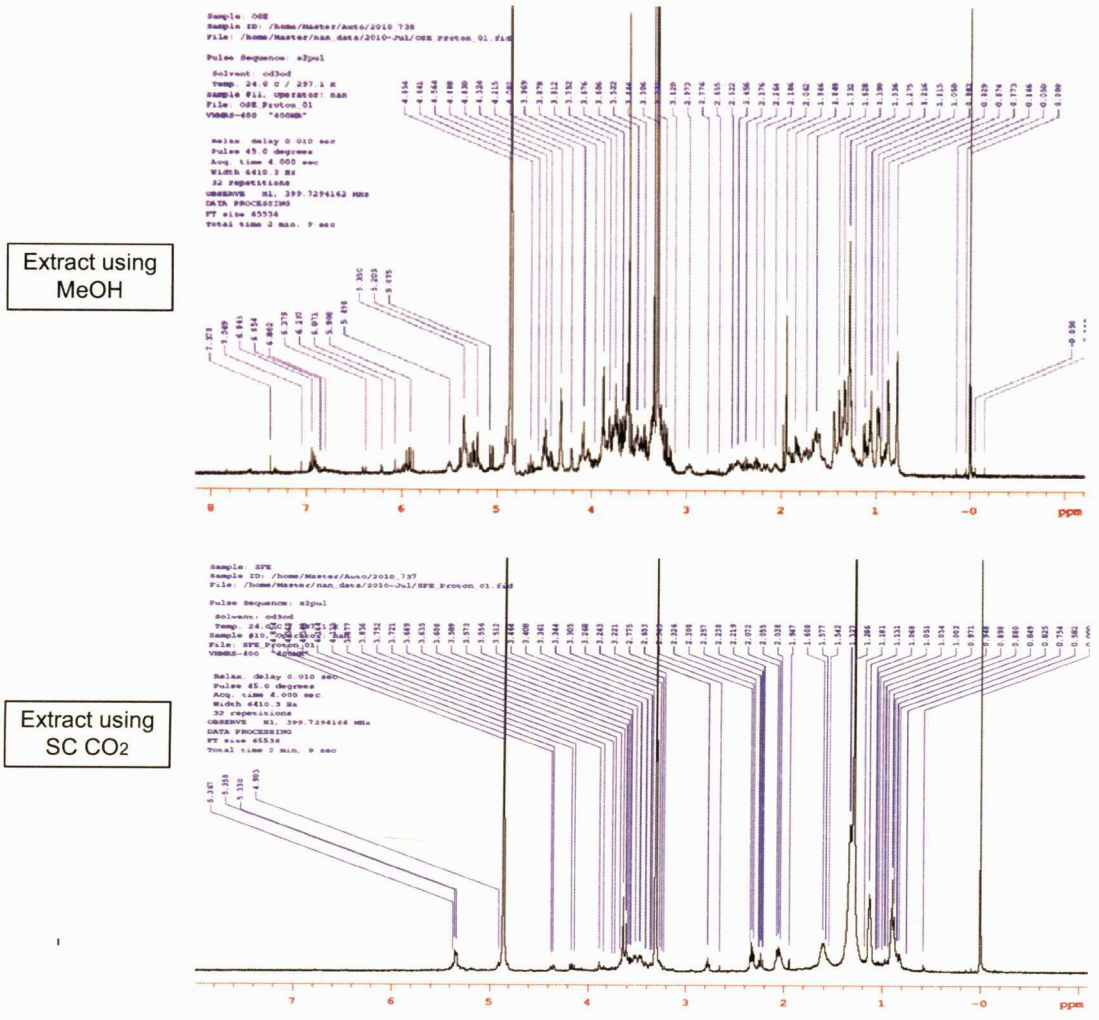


Figure 5-14 NMR spectra of extract using MeOH and SC CO_2

5.6 Summary

An environmentally friendly method was developed to extract active components, especially triterpenoids saponins, from *A. victoriae*. This study is the first attempt to use supercritical fluid extraction as an alternative method to replace conventional solvent extraction from *Acacia* plant. SFE was efficient for the extraction of triterpenoids saponins from *A. victoriae* that have anticancer activity. SFE is rapid,

and highly efficient for the selective extraction of active compounds from *A. victoriae*. The fractions obtained can be subjected to further purification to obtain pure single components to serve as reference standards for the purpose of standardization and quality control of commercial product preparation. The optimum conditions for the extraction of anticancer active compounds within the conditions examined, was 31 °C, 90 bar using 5 mol% MeOH. Other parameters such as particle size of samples, fluid flow rate, etc., may require further optimization.

6. Conclusion and Recommendations

Acacia victoriae is commonly used in Australia as a health supplement because of its medicinal value. Commercial products have been developed using different parts of this plant; Acacia seed powder has been used as a flavor. Chemical and pharmacological investigations conducted during the last decade on *A. victoriae* demonstrated the presence of active compounds that have therapeutic effects. These data corroborate the potential of using extracts from Acacia plant as nutraceutical and even pharmaceutical formulation for prevention and treatment of cancer.

The active compounds in Acacia plant are various types of triterpenoids saponins. These groups of compounds are very complex and because of similarity in molecular structure, their separation is very time consuming, involving many stage and large amount of organic solvents. Since the demand for *A. victoriae* as a health enhancer is increased, one of the urgent issues is to use a less toxic solvent in extraction and purification of this active compound. Recently, many new technologies have gradually been introduced in the modernization and development of traditional herbal medicines. Undoubtedly, supercritical fluid extraction using CO_2 is highly promising. Supercritical CO_2 has many advantages over conventional extraction methods, for example, low temperature, high selectivity in extraction and environmental friendliness. However, there has been no report on the application of SFE in the extraction of *A. victoriae*. Therefore, in this study, a methanol modified SFE process was explored and developed to extract triterpenoids saponins from *A. victoriae*. The aim of this project was to investigate the feasibility of extracting triterpenoids saponins selectively from Acacia plant using SFE. Furthermore, conventional

multistage fractionation was used to separate extract based on their different polarities and assess the biological activity.

TLC and proton NMR analysis were performed to identify the characteristic of extracted group. *In vitro* MTT assay was conducted to determine the cytotoxic effect of the extract on A2780 cell line that is ovarian cancer cell.

In the study of conventional organic solvent extractions, crude triterpenes were isolated from MeOH and further fractionated by using solvents with different degree of polarity. The results of TLC and NMR analysis demonstrated that triterpenoids saponins extracted from *A. victoriae* were relatively polar compared with TN-1 saponins. NMR spectrum confirmed the presence of various types of triterpenes in extracts from all fractions. However, proton NMR used in this study was unable to determine the molecular structure of these saponins. Addition of several stages of fractionation using liquid chromatography substantially increased the cost of process, and the result of this study demonstrated it eventually did not enhance the cytotoxic activity of separated fractions. It is, therefore, not necessary to further fractionate saponins for the production of an anticancer formulation. It is confirmed that crude triterpenoids saponins extracted from *A. victoriae* demonstrated higher cytotoxic activity than fractions obtained by liquid-liquid partitioning. Various organic solvent systems were used to extract active compounds from *A. victoriae*. Methanol was superior compared with other organic solvents for extraction active compounds. MTT assay for inhibition activity on cancer cell line was used; its IC_{50} was 19.24±0.32 μg/ml, which was 7 fold higher than using extract from ethyl acetate.

SFE was efficient for the extraction of triterpenoids saponins that have anticancer activity from *A. victoriae*. By varying the methanol mole fraction, temperature and pressure of the SFE process, higher selectivity and yield of the products were attained. The optimum conditions for the extraction of anticancer active compounds within the conditions examined, was 31 °C, 90 bar using 5 mol% MeOH, and the IC_{50} value at this condition was 0.05±0.03 μg/ml. A higher selectivity and anticancer activity of the active compounds were obtained when using SFE in comparison with conventional extraction methods. Extract from SFE (IC_{50} =371.28 μg/ml) was approximately 9 fold less toxic to human cell than extract from methanol (IC_{50} =42.77 μg/ml). NMR analysis indicated the presence of larger amount of triterpenoids saponins in extract by SFE than MeOH extract, which confirmed that the extract from SFE contained selective groups of triterpenoids saponins that contributed to higher biological activity. On the contrast, the extract from MeOH was more complicated and had larger sugar content than the one from SFE. The results suggest that supercritical CO_2 technology could be effectively used for the extraction of active compounds from *A. victoriae*.

On the basis of this work, a series of suggestions were proposed for the future work to further enhance the efficiency of SFE and obtain a deep understanding on the biological anticancer mechanism of triterpenoids saponins from *A. victoriae*.

Firstly, it is recommended to conduct further and determine the molecular structure of triterpenoids saponins from *A. victoriae*. Purification and identification combined with LC-MS, ^{13}C NMR and 2D NMR are recommended to be conducted to identify these active compounds. Other cancer cell lines can be involved in the following study to compare with A2780 in terms of anticancer activity.

Secondly, more biological technologies, such as enzyme linked immunosorbent assays (ELISAs) and radioimmuno assays (RIA) could be used to investigate the biological anticancer mechanism of triterpenoids saponins. Meanwhile, *in vivo* studies can be involved in various animal models. Other biological activity of extracted saponins such as anti-inflammatory effect can also take into consideration for future study.

Further optimization should be considered in future work for SFE to tune the physio-chemical properties of SCF for the extraction and fractionation of saponins. The effect of other modifiers such as ethanol, water and their composites, pressure, temperature, particle size of matrix, mixing protocols, static and dynamic mode of extraction on the extraction and fractionation efficiency of SCF should be investigated thoroughly. The optimum conditions can be used for the extraction of saponins from other plants.

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Appendices

Appendix A: MTT assay protocol description

Protocol name ..... MTT@ 600nm (1.0s,shake)

Protocol number ..... N/A

Name of the plate type ..... Generic 8x12 size plate

Number of repeats 1

Delay between repeats ..... 0 s

Measurement height ..... 3.00 mm

Protocol notes .....

Shaking duration ..... 60.0 s

Shaking speed ..... Normal

Shaking diameter ..... 0.10 mm

Shaking type ..... Double orbit

Repeated operation ..... Yes

Name of the label ..... MTT@600

Label technology ..... Photometry

CW-lamp filter name P600

CW-lamp filter slot A7

Measurement time ..... 1.0 s

Absorbance Mode ..... Visible

Excitation Aperture ..... Normal

Plate map of plate

A	M	M	M	M	M	M	M	M	M	M	M	M
B	M	M	M	M	M	M	M	M	M	M	M	M
C	M	M	M	M	M	M	M	M	M	M	M	M
D	M	M	M	M	M	M	M	M	M	M	M	M
E	M	M	M	M	M	M	M	M	M	M	M	M
F	M	M	M	M	M	M	M	M	M	M	M	M
G	M	M	M	M	M	M	M	M	M	M	M	M
H	M	M	M	M	M	M	M	M	M	M	M	M

Appendix B: ANOVA test

Ic50

Number of obs = 36 R-squared = 0.9989
Root MSE = 1.13069 Adj R-squared = 0.9984

Source	Partial SS	df	MS	F	Prob > F
Model	28757.1337	11	2614.28488	2044.87	0.0000
MeOH	5219.5855	3	1739.86183	1360.90	0.0000
T	2348.52197	5	469.704393	367.40	0.0000
P	.636993	3	0.212331	0.17	0.9182
Residual	30.6830287	24	1.27845953		
Total	28787.8167	35	822.50905		

Recovery

Number of obs = 36 R-squared = 0.9912
Root MSE = .023577 Adj R-squared = 0.9871

Source	Partial SS	df	MS	F	Prob > F
Model	1.49882993	11	0.136257267	245.11	0.0000
MeOH	0.827676565	3	0.275892188	496.30	0.0000
T	0.064068262	5	0.012813652	23.05	0.0000
P	0.035832202	3	0.011944067	21.49	0.0000
Residual	0.013341496	24	0.000555896		
Total	1.51217143	35	0.043204898		