CHEMICAL STUDY OF BIOACTIVE CONSTITUENTS FROM *TRICHOSANTHES CUCUMERINA* ROOT AND FRUIT JUICE

SUMONTHIP KONGTUN

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Thesis
Entitled

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CHEMICAL STUDY OF BIOACTIVE CONSTITUENTS FROM *TRICHOSANTHES CUCUMERINA* ROOT AND FRUIT JUICE

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ABSTRACT

Trichosanthes cucumerina L. seeds have been used as remedies for dysentery, coughs and as an emetic. A previous phytochemical investigation in cucurbitaceous plants revealed that the triterpenes have interesting bioactivities, For example, cucurbitacins elicit catharsis and cytotoxicity, and bryonolic and bryononic acids possess anti-inflammatory property. This research aimed to find the bioactive constituents from *T. cucumerina* root and to establish a validated method for quantitative High-performance liquid chromatography (HPLC) analysis of the isolated cucurbitacins.

The dichloromethane extract of the root was subject to chromatographic separation, which the isoprenoid derived compounds could be isolated. The structures were identified using NMR spectroscopy. They were bryonolic acid (SK1), chondrillasteryl-3β-*O*-glucopyranoside (SK2), bryononic acid (SK3), cucurbitacin B(SK4) and dihydrocucurbitacin B (SK5). The dichloromethane root extract, the isolated compounds and the fruit juice were tested for antimalarial activity. Compound SK4 and the fruit juice were antimalarial active with IC_{50} value of 0.625 μg/ml and 0.089 μg/ml, respectively. In addition, compound SK1 was proved as an antipapilloma viral agent with the ED_{50} value of 1.5 ng/ml. This is the first report on the antiviral activity of bryonolic acid, which is the main constituent in *T. cucumerina* root.

The quantitative HPLC analysis was performed on the isolated cucurbitacin B (SK4). This analysis used the 5 μm Hypersil BDS C18 column (250 x 4.6 mm, i.d.), the solvent system of acetonitrile-water (40:60) and a UV detector at 210 nm. The HPLC analytical method was validated according to the USP 26 requirement.

KEY WORDS : *TRICHOSANTHES CUCUMERINA*/ CUCURBITACIN/ BRYONOLIC ACID/ BRYONONIC ACID/ ACTIVITY

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การศึกษาเคมีของสารที่มีฤทธิ์ทางชีวภาพจากรากบวบขมและน้ำคั้นจากผลบวบขม (CHEMICAL STUDY OF BIOACTIVE CONSTITUENTS FROM *TRICHOSANTHES CUCUMERINA* ROOT AND FRUIT JUICE)

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บทคัดย่อ

บวบขม (*Trichosanthes cucumerina*) เมล็ดมีสรรพคุณรักษาโรคบิด ขับเสมหะ และทำให้อาเจียน ในการศึกษาด้านพฤกษเคมีในพืชวงศ์ Cucurbitaceae พบว่า สารที่แสดงฤทธิ์ทางชีวภาพเป็นสารจำพวกไทรเทอร์ปีนส์ ได้แก่ cucurbitacins แสดงฤทธิ์ต้านมะเร็ง bryonolic และ bryononic acids มีฤทธิ์ต้านอักเสบ ในงานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาองค์ประกอบทางเคมีของสารที่แสดงฤทธิ์ทางชีวภาพ ที่พบในสารสกัดไดคลอโรมีเทนจากส่วนรากและน้ำคั้นจากผล รวมถึงพัฒนาวิธีวิเคราะห์ปริมาณสารสำคัญ กลุ่ม cucurbitacins ในสารสกัดและในน้ำคั้นจากผล ไทรเทอร์ปีนส์ 5 ชนิดที่แยกได้จากสารสกัดไดคลอโรมีเทนจากรากในงานวิจัยนี้ ได้แก่ bryonolic acid (SK1), chondrillasteryl-3β-*O*-glucopyranoside (SK2), bryononic acid (SK3), cucurbitacin B (SK4) และ dihydrocucurbitacin B (SK5) การทดสอบฤทธิ์ต้านเชื้อมาลาเรียของสารสกัดไดคลอโรมีเทนจากส่วนราก สารสกัด และน้ำคั้นจากผล พบว่า มีค่า IC_{50} 0.625 ไมโครกรัม/มิลลิลิตร และ 0.089 ไมโครกรัม/มิลลิลิตร ตามลำดับ นอกจากนี้ ยังพบว่า SK1 ซึ่งเป็นสารหลักที่พบในสารสกัดราก แสดงฤทธิ์ต้านไวรัสปาปิโลมาซึ่งเป็นสาเหตุสำคัญของมะเร็งที่กล่องเสียง (laryngeal cancer) โดยมี ED_{50} 1.5 นาโนกรัม/มิลลิลิตร นี้เป็นรายงานฤทธิ์ต้านไวรัสปาปิโลมาครั้งแรกของสาร bryonolic acid

ในการพัฒนาวิธีวิเคราะห์ปริมาณ cucurbitacin B (SK4) พบว่า สภาวะที่เหมาะสมในการวิเคราะห์ประกอบด้วยคอลัมน์ที่บรรจุ Hypersil BDS C18 ขนาด 5 ไมโครเมตร (250 x 4.6 มิลลิเมตร) อะซิโตไนไตล์และน้ำในอัตราส่วนคงที่ 40:60 เป็นตัวทำละลายเคลื่อนที่ และ UV detector ที่ความยาวคลื่น 210 นาโนเมตร ได้ใช้เกณฑ์ของ USP 26 ในการประเมินความถูกต้องและเที่ยงตรงของวิธีวิเคราะห์ ซึ่งวิธีวิเคราะห์ที่ผ่านการประเมินตามเกณฑ์ของ USPให้ผลวิเคราะห์ปริมาณสาร cucurbitacin B ในน้ำคั้นจากผลและสารสกัดที่ถูกต้องและเที่ยงตรง

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LIST OF ABBREVIATIONS

cm	=	centimeter(s)
$^{13}C\{^{1}H\}$-spectrum	=	^{13}C ^{1}H-broadband decoupled-NMR
$CDCl_3$	=	deutero chloroform
CD_3OD	=	deutero methanol
COSY	=	correlated spectroscopy
DEPT	=	distorsionless enhancement by polarization transfer
DMSO	=	dimethyl sulfoxide
ED_{50}	=	dose level giving 50% effectiveness
EIMS	=	electron impact mass spectrum
Fig.	=	figure
g	=	gram
g	=	gravity
HMBC	=	hetero-multiple broadening coherence
HMQC	=	hetero-multiple quantum coherence
HPLC	=	high-pressure liquid chromatography
h	=	hour(s)
IC_{50}	=	concentration giving 50% inhibition
i.d.	=	inner diameter
IR	=	infrared
J(Hz)	=	coupling constant (hertz)
kg	=	kilogram(s)
l	=	liter(s)
LPLC	=	low- pressure liquid chromatography
MPLC	=	medium-pressure liquid chromatography
mg	=	milligram
μg	=	microgram
min	=	minute(s)

LIST OF ABBREVIATIONS

(continued)

ml	=	milliliter(s)
mm	=	millimeter(s)
m.p.	=	melting point
M_R	=	relative mass
m/z	=	mass to charge ratio
MHz	=	megahertz
ng	=	nanogram
nm	=	nanometer
NMR	=	nuclear magnetic resonance spectroscopy
No.	=	number
ppm	=	part per million
Q	=	quartet
RDA	=	Retro-Diel-Alder
Rf	=	mobility relative to front
s	=	symmetrical
as	=	asymmetrical
TLC	=	thin-layer chromatography
UV	=	ultraviolet
$^{\circ}C$	=	degree Celcius
λ	=	wavelength
ν	=	stretching vibration
m	=	multiplet
δ	=	chemical shift (NMR)
δ	=	deformation vibration (IR)
sn	=	stereospecific numbering
S, *s*	=	singlet

LIST OF ABBREVIATIONS

(continued)

D, *d*	=	doublet
T, *t*	=	triplet
Q, *q*	=	quartet
br, *s*	=	broad singlet
ax	=	axial
eq	=	equatorial

CHAPTER I
INTRODUCTION

Trichosanthes cucumerina L.,(Thai name: Buap khom) belongs to the family Cucurbitaceae and has been used in Thai folklore medicine. The seed was an emetic, expectorant and antidysenteric. It was a substitute for ipecauanha. The fruit was purgative. The dried fruit pulp was sliced into pieces and smoked to relieve the sinusitis. The fresh fruit pulp was used as antidandruff shampoo (1).

The previous study revealed that the immature fruit contained cucurbitacins and phytosterols as free and glycoside forms. The main cucurbitacin was cucurbitacin B. The aqueous extract of the fruit and the isolated cucurbitacins exerted a very strong cytotoxicity against nasopharynx carcinoma (KB cell) with the ED_{50} value of < 0.1 μg/ml. (2). Cucurbitacin B and dihydrocucurbitacin B were isolated from the fruit juice. The MIT colorimetric assay for cytotoxicity of cucurbitacin B, dihydrocucurbitacin B and a mixture of cucurbitacin B and dihydrocucurbitacin B showed the ED50 against breast cancer cell line (SK-BR-3) at 0.05 μg/ml, 0.40 μg/ml and 0.48 μg/ml, respectively (88, 89).

The bioactivities of cucurbitacins have been reviewed (41). They possessed hepatoprotective, antitumour, antiinflammatory, antifertility, antimicrobial, antihelminthic properties and so on. The ethonomedicinal value of the plant has guided the bioactivity work in the direction of antimalarial and antipapilloma virus.

Malaria is the still considned as important parasitic infection in the world. It is one of the planet's diseases and one of the leading causes of sickness and death in the developing world. According to the World Health Organization (WHO) there are 300 to 500 million clinical cases of malaria each year resulting in 1.5 to 2.7 million deaths. Children aged one to four are the most vulnerable to the infection. Malaria is the the main cause of the deaths of African children under the age of five. Malaria, which is characterised by intermittent fevers, is a disease caused by protozone parasites of the genus *Plasmodium*. In Thailand, malaria is the most prevalent along

the Thai-Myanmar and Thai-Cambodia borders.It has been reported that malaria in Thailand is forest-related and is the most prevalent along the borders. In 2001, the overall malaria cases were 126,659. Among Thai cases, 46.3% was caused by *P. falciparum*. This disparity in the risk of acquiring malaria reflects the fact that the predominant species of malaria transmitted in urban areas (3).

Cancer, a major killer throughout human history, changed its group as humankind advanced industrially and technologically. Cancers of the mouth and pharynx account for 363,000 annual new cases worldwide and almost 200,000 deaths. They are 2.5 times more common in men than in women. The risk is similar in developed and developing countries; the age-standardized incidence rates are 13.5 versus 11.5 for males and 3.0 versus 5.1 for females in developed and developing countries, respectively.

Cancers of the mouth and pharynx are a heterogeneous group of neoplasms, among which nasopharyngeal cancer (NPC) has the least common with the others. Areas of high and intermediate risks for NPC are Southeastern China and Northern Africa. Genetic susceptibility in these high-risk populations is strongly suspected to interact with known environmental causes. The lower survival rate estimated for China and Eastern Asia (lower than that of Africa) is partly the result of the fact that at least 70% of these cancers are localized to the pharynx and nasopharynx, and cancers in these areas have a poorer prognosis. Other cancer causing agents, such as some viruses, by accelerating the rate of cell division (4).

Among the interesting bioactivities antimalarial and antiviral properties of the root extract, the fruit juice and the isolated compound have been nvestigated in this study. The isolation methods and the validated quantitative analysis of the isolated compound have been established, as well as the structure elucidation using NMR nuclear magnetic resonance spectroscopy.

CHAPTER II
OBJECTIVE

1. To isolate and identify the cucurbitacins from *Trichosanthes cucumerina* root and fruit juice
2. To establish the HPLC analytical method of the isolated cucurbitacin
3. To verify the bioactivity of the isolated compounds from the root and the fruit juice
4. To determine the amount of the main cucurbitacin in the root extract and the fruit juice

CHAPTER III
LITERATURE REVIEW

1. The Family Cucurbitaceae

1.1 Botany

The plants in Cucurbitaceae family compose of many genera, occuring mostly in the tropic and the temperate regions. The Cucurbitaceae family comprises about 110 genera and about 640 species(5). Genera include *Cucurbita* (5 spp.), *Cucumis* (25 spp.), *Ecballium* (15spp.), *Citrullus* (3 spp.), *Luffa* (6 spp.), *Bryonia* (4 spp.), and *Momordica* (45 spp.) (5). The plants are woody or herbaceous mostly with climbing or trailing stems bearing tendrils and often arising from a tuberous rootstock. They are rarely without tendrils (6). The leaves are alternate, simple and palmately veined or penately compound with 3-25 leaflets. When they are simple, they are usually very variable and petiolate. The flowers are usually unisexual with three to five petals. These are free or united into a regular corolla. The stamens are basically five, alternate with the petals; staminodes are often present in the pistillate flowers. The ovary is usually inferior, generally unilocular, composed of one to five (most often three) united carpals. The ovules are anatropous and range from one to many. The style is single with usually three stigma-lobes. The fruits are dry or fleshy capsules, berries or hard-shelled pepos; they are indehiscent or dehiscent by valves, an operculum. Slits or apical pores (6).

Early chemical studies revealed that, in addition to a number of tetra-and pentacyclic triterpenes, the toxic bitter principles, cucurbitacins, a group of often highly oxygenated tetracyclic compounds with a unique carbon skeleton and almost always a carbonylgroup in ring C, could be considered as a taxonomic character in the Cucurbitaceae (7). However, the cucurbitacin have more recently found in certain members of other families, including the Cruciferae, Begoniaceae, Scrophylariaceae, Euphorbiaceae(8), Rosaceae(9) Elaeocarpaceae(2,10), Datiscaceae(2), Primulaceae (11) and Liliaceae (12).

1.2 Traditional use

In Thailand some of cucurbitaceous plants are edible, e.g. *Coccinia indica* Wight. and Arm.(ivy gourd), *Cucurbita maxima* Duchesne.(pumpkin), *C.pepo* L.var *Ovifola bailey*, *Cucumis sativus* L. (cucumber), *Citrullus vulgaris* Schard. (water melon), etc. Some are very bitter and not edible, e.g. *T. cucumerina*, *Bryonia dioica* Jacq. and *Ecballium elaterium* L. The traditional uses of the Cucurbitaceae in Thailand ranged from cathartic, anthelmintic, lice-killing, anti-malarial, antidiabetic, antiinflammatory to wart removing (2).

The constituents in the Cucurbitaceae have been known for a long time for their purgative action (2). The plants produced glycosides in their tissues, such as squirting cucumber and *E. elaterium* L., which release a purgative glycosidic compound, elaterin. Currently the interest in Cucurbitaceous plants persists for several reasons. The sporadic occurrence of toxic principles in cultivated species constitutes a human health harzard and causes cattle mortality (13). In addition the search for substances with antineoplastic (2,14,15) and antidiabetic (16) properties has been carried out in this family. The Chinese use the seeds and the fruit peel of *T. kirilowii* Maxim., and *T. multiloba* Mig., as tonics and ingredients in astringent remedies. There were over twenty plant species in the publication (17).

1.3 *Trichosanthes cucumerina*

Trichosanthes, a genus of climbing herbs in Cucurbitaceae family, are found in Southeast Asia and Australia. There are more than thirty species in this genus (17). Six species of *Trichosanthes* are found in Thailand (Table 1).

Table 1 *Trichosanthes* plants in Thailand (1)

Plant	Local name
*T. anguina*L.	Buap nguu
T. bracteata (*T.tricuspidata)*	Kradueng chaang phueak, Khi ka yai
T. cordata	Khi ka khao
T. cucumerina	Buap khom
T. integrifolia (Gymnopetalum integrifolia)	Khi ka daeng
T. wawraei	Khi ka din

T. cucumerina **(**Local names **:** Nom phichit, Buap Khom(central), Manoi chaa (northern)(18). An annual, climbers; tendrils trifid. Leaves deeply 5 lobed, toothed. Flowers irregularly divied into narrow, short lobed, small, white, dioecious; male flowers in recemes; female flowers solitary. Berries up to 3 in long, spindle sharp, smooth, orange or scarlet coloured when ripe, green and striped when immature.

The plants are wildly grown on the tree along the river throughout Thailand. It has been as cardiotonic, laxative, alterative, and antifebrile; its decoction is given in bilious fever as febrifuge and laxative (2). The leaves are febrifuge, laxative and emetic; a decoction of the leaves made with the addition of coriander is given in bilious fever; the leaf juice is used as an emetic.

The leaf juice is locally applied as a liniment if the liver is congested; in remittent fever it is applied over the whole body; the juice is rubbed over the head for the cure of alopecia. The root is purgative and tonic; its juice is a strong purgative and has a strong irritant action on the gastrointestinal system. An infusion of the dried fruit is an aperient; it also aids digestion. The seed are antifebrile and anthelmintic.

Chemical composition : The fruit has been previously found to contain cucurbitacin B, dihydrocucurbitacin B, dihydrocucurbitacin D, 24ξ-ethyl-5α-cholesta-7-en-3β-ol, 24β-ethyl-5α-cholesta-7,22-dien-3β-ol, 24β-ethyl-5α-cholesta-7,22,25-

trien-3β-ol, 3-*O*-β-D-glucopuranosyl-cucurbitacin B, 3-*O*-β-D-glucopuranosyl-(6′→1″)-octadeca-nost-24ξ-ethyl-5α-cholesta-7,22-dien and sphingoglycolipid (2). The roots were previously found to contain SC1, SC2, SC3, and SC4 (3β-hydroxy-olean-13(18)-ene-28-oic, 24β-ethyl-cholest-7,22-diene-3β-ol, 24ξ-ethyl-cholest-7-en-3β-ol and 3-oxo-olean-13(18)-ene-30-oic, respectively (19).

Bioactivities : The fruit juice showed antimicrobial activity against gram positive and acid fast bacterial and (*in vitro*) cytotoxic activity (19). The main cucurbitacin was cucurbitacin B. *T. cucumerina* root has contained two pentacyclic triterpenes, i.e. 3β-hydroxy-olean-13 (18)-ene-28-oic (I) and 3-oxo-olean-13 (18)-ene-30-oic (II) and two phytosterols, i.e. 24β-ethyl-cholest-7,22-diene-3β-ol (III) and 24ξ-ethylcholest-7-ene-3β-ol (IV). The above four compound have not shown antimicrobial activity, only the pentacyclic triterpene II and the crude alcohol extract of the root possess the in vitro cytotoxic activity against the leukemia cell culture (P388) with ED50 60 μg/ml and 0.3 μg/ml, respectively. The cucurbitacins and phytosterols are isolated from the fruit. They are: cucurbitacin B (V), dihydrocucurbitacin B (VI), dihydrocucurbitacin D (VII), phytosterol III, IV and 24β-ethyl-5α-cholest-7,22,25-triene-3β-ol. The cucurbitacin B and dihydrocucurbitacin B are tested for the cytotoxicity. The MTT colorimetric assay for cytotoxicity are carried out. It indicates the highly toxic action of cucurbitacin B and dihydrocucubitacin B against breast cancer cell line (SK-BR-3) with ED50 0.05 μg/ml and 0.40 μg/ml, respectively (17).

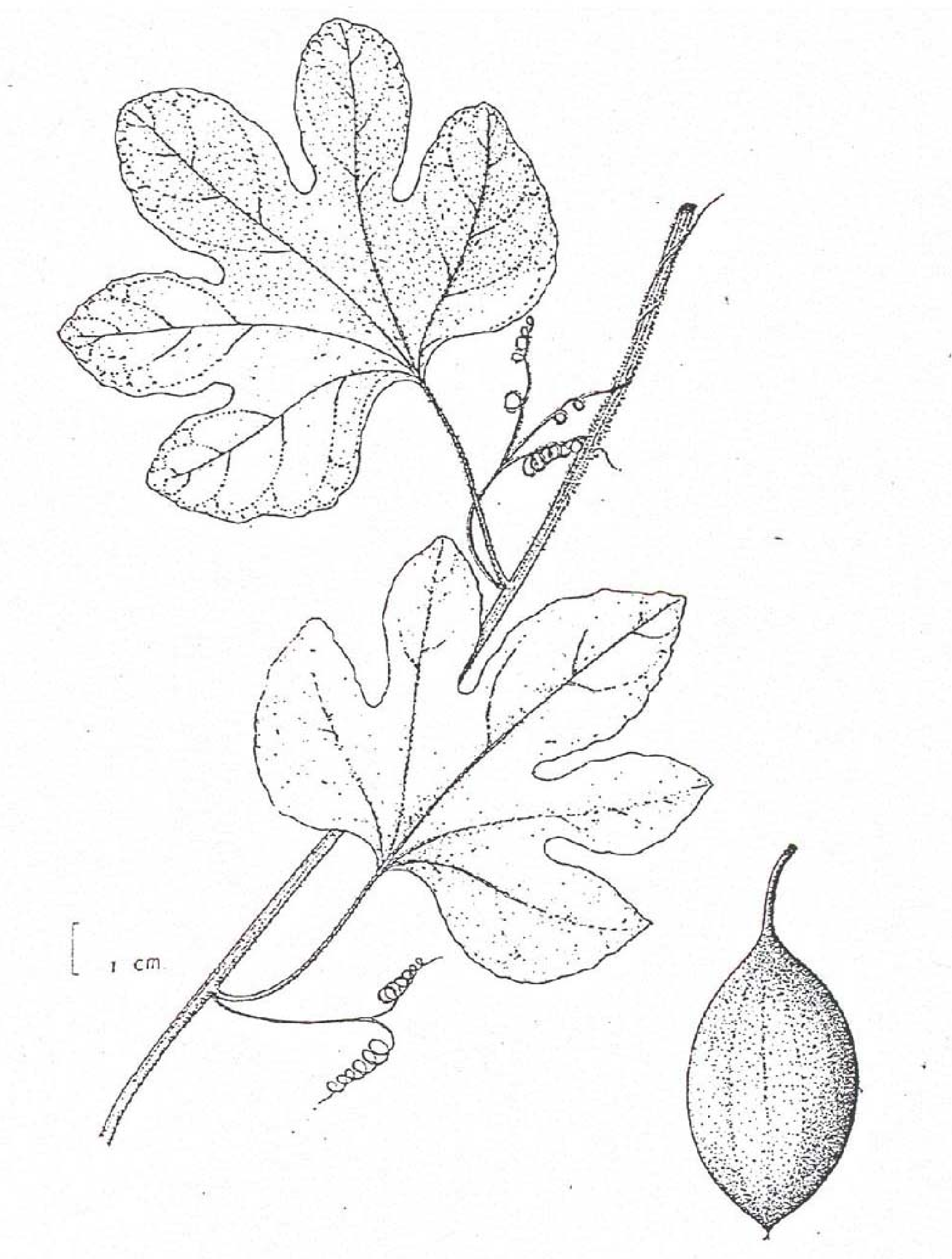


Figure 1 *Trichosanthes cucumerina* L.(18)

2. Extraction and Isolation of isoprenoid derived compounds.

The extraction method normally depends on the chemical constituents, the texture and the water content of the plant material being extracted. Various extraction methods have been described for sterols from plant material. Generally, the dried plant powder is continuously extracted in a Soxhlet apparatus with a range of solvents, starting with petroleum ether and chloroform (for lipids and terpenoids) and then using ethyl acetate and alcohol (for more polar compounds). This method is useful when working on the gram scale (20). Moreover, the MCI gel CHP20P, the modified resin, can be used as partition chromatography to fractionate the phytosteryl glycosides using methanol as an eluant (21). The chloroform extract of leaves is performed on the Sephadex LH-20 column, and the chlorophyll is removed by eluting the column with cholroform or chloroform-methanol (9:1) (22, 23, 24).

The isolation is usually carried out using silica gel column chromatography as the first column, and gradually increasing the polarity of the eluting solvents. The solvent system for phytosterols includes a mixture of hexane and ethyl acetate while that for phytosteryl glycosides is a mixture of chloroform and methanol. A clearly related phytosterols are occasionally found in particular plant tissue, and the elaborated separation procedures is needed.

2.1 Chromatographic material (24)

2.1.1 Silica gel

Silica gel is a polar sorbent derived from polymerization of silicic acid. It has been widely used as a stationary phase for conventional chromatographic separations of relatively low polar compounds. The mode of separation is based on adsorption onto its polyhydroxyl active site. The gel itself may be rather acidic (pH 4-5) and it is then suitable for the separation of acidic or neutral compounds. Less polar solvents such as hexane, diethylether, chloroform, and ethylacetate can be used as a mobile phase. Silica gel is not recommended for separations of very polar compound since irreversible adsorption may occur.

2.1.2 Sephadex LH-20

Sephadex LH-20 is a hydroxypropyl derivative of a cross-linked dextran gel modifield from sephadex G-25. It can be used with both aqueous and

organic solvents. It acts as molecular sieve for the removal of high molecular mass impurities and oligomeric materials from the sample to ease further purification. Not only is Sephadex LH-20 column chromatography useful as a preliminary fractionation step but it is employed also as the very last step in the isolation process to remove the traces of solid salts and other extrageneous matter. In fact, the interactions of solutes are not on the molecular size. The affinity of the solutes towards the gel depends largely upon the solvent and may therefore be suppressed by the proper choice of the conditions or utilized in the separations which are not based on the molecular size difference.

Sephadex LH-20 is commonly employed in the separation of hydrophilic compounds from various plant extracts, chiefly using aqueous methanol or methanol as an eluent. Other solvent such as dichloromethane, can also be used provided that they can solved the substances to be separated. Sephadex LH-20 is frequently used since no material is lost after separation.

2.1.3 MCI gel CHP 20P

MCI gel CHP 20P is a highly porous styrene-divinylbenzene copolymer carrying macropores. Due to its large surface area, this resin is a very effective adsorbent. As it is a nonpolar polymeric resin, it is suitable for the adsorption of nonpolar materials from polar solvent solutions. It is used particularly in industry, e.g. adsorption of vitamins, enzymes, steroids and other substances from fermentation liquors. Nowaday this resin plays an important role in the separation of natural products of high polarity, such as saponins or polar flavonoids. The mode of separation is similar to that of reversed phase chromatography with water and increasing amounts of methanol as solvent.

2.2 Separation method with low pressure column chromatography (LPLC)

The conventional column liquid chromatography is often not efficient enough to resolve substances of closely related chemical structures. Low pressure liquid chromatography (LPLC) is among the popular techniques employed for solving the difficulties. The pressure used in LPLC is about 2-4 bars for prepacked lobar columns with different packing materials and different particle size which are commercials available. A methanol-water mixture is the most common solvent system

used with reversed phase column. Lobar column is generally applied at the final purification step. It can accommodate the separation of gram quantities with the resolution sometimes approaching that of high-pressure liquid chromatography (HPLC). The simplicity of set up and operation as well as the economic cost makes LPLC a widely used technique.

Individual sterols in a mixture can often be successfully isolated on the basis of interaction of silver ion with double bonds in the molecules. Separations are usually performed after the conversion into the steryl acetate by treatment with acetic anhydride-pyridine. The steryl acetate mixtures are chromatograhed on TLC plates of silica gel impregnated with 25% of silver nitrate (24).

Sterols with isolated double bonds absorb more weakly in the range 190-215 nm which permits the detection by UV-monitoring during HPLC separation of sterol mixtures. HPLC, especially reversed phase has proved to be the most advantageous separation of sterol mixtures. The developing solvent is generally based upon acetonitrile or methanol aqueous mixtures with varying additions of water (24).

The phytosterols content of the cucurbitaceous plants were investigated and summerized in Table 2 (21).

Thin-layer chromatography (TLC) was applied for the determination of the isolated phytosterols, and the isolations were accomplished with repeated column chromatography. The overview of TLC and column chromatography were summarized in Table 3-6.

Table 2 The phytosterol contents of some cucurbitaceous plants (21)

Plants	Sterols																							
	a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	p	q	r	s	t	u	v	w	x
Apodenthera undrilata Gray (seed)	+	+	+	+																				
Cucumis melo L. (melon) (seed)	+	+			+	+																		
Cucurbita digitata	+	+			+	+	+	+																
C. foetidissima HBK (baffalo gourd)	+	+			+	+																		
Lagenaria leucantha Rusby							+	+																
Luffa acutangula Roxb.							+	+																
T. japonica Regel.	+	+					+	+																
Coccinea grandis Voigt (ivy gourd)	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+								
Gynostemmma pontaphyllumt				+					+	+	+	+	+			+		+	+	+	+			
Makino (aerial parts)				+	+																			
Momordica cochinchinensis	+	+																				+	+	
Sicyos angulatus L. (bur. Cucumber)	+	+	+	+	+	+												+	+					
T. dioica	+	+																						

a = 24α-ethylcholest-7-enol
b = 24β-ethylcholest-7-enol
c = 24α-ethylcholesta-7,22-dienol (spinasterol)
d = 24β-ethylcholesta-7,22-dienol (chondrillasterol)
e = 24α-methylcholest-7-enol (stellasterol)
f = 24β- methylcholest-7-enol
g = 24α-ethylcholesta-5,22-dienol (stigmasterol)
h = 24β-ethylcholesta-5,22-dienol (poriferasterol)
i = 24α-ethylcholest-5-enol (sitosterol)
j = 24β-ethylcholest-5-enol (clionasterol)
k = 24α-methylcholest-5-enol (campesterol)
l = 24β-methylcholest-5-enol
m = 24α-methylcholesta-5,22-dienol
n = 24β-methylcholesta-5,22-dienol (brassicasterol)
o = 24β-ethylcholesta-5,22-dienol (clerosterol)
p = 24α-methylene-25-mehylcholest-5-enol
q = 24α-methylcholestanol (campestanol)
r = 24β-methylcholestanol
s = 24α-methylcholesta-7,22-dienol
t = 24β-methylcholesta-7,22-dienol
u = 24β-ethylcholesta-7,25-dienol
v = 24β-ethylcholesta-7,22,25-trienol
w = 24α-ethylcholesta-8,22-dienol
x = 24β-ethylcholesta-8,22-dienol

Table 3 TLC and column chromatography of phytosterols and triterpenes

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	hRf	Adsorbent	Solvents system	Detection	
spinasterol	silica gel	$CHCl_3$	30	Silica gel	$CHCl_3$	10% H_2SO_4	25
spinasteryl glycoside	silica gel	$CHCl_3$-MeOH 9:1	76	Sephadex LH20	$CHCl_3$-MeOH 9:1	10% H_2SO_4	25
sterol	silica gel	$CHCl_3$-EtoAc-Formic acid 48:48:4	87	Silica gel	Diethyl ether-$CHCl_3$-MeOH 20:10:1	UV light	27
4,4-dimethylsterol	silica gel	$CHCl_3$	27	-	-	-	27
4α-methyl sterol	silica gel	$CHCl_3$	22	-	-	-	27
4-dimethyl sterol	silica gel	$CHCl_3$	16	-	-	-	27
24S-ethyl-25,22-dien-3-yl-acetate	silica gel	$CHCl_3$	41	-	-	-	27
24-ethyl-25-oxo-26-norcholesta-5,23-dien-3β-yl acetate	silica gel	$CHCl_3$	28	-	-	-	27
Δ^7-stigmasteryl acetate	-	-	-	20%$AgNO_3$ silica gel	ether-hexane 1:49	-	28
α-spinasteryl acetate	-	-	-	20%$AgNO_3$ silica gel	ether-hexane 1:49	-	28
Δ^5-sterols and Δ^7-sterols	silica gel	Et_2O-C_6H_6 1:9	-	20%$AgNO_3$ silica gel	ether-hexane 1:49	-	29
mixture of 24-ethyl cholest-5-en-3β-ol and 24-ethylo-5α-cholest-7-en-3β-ol	$AgNO_3$ silica gel	CH_2Cl_2-CCl_4 1:5	-	20%$AgNO_3$ silica gel	ether-hexane 1:49	-	30

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	hRf	Adsorbent	Solvents system	Detection	
mixture of stellasteryl acetate and 24-ethyl-5α-cholesta-7,22-dien-3β-ol	$AgNO_3$ silica	CH_2Cl_2-CCl_4 1:5	56	-	-	10% H_2SO_4	30
24β-ethyl-5α-cholesta-7,25 (27)-dien-3β-yl acetate	$AgNO_3$ silica	CH_2Cl_2-CCl_4 1:5	49	-	-	10% H_2SO_4	30
24β-ethyl-5α-11-cholesta-7,22,25 (27)-trien-3β-yl acetate	$AgNO_3$ silica	CH_2Cl_2-CCl_4 1:5	10	-	-		30
sitosterol-3-O-α -D-riburono turanoside	silica gel	CH_2Cl_2- Et_2O	50	-	-		31
sitosterol-3-O-β -D-xylopyranoside	silica gel	CH_2Cl_2- EtOAc	6	-	-		31
sitosterol-3-O-α -D-xylonofuranoside	silica gel	CH_2Cl_2- EtOAc	15	-	-		31
steryl from cucurbitaceae	silica gel	n-hexane-EtOAc 6:1	41	-	-	UV (210 nm) Detector	31
steryl acetate	$AgNO_3$ silica	CCl_4 - CH_2Cl_2 5:1	28	-	-		32
Δ^7-sterols accompanied by saturated sterols	silica gel	n-hexane-EtOAc 6:1	-	-	-	UV (210 nm) Detector	33
Δ^7- sterols accompanied by Δ^8-sterols	silica gel	n-hexane-EtOAc 6:1	-	-	-		33

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	hRf	Adsorbent	Solvents system	Detection	
cucurbitacin B	silica gel GF_{254}	diisopropyl ether-acetone 5:2	48	-	-	2% vanillin in ethanol + 85% orthophosphoric acid, dried 20 min at 120 °C; examined in day light and UV at 365 and UV 254 nm.	35 36
	silica gel GF_{254}	C_6H_6-di-EtOAc 2:1:1	90	-	-		
	silica gel GF_{254}	trichloroethylene-isopropyl alcohol 10:1	41	-	-		
	silica gel GF_{254}	Et_2O-C_6H_6 8:2	-	silica gel	$CHCl_3$-MeOH 100:1		
	silica gel GF_{254}	ether-hexane-MeOH 70:30:5	38	silica gel	$CHCl_3$-MeOH 100:1		
		$CHCl_3$-MeOH 95:5	90	silica gel	$CHCl_3$-MeOH 100:1	5%ferric chloride in EtOH(violet sport with cucurbitacins containing the diosphenol grouping(e.g. E,I,J,K,L)	35, 36
cucurbitacin D	silica gel GF_{254}	diisopropyl-acetone 5:2	33	-	-	5%ferric chloride in EtOH(violet sport with cucurbitacins containing the diosphenol grouping(e.g. E,I,J,K,L)	35, 36
	silica gel GF_{254}	C_6H_6- dioxane-EtOAc 2:1:1	51	-	-		
	silica gel GF_{254}	ether-hexane-MeOH 70:30:5	26	silica gel	$CHCl_3$-MeOH 100:1		
	silica gel GF_{254}	$CHCl_3$- MeOH 95:5	82	silica gel	$CHCl_3$-MeOH 100:1		

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	hRf	Adsorbent	Solvents system	Detection	
cucurbitacin E	silica gel GF_{254}	diisopropyl- acetone 5:2	76	-	-	Vanilin *p*-dimethyla min-*O* -benzaldehy devapour of I_2	35
	silica gel GF_{254}	C_6H_6 –dioxane-EtOAc 2:1:1	90		-		
	silica gel GF_{254}	ether-hexane-MeOH 70:30:5	49	silica gel	$CHCl_3$-MeOH 100:1		
	silica gel GF_{254}	$CHCl_3$- MeOH 95:5	93	silica gel	$CHCl_3$-MeOH 100:1		
cucurbitacin F	silica gel GF_{254}	n-BuOH-Et_2OH-HOAc 20:60:0.8	97	Silica gel	n-BuOH-Et_2OH-HOAc 20:60:0.8		36
hexacucurbitacin F	silica gel GF_{254}	n-BuOH-Et_2OH-HOAc 20:60:0.8	71	Silica gel	n-BuOH-Et_2OH-HOAc 20:60:0.8		36
23, 24 – dihydrocucurbitacin F	silica gel GF_{254}	n-BuOH-Et_2OH-HOAc 20:60:0.8	97	Silica gel	n-BuOH-Et_2OH-HOAc 20:60:0.8		36
cucurbitacin I	silica gel GF_{254}	diisopropyl ether - acetone 5:2	45	Silica gel	n-BuOH-Et_2OH-HOAc 20:60:0.8		36
	silica gel GF_{254}	C_6H_6 - dioxane - EtOAc 2:1:1	79	Silica gel	n-BuOH-Et_2OH-HOAc 20:60:0.8		
	silica gel GF_{254}	trichloroethylene – isopropyl alcohol 10:1	33	Silica gel	n-BuOH-Et_2OH-HOAc 20:60:0.8		
	silica gel GF_{254}	ether-hexane-MeOH 70:30:5	30	Silica gel	$CHCl_3$-MeOH 100:1		
	silica gel GF_{254}	$CHCl_3$-MeOH 95:5	84	Silica gel	$CHCl_3$-MeOH 100:1		

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	hRf	Adsorbent	Solvents system	Detection	
tetrahydrocucurbitacin I	silica gel GF_{254}	diisopropyl ether - acetone 5:2	26	Silica gel	$CHCl_3$-MeOH 100:1		34
	silica gel GF_{254}	C_6H_6 - dioxane - EtOAc 2:1:1	47	Silica gel	$CHCl_3$-MeOH 100:1		
	silica gel GF_{254}	trichloroethylene – isopropyl alcohol 10:1	21	Silica gel	$CHCl_3$-MeOH 100:1		
cucurbitacin J	silica gel GF_{254}	diisopropyl ether - acetone 5:2	30	Silica gel	n-BuOH-Et_2OH-HOAc 20:60:0.8		34
	silica gel GF_{254}	C_6H_6 – dioxane - EtOAc 2:1:1	90	-	-		
	silica gel GF_{254}	trichloroethylene – isopropyl alcohol 10:1	21	-	-		
cucurbitacin K	silica gel GF_{254}	diisopropyl ether – acetone 5:2	21	-	-		34
	silica gel GF_{254}	C_6H_6 – dioxane - EtOAc 2:1:1	47	-	-		
	silica gel GF_{254}	trichloroethylene – isopropyl alcohol 10:1	15	-	-		

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	hRf	Adsorbent	Solvents system	Detection	
cucurbitacin L	silica gel GF_{254}	diisopropyl ether - acetone 5:2	41	-	-	10% H_2SO_4	34, 36
	silica gel GF_{254}	C_6H_6 – dioxane - EtOAc 2:1:1	73	-	-		
	silica gel GF_{254}	trichloroethylene – isopropyl alcohol 10:1	30				
	silica gel GF_{254}	ether-hexane-MeOH 70:30:5	28	-	$CHCl_3$-MeOH 100:1		
	silica gel GF_{254}	CHCl3-MeOH 95:5	75	-	$CHCl_3$-MeOH 100:1		
cucurbitacin O	-	-	-	Silica gel	$CHCl_3$-MeOH 95:5		26
cucurbitacin P	-	-	-	Silica gel	$CHCl_3$-MeOH 95:5		26
cucurbitacin Q	florisil	ether-EtOAc 2:1		Silica gel	$CHCl_3$-MeOH 95:5		36
3β-hydroxy-16-oxo-12-ene-28-noroleanane (maragenin I)	-	-	22	Silica gel	10% EtOAc in light petroleum		36
3β-acetoxy-16-oxo-12,17-diene-28-noroleanane (maragenin II)	20%$AgNO_3$ in silica gel	cyclohexane EtOAc 95:5	90	-	-		36
lupeol	silica gel GF_{254}	hexane-EtOAc 6:4	32	-	-		26
	silica gel GF_{254}	hexane-Et_2O-HOAc 80:20:1					

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	hRf	Adsorbent	Solvents system	Detection	
lupeol acetate	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	29	-	-		37
	silica gel	hexane-EtOAc 6:4	68	-	-		
	silica gel	hexane-Et_2O-HOAc 80:20:1	68	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	88	-	-		
betulin	silica gel	hexane-EtOAc 6:4	54	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	11	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	7	-	-		
betulin-3-acetate	silica gel	hexane-EtOAc 6:4	88	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	24	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	11	-	-		
betulin diacetate	silica gel	hexane-EtOAc 6:4	62	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	84	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	50	-	-		
calenduladiol	silica gel	hexane-EtOAc 6:4	47	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	8	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	8	-	-		

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	hRf	Adsorbent	Solvents system	Detection	
calenduladio-diacetate	silica gel	hexane-EtOAc 6:4	60	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	55	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	35	-	-		
betulinic aldehyde	silica gel	hexane-EtOAc 6:4	82	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	24	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	27	-	-		
betulinic aldehyde acetate	silica gel	hexane-EtOAc 6:4	64	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	93	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	80	-	-		
betulinic aldehyde semicarbazone	silica gel	hexane-EtOAc 6:4	9	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	7	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	1	-	-		
betulinic acid	silica gel	hexane-EtOAc 6:4	54	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	17	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	4	-	-		
methyl betulinate	silica gel	hexane-EtOAc 6:4	71	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	26	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	13	-	-		

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	hRf	Adsorbent	Solvents system	Detection	
betulinic acid acetate	silica gel	hexane-EtOAc 6:4	92	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	48	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	23	-	-		
methyl betulinate acetate	silica gel	hexane-EtOAc 6:4	64	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	88	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	77	-	-		
thurberogenin	silica gel	hexane-EtOAc 6:4	73	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	16	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	30	-	-		
thurberogenin diacetate	silica gel	hexane-EtOAc 6:4	92	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	42	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	90	-	-		
lup-2(29)-en-3β-28-triol (lupenetriol)	silica gel	hexane-EtOAc 6:4	25	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	16	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	30	-	-		
lupenetriol triacetate	silica gel	hexane-EtOAc 6:4	26	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	28	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	26	-	-		

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	hRf	Adsorbent	Solvents system	Detection	
lupentriol tribenzoate	silica gel	hexane-EtOAc 6:4	64	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	94	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	93	-	-		
16β,28′-ethylidene lupenetriol	silica gel	hexane-EtOAc 6:4	71	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	18	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	10	-	-		
erythrodiol	silica gel	hexane-EtOAc 6:4	55	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	11	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	19	-	-		
erythrodiol diacetate	silica gel	hexane-EtOAc 6:4	62	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	84	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	63	-	-		
oleanolic aldehyde	silica gel	hexane-EtOAc 6:4	69	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	22	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	40	-	-		
oleanolic aldehyde acetate	silica gel	hexane-EtOAc 6:4	64	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	96	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	98	-	-		

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	hRf	Adsorbent	Solvents system	Detection	
oleanolic aldehyde acetate oxim	silica gel	hexane-EtOAc 6:4	57	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	51	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	11	-	-		
oleanolic acid	silica gel	hexane-EtOAc 6:4	49	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	14	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	7	-	-		
methyl oleanolate	silica gel	hexane-EtOAc 6:4	80	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	23	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	40	-	-		
oleanolic acid acetate	silica gel	hexane-EtOAc 6:4	90	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	41	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	41	-	-		
methyl oleanolic acetate	silica gel	hexane-EtOAc 6:4	96	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	62	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	66	-	-		
oleanolic acid acetate imidazolide	silica gel	hexane-EtOAc 6:4	37	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	8	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	1	-	-		

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	HRf	Adsorbent	Solvents system	Detection	
oueretaroic acid	silica gel	hexane-EtOAc 6:4	8	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	1	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	0	-	-		
methyl queretarate	silica gel	hexane-EtOAc 6:4	26	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	2	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	6	-	-		
queretaroic acid diacetate	silica gel	hexane-EtOAc 6:4	43	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	12	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	9	-	-		
methyl queretarate diacetate	silica gel	hexane-EtOAc 6:4	69	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	27	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	43	-	-		
queretarol	silica gel	hexane-EtOAc 6:4	8	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	1	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	0	-	-		
queretarol triacetate	silica gel	hexane-EtOAc 6:4	56	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	6	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1		-	-		

Table 3 TLC and column chromatography of phytosterols and triterpenes (continued)

Compound	TLC condition			Column chromatography condition			Ref.
	Adsorbent	Solvent system	HRf	Adsorbent	Solvents system	Detection	
longispinogenin	silica gel	Hexane-EtOAc 6:4	30	-	-		37
	silica gel	Hexane-Et_2O-HOAc 80:20:1	3	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	2	-	-		
longispinogenin triacetate	silica gel	Hexane-EtOAc 6:4	87	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	40	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	80	-	-		
longispinogenin triformate	silica gel	Hexane-EtOAc 6:4	87	-	-		37
	silica gel	hexane-Et_2O-HOAc 80:20:1	54	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	56	-	-		
6β,28-ethyliene – longispinogenin	silica gel	Hexane-EtOAc 6:4	79	-	-		37
	silica gel	Hexane-Et_2O-HOAc 80:20:1	21	-	-		
	silica gel +10%$AgNO_3$	CH_2Cl_2 – $CHCl_3$ 1:1	43	-	-		

Table 4 TLC-spray reagent for triterpenes

Compound	Reagent	Colour	Ref.
3- hydroxy steroids	2% phosphomolybdic acid in EtOH	Dark blue on a plate yellow green background developed after heating the plate at 110 °C, a few minutes	26
Δ^5, Δ^7 and $\Delta^{5,7}$ sterol	Liebermann Burchard (1 ml H_2SO_4, 20 ml acetic acid and 50 ml $CHCl_3$)	blue-green after heating the plate at 85-90 °C, 15 minutes	20
triterpenes	Carr-Price (20% antimony chloride in CCl_4)	a ranges of colors developed on heating the plate at 110 °C, for 10 minutes	20
α-ketols	fleshly prepared 0.2% solution of triphenyltetrazolium chloride in N KOH (made up in 50% aq.MeOH)	red spots revealed over the stream	29
diosphenol system	5% ethanolic solution of ferric chloride	brown spots	38
cucurbitacins	vanillin-phosphoric acid (1 vol. 2% solution of vanillin in EtOH, 2 vol. 85% orthophosphoric acid and 8 vol. EtOH) saturated aqueous solution (NH_4) H_2SO_4	pink or yellow colors revealed after heating the plate at 120 °C for 20 minutes dark spots appeared after drying the plate with hot air and heating at 400 °C	38 38
cucurbitacin B (dark purple) 23,24-dihydrocucurbitacin B (yellow)	successively sprayed with 2% solution of vanillin in $CHCl_3$ and 5% solution of bromine in CCl_4	dark purple and yellow colors developed on heating the plate at 70-75 °C for 3-5 minutes	39
α,β- unsaturated ketones	iodine vapour on kieselgel GF254 plate	brown color	38
triterpenes in general	-	dark spots appeared in the UV light at 254 nm	38

Table 5 TLC-solvent systems for plant steroids (40)

Compound	TLC type	Example of solvents
VS	silica gel	hexane-Et2O
CA, CU		toluene-EtOAc
CA, WI, ES, SA, SS		$CHCl_3$-MeOH
VS		CH_2Cl_2-MeOH (97:3)
CA		$CHCl_3$-MeOH-formamide (93:6:1)
BU		EtOAc-MeOH-water (81:11:8)
CA		EtOAc-MeOH (97:3)
SS, SA		$CHCl_3$-MeOH-water (14:6:1)
CU, ES, WI	non-polar boned silica	MeOH-water (7:3)

Abbreviations for the various classes of steroids: BS: brassinosteroids; BU: bufadienolides; CU: cucurbitacin; ES: ecdysteroids; SA: steroidal alkaloids; SS steroidal saponin; VS: vertebrate-type steroids; WI: withanolides

Table 6 Various methods for steroid visualisation on TLC (40)

Method	Spray reagent	Compounds[a]
Fluorescence quenching	ZnS-containing silica	ES, CA, WI, VS
Non-specific colour reaction	Anisaldehyde	ES, SS
	H_2SO_4	SS,SA,SS
	$SbCl_3$	BU, WI, SS, SA,WI
		CA, ES, SS, VS
	Vanillin-95% EtOH- H_2SO_4	SA, VS
	Chloramine T-H_2SO_4	VS
	Carbazole- H_2SO_4	
“Specific” reactions	3,5-dinitrobenzoic acid in alcohol	CA
	$CeSO_4$ in H_2SO_4	CA
	2,4,2′,4′-tetradinitrophenyl in toluene	CA
	Various method	CU
	$(NH_4)_2CO_3$ (fluorescence induction)	ES
	Folin-Ciocalteu→blue (for 3-keto group)	ES
	2,4-dinitrophenylhydrazine→yellow (for keto group)	ES
	Triphenyltetrazolium chloride→ red (for keto group)	ES
	Dragendorff’s reagent	WI, SA
	4-(4-Nitrobenzyl)-pyridine (for epoxides)	WI

[a] See the abbreviation for the steroid classes from Table 5.

3. Chemical constituents of cucurbitaceous plants

The chemical composition of cucurbitaceous plants belongs to amino acids, fatty acids, sterols and triterpenes. The characteristic phytosterols occuring in this family are 24-ethyl-Δ^7-sterols such as 24-ethyl-cholest-7-en-ol, 24-ethyl-cholesta-7,22-diene-ol, 24-ethyl-cholesta-7,25-diene-ol and 24-ethyl-cholesta-7,22,25-triene-ol, as the major sterol components (21)(Fig.2) and cucuribitacins are predominantly found in the Cucurbitaceae family, but are also present in several other families of the plant kingdom (41). The cucurbitacins may result in serious poisoning and even death. Despite their toxicity, species in which they are found have been used for centuries in various pharmacopoeia (41).

3.1 Phytosterols

Sterols are triterpene derivatives, based on the cyclopentanoperhydro-phenanthrene ring system (Fig.2). The commonly occuring phytosterols in higher plants included stigmasterol, sitosterol, compesterol, as both free and glycoside forms (20, 24).

Figure 2 Structure of cyclopentanoperhydrophynanthrene ring system

Phytosterols arise from the isoprenoid pathway. In the Eucaryotes, which have the capability of photosynthesis, all sterols (phytosterols, cardenolides, spiro-stanes, solanidanes) arise from the stepwise demethylation of cycloartenol together with the opening of 9β,19-cyclopropane ring (42). Phytosterols differ from zoosterols, in having extra carbon atom at the side chain (20).

Formation of the steroids

The conversion of a C30 skeleton to a C27 of smaller skeleton involves a stepwise demethylation at the 4 and 14-positions. Both methyl groups in the 4-position

are loss through a series of oxidations ($CH_3 \rightarrow CH_2OH \rightarrow CHO \rightarrow CO_2H$), which enol up with a decarboxylation (42).

Phytosterols have one or two extra carbons, i.e. methyl, methylene, ethyl, ethylidene groups, at the side chain (C-24). Such characteristic side chains are also found in algae (fucosterol), fungi(ergosterol), and marine organisms.

3.2 Triterpenes

The bitter principles in cucurbitaceous plants include the chemotaxonomic cucurbitacins. They are highly oxygenated, mainly tetracyclic, triterpenenic compounds and derived from the cucurbitane skeleton [(19-10→9β)-abeo-10α-lanost-α-en]. They cannot be considered as steroidal since the methyl from C10 has moved to C9 (Fig.3).

The general structure of the cucurbitacins is triterpenic signifying that they have 30 carbons. The C4 carries a gem-dimethyl group and the C24 an isopropyl, other methyls are present on carbons C9, C13, C14 and C20. All of them have an unsaturated carbon C5. The most common in the plant kingdom are certainly cucurbitacin B and D. Now that the general structure of these compounds has been described with the most common substituents which are as follows:

C1 : some present an unsaturation (cucurbitacins E, I, J, K and L)

C2 : those are usually hydroxylated except in their isoforms. Cucurbitacin C has no substituents.

C3 : hydroxy or ketonie

C9 : usually methylated (hydroxymethylated in cucurbitacins A and C)

C11 : ketonie except in 11-deoxo-cucurbitacin I.

C16 : hydroxy (α).

C20 : hydroxy (β).

C22 : ketonic except in 11-deoxo and 22-deoxo-isocucurbitacin D.

C23 : an unsaturation can be present (cucurbitacins A, B, C, D, E, F, I, O and Q).

C16 : not usualy substituted except for a hydroxyl on cucurbitacins G, H, J and K.

C25 : can be hydroxylated

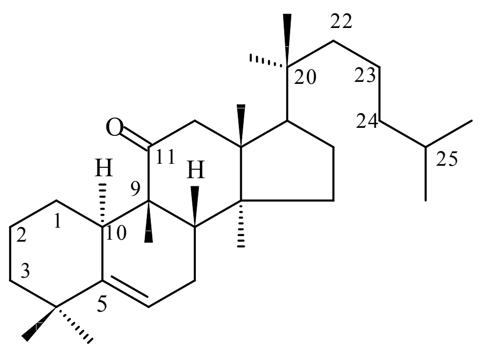


Figure 3 General structure of cucurbitacins

They are predominantly found in the Cucurbitaceae family. Their toxicity, species in which they are found have been used for centuries in various pharmacopoeia. Biological effects of the cucurbitacins and the plants containing them are very varied. The bioactivity of cucurbitacins include strong purgative activity, cytotoxicity, hepatoprotective effect, antiinflammatory and antifertility in female mice. They can also play other biological roles such as plant growth regulators and insect feedant or antifeedant (5).

Cucurbitacin B and iso-cucurbitacin B are found in *Helicteres isora* (43) and the derivative cucurbitacin B derivative (3-epi-iso hydroxyl in β) has been isolated from *Ipomopsis aggregata* (44).

Arevenin I (2β-glucoside of cucurbitacin B) and II (2β-glucoside of 23,24-dihydrocucurbitacin B) and cucurbitacins B, D, E, I and R (45) have been isolated from *Anagallis arvensis.* In the same year there authors published the isolation of arvenins III (2β-glucoside of cucurbitacin D and IV) (2β-glucoside of cucurbitacin R) (46).

Arevenin I has also been isolated recently from *Cayaponia angustiloba* and *Cyaponia racemosa*. These species like *Gurania subumbellata* contain cucurbitacins B, D, dihydro-D, F and dihydro-F (47).

The roots of *Picrorhiza kurrooa* also contain Arvenin I and other heterosides, such as 2*β*-glucoside of cucurbitacin), 23,24-dihydro cucurbitacin B and deacetoxy-B. Datiscocide was obtained from *Datisca glomerata,* which is the 16β-glucoside of cucurbitacin D, and also datiscacin (20-acetoxy- cucurbitacin I). This appears to be

the first natural cucurbitacin isotated with an acetate ester on C20. More recently cucurbitacin F has also been isolated.

Several cucurbitacins have also been isolated from *Desfontaini spinosa* (48) such as 11-deoxo-I, 23,24-dihydro-11-deoxo-I and spinoside A (4-diacetyl-α-arabinoside of cucurbiacin I) and B(2-acetyl-α-arabinoside of cucurbitacin D).

One group of cucurbitacins with the general structure described above but lacking in some common substituents are bryogenin, bryosigenin and bryodulcosigenin isolated from *B. dioica* (49). The lack of the substitutions at C2, C11, C16 ,C20 and C22 positions and also the unsaturations at carbons C1 and C23, and the presence a hydroxyl at C3 and a methyl at C9 exist. Bryogenin has a ketonic group on C24, bryosigenin has an additional hydroxyl at C25 and bryoidulcosigenin has hydroxyls on both carbons. Hylands and Salama isolated cucurbitacin S (Fig. 4) from the same species. This presented a new heptagonal ring between carbons C16 and C24. The same ring also occured in cucurbitacin T which differs from S in having an OH at C20 and a methoxyl at C25 (50).

Figure 4 Structure of cucurbitacin S

Two non-cucubitacins (2β-glucosides (WG1 and WG2)) have recently been isolated from the rhizomes of *Wilbrandia species* (51). The structures are very similar to cucurbitacin P and Q, respectively, but they have only one methyl group at C4, the aromatic A-ring, and a double bond at C6.

Some hexanor cucurbitacin derivatives are also found in their natural form such as hexanor-cucurbitacin I and 16-deoxy-Δ^{16}-hexanor-cucurbitacin O, which isolated

are from *E. elaterium* (52) together with cucurbitacin R and anhydro-22-deoxy-3-epi-isocucubitacin D. Their carbons C16 and C23 are cyclized via an oxygen atom. Hexanor-cucurbitacin I has also been isolated from *Citrullus colocynthis* in the from of a 2β-glucoside together with 2β-glucosides of cucurbitacin I, E and L (53).

Hexanor cucurbitacin F is found together with cucurbitacins F and 23,24-dihydro cucurbitacin F in the stem bark of *Elacocarpus doliehostylus*. The latter two compounds have also been isolated from *Crinodendron hookcrianum*.

Cucurbitacins from *Momordica charantia* fruit and seed have been isolated. Some are bitter (momordicosides K and L) and some not bitter (momordicosides G, F_1, F_2 and I) (Fig. 5). The non-bitter one extra ring arising from the bonding between C5 via an oxygen and C19. Momordicosides differ from the usual cucurbitacins in having an aldehydic group at C9 other with a 7β-glucosidic link at C7. Momordicins I and II have the glucosylation at C23 (54).

Momordicoside K

Momordicoside L

Momordicoside G : R_1 = β-D-allose; R_2 = CH_3

Momordicoside F1 : R_1 = β-D-glucose; R_2 = CH_3

Momordicoside F2 : R_1 = β-D-allose; R_2 = H

Momordicoside I : R_1 = β-D-glucose; R_2 = H

Momordicins I (R = H) and II (R = β-D-glucose)

Figure 5 Cucurbitacins from *Momordica charantia* (54)

Cucurbitacin A : R = OAc

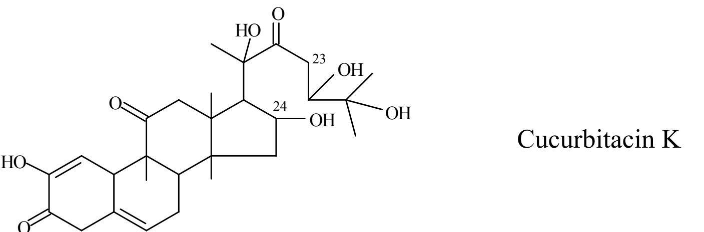
Cucurbitacin B : R = O
Cucurbitacin D : R = O

Cucurbitacin C : R = OAc

Cucurbitacin E : R = OAc
Cucurbitacin I : R = OAc

Cucurbitacin L : R = OH
23, 24-Dihydrocucurbitacin E : R = OAc

Tetrahydrocucurbitacin I (Tet I) : R = OH
23, 24-Dihydrocucurbitacin B (D;B) : R = OAc



Cucurbitacin K .

Figure 6 Partial formula of cucurbitacins (54)

3.3 Biosynthesis

Triterpenes and phytosterols generated from the isoprenoid pathway. Various modes of cyclization of the squalene the precursor generated by this pathway, result the main classes of triterpenes. The usual biosynthetic route of tetracyclic triterpenes in plants involves the loss of one 10-methyl hydrogen to give cycloartenol (A), the ubiquitous plant substance, which then replaces lanosterol as a precursor of plant sterols. The commonly occuring plant sterols, such as β-sitosterol are formed via cycloartenol, 24-methyleneartenol citrostadienol, etc. The sequence of reactions involve the introduction of two extra carbon atoms at the side chain initially producing a 24-methylene and then a 24-ethylidene group. They may either be saturated to the corresponding 24-α or 24-β alkyl group, or the double bond may migrate to other positions, e.g. Δ^{22} stigmasterol (F). In addition, the unsaturation in ring B formed by the opening of cyclopropane ring usually migrates from its initial position at Δ^{7} to Δ^{5} (Fig. 7,8,9) (55).

However, the cucurbitacins represent an interesting variation in the biogenetic pathways leading to the tetracyclic triterpenes. A migration of one methyl group from C-10 to C-9 in a lanostane precursor. The isolation of the only compound with a cucurbitane carbon skeleton but no carbonyl group at C-11,litsomentol (G) from *Listea tomentosa*, support the routh involving the complete rearrangement of a lanostane. However, other routes are also possible. The almost universal presence of an oxygen at 11 could be taken as a good evidence for the cycloartenol intermediate since in cycloartenol C-11 is activated as being α-situated to the cyclopropane ring, and so is readily oxidizable.

This is particularly attractive since cycloartenol is known to be the precursor of tetracyclic triterpene in higher plants. Zander and Wigfield investigated the pathway but their evidence could not distinguish the intermediate between cycloartenol and parkeol (A to H, Fig. 8), in which the unsaturation at C9 (2) could be epoxidized and cleaved resulting a carbonium ion. It could be further isomerised to give the skeleton of cucurbitacins. They were able to show, however, that lanosterol was not involved.

H^+

cycloartenol

1. loss methyl at C-14
2. lose gem-dimethyl at C-4
3. double bond migration

desmosterol

>SCH_2

>SCH_2 2H

>SCH_2 2H

R

sitosterol

fucosterol

ergosterol

stigmasterol

Figure 7 The routes generating phytosterols

cycloartenol (A)

lanosterol (B)

24-methylenecylcloartenol (C)

citrostdienol (D)

β-sitosterol (E)

stigmasterol (F)

litsomentol (G)

parkeol (H)

Figure 8 Structure of the tetracyclic triterpenes in plants (55)

Figure 9 Possible routes generating the cucurbitacins (55)

$CH_3-CO_2^{\ominus} \longrightarrow$ 173 $\equiv$

[1,2-^{13}C]- acetate

(Chair-chair-chair-boat conformation)

(175)

Via

α-amyrin

β-amyrin

Figure 10 Possible routes generating the other pentacyclic triterpenes (C_{30}) (55)

4. Bioactivity of isoprenoid derived compounds in cucurbitaceous plants

4.1 Bryonolic acid

Bryonolic acid was first isolated from the roots of *B dioica* JACQ., Tabata *et al*. reported the isolation of bryonolic acid from the cultured cells of *Luffa cylindrica* (Cucurbitaceae) and the anti-allergic activity of this compound against both homologous passive cutaneous anaphylaxis and hypersensitivity in rodents. They found that cultured cells are capable of producing a large amount of bryonolic acid (ca. 3% of dry weight), which may be considered a promising natural product for the treatment of a delayed-type allergy for their unique properties. The effect of bryonolic acid on the growth of B-16 melanoma cells *in vitro* according to the MTT method. Bryonolic acid significantly inhibited the cell growth above 5 μg/ml (87).

4.2 Phytosterols

Phytosterols are essential components of the plant membrane and play an important role in plant cell growth. However, some biological activities of phytosterols have been reported recently including anticomplementary activity (56), anti-inflammatory activity (57), cytotoxic and antiviral activity (58,59). Chondril-lasterol and their glyclosides have also been reported as antinutrients (60).

4.3 Cucurbitacins

- Cytotoxic and antitumor activity

The cucurbitacin are of great interest because of the wide range of biological activity they exhibited in plants and animals. They are predominantly found in the family Cucurbitaceae but are also present in several other families of the plant kingdom. Despite their toxicity, species of the plants in which they are found have been used for centuries in various pharmacopoeias. A number of compounds of this group have been investigated for their cytotoxic, hepatoprotective, antiinflammatory, cardiovascular effects (41). Cucurbitacin B and dihydrocucurbitacin B were isolated from the fruit juice. The MTT colorimetric assay for cytotoxicity of cucurbitacin B, dihydro-cucurbitacin B and a mixture of cucurbitacin B and dihydrocucurbitacin B showed the ED_{50} against breast cancer cell line (SK-BR-3) at 0.05 μg/ml, 0.40 μg/ml and 0.48 μg/ml, respectively. Cucurbitacins have strong cytotoxic and antitumour activity. Cucurbitacin B, iso-cucurbitacin B and the plant species in which the are found strongly cytotoxic (ED_{50} 10^{-5} μg/ml and 7.6 x 10^{-5} μg/ml, respectively) when

tested on KB cell cultures proceeding from human nasopharyngeal carcinomas (39). Cucurbitacins B, D, E, I, J, K, L and tetrahydro-I isolated from *Bryonia alba* roots all revealed cytotoxic activity in Hela and KB human cell cultures (ED_{50} = 10^{-5} μg/ml) with the first four substances being the most effective.

- Gastrointestinal effects

Species in which cucurbitacins are found, mainly those belonging to the Cucurbitaceae family (*Citrullus colocynthis* seeds, *Ecballium elaterium* fruits, *Bryonia* roots) have been used for centuries for their purgative properties. Cucurbitacin D increases intestinal movement *in vivo* but not in an isolated intestine.

- Hepatoprotective and hepatocurative activities

Cucurbitacin species have been used in folk medicine for the treatment of liver ailments for a long time. *Anagallis arvensis* is used as a hepatic therapy in Taiwan (59). Elaterium, obtained from *Ecballium elaterium* (a species mentioned in Ebers' Papyrus) has been used since the times of Dioscorides meixed with milk for treating jaundice (61). Cucurbitacin B has been shown to be effective chronic hepatitis by normalizing hepatic protein levels, stimulating cellular immunity functions and increasing the AMPc/GMPc ratio in the plasma of experimental animals. These effects are all beneficial in the prevention or cure of hepatitis. Cucurbitacin B (0.2 mg/kg) decreases levels of GPT, hepatic collagen and β-lipoproteins in rats with experimental fatty liver (CCl_4-induced) thus decreasing hepatic damage. In chronic experiments it has been shown to prevent hepatitis and cirrhosis induced by the same hepatotoxin (62).

- Antiinflammatory activity

Several species with cucurbitacins are commonly used as anti-inflammatories. *Ecballium elaterium*, *Bryonia alba* and *Cayaponia tayuya* are applied topically in popular medicine to cure problems of sinusitis or rheumatism (63). The purified fraction of the rhizomes of *Wilbranolia sp*. produces at 200 mg/kg a significant inhibition of acute inflammatory carrageenan-induced rat hind paw oedema and granulomatous lesion, comparable to that of 100 mg/kg of phenylbuta zone (64).

- Cardiovascular effects

Cucurbitacin D induces a strong increase in capillary permeability which brings about hypovolaemia thus a decrease in arterial pressure. The LD_{50} of

cucurbitacin D is 1 mg/kg with death being caused by respiratory-cardiac failure after high dose over a long period of time (64).

- Effect on central nervous system

Cucurbitacin D has been shown to increase the hypnotic effect of sodium penthobarbital in mice, which could indicate a depressive effect on the central nervous system (64).

- Antimicrobial activity

Some cucurbitacin containing species such as *Momordica charantia* present antibacterial activity (21). Cucurbitacin C at a concentration of 10 mg/ml inhibits growth of *Phytophtora cactorium* (65).

- Effects on insects

The cucurbitacins of these plants are more feeding than their fatty acids (palmitic and linoleic) and their total sugar content. At cucurbitacin levels of more than 0.2 mg/g fresh weight, they can have a prejudicial effect on the insects (66, 67). Cucurbitacin E and I are strong antifeedants of the flea beetle (*Phyllotreta nemorum*) (68).

According from the bioactivity which have been reported from the literature, cucurbitacins possess the main active compounds in *T. cucumerina*. The phytochemical study in the *T. cucumerina* root extract have been revealed some interesting isoprenoid derivatives but no bioactivity study report.

5. Diseases related to bioactive isoprenoid derived compounds in cucurbitaceous plants

5.1 Malaria

Malaria is still major public health problem in Thailand with 300,000 – 500,000 malaria cases being reported annually. Four species of malaria parasite are found in Thailand; the most comman species are *Plasmodium falciparum* (68.5%) and *P. Vivax* (31.4%) white *P.malaria* (0.1%) and *P. ovale* (reported 20 years ago) are rare.

P. falciparum, the most dangerous and life threatening species of malaria parasite. This species also accounts for an important share of mortality in 3,000

malaria cases, most of which are cerebral malaria, It is the species that is most virulent and potentially lethal to humans.

A culture of the K1 strain of *P. falciparum* was supplied by Prof. Dr. Peerapan Tan-ariya, the Department of Microbiology, Faculty of Science, Mahidol University. The K1 strain was first isolated in 1979 by Prof. Sodsri Thaithong (Department of Biology, Faculty of Science, Chulalongkorn University) from a malaria patient who was located at Kanchanaburi Province, West Thailand near the Thai-Mianmar border. The area is rural, hilly and semi-forested. The K1 strain is highly to both chloroquine and pyrimethamine but remains sentitive to mefloquine, ART, ARS and DHA (69). It has been maintained in continuous culture by the candle jar technique at the Department of Microbiology, Faculty of Science, Mahidol University since 1979 (70).

5.1.1 Geographical distribution

Endemic malaria occurs in the tropics and subtropics. High transmission areas are found on the fringes of forests in Southest Asia, South America and part of Sub-Sahara Africa. Sub-Sahara Africa is the region with the highest malaria infection rate. Hear alone, the diseases kills at least one million people each year. According to some estimates, 275 million out of a total of 530 million people have malaria parasites in their blood, although they may not develop symptoms. About 40% of the world's population about two million people are at risk in about 90 countries are territories. So to human are generally host to four species of malaria parasites : *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. Of the four human malaria strains, *P. falciparum* causes the most dangerous complications, such as cerebral malaria. It is the species that is most virulent and potentially lethal to humans. It is responsible for about 95% of malaria deaths worldwide and has a mortality rate of 1-3%.

5.1.2 Mode of transmission

Malaria is transmitted by an infected, female mosquito, *Anopheles gambiae*. It can also be acquired from an infected blood transfusion or even from the shared needles of drug addiets.

Human malaria parasite only develop in *Anopheles* mosquitoes. The parasites move to the salivary glands of the mosquito and are injected into human host by the feeding insect. The *Anopheles* mosquito only feeds in the evening. The para-

site is injected into the host when the mosquito feeds and then progresses through a number of stages and transformations. The threadlike malaria parasites enter the bloodstream and are carried to the liver where they invade liver tissue cell, transform into spores and replicate repeatedly. It also has two main stage in the human (Fig.11), first in the liver and then in the blood. The liver stage, which has no symptoms, lasts for about a week to ten days after the bite of the infected mosquito. Thereafter the parasites emerge into the blood, with the most of symptoms. Malaria treatment is therefore directed at the blood stage. Blood stage malaria parasites live inside human red blood cells. This helps them to conceal themselves from the immune system. Nonetheless, a red blood cell is a harsh environment. Almost the only source of nourishment available inside the red cell is haemoglobin, a protein responsible for transporting oxygen in humans. This protein has attached to it an iron containing substance called haem. It is actually this iron that bonds to oxygen. The parasite uses the protein part of haemoglobin as food, but haem is a waste product from its point of view.

A part at the haem is incorporated into an insoluble crystalline substance called malaria pigments. This pigment acts as a sink for removal of haem because it is very insoluble (71). The spores, formed within cyst-like structures in the liver, are released into the bloodstream where they attack and destroy red blood cells. In the process, they undergo another transformation that allows a form of the parasite to attack and invade new red blood cells.

The synchronized development of different stage of the parasite is responsible for the characteristic cycle of fever in infected human. A form of the parasite periodically bursts from demolished cells and is released into the blood stream to invade new blood cells. Different species produce different fever cycles.

P. falciparum has a forty-eight hour period between fever peaks. The process is repeated over and over until natural or acquired immunity, or antimalarial chemotherapy, or death brings the repetitive process to an end.

When an *anopheline* mosquito takes blood from a malaria human, the parasite enters the mosquito and goes through a number of complex changes over a 14 to 21 day period. It becomes an infections form and moves to the mosquito salivary glands where it is ready to re-infect and complete the cycle.

5.1.3 Clinical features

P. falciparum is the most lethal of malarias. The symptoms appear 7-14 days after the bite of an infected mosquito. Most patients present with fever, chills nausea and vomiting. Epigastric discomfort, anorexia and dyspepsia are common. Waterry diarrhea is frequent with certain strains of *P. falciparum*. Patient may not seem to be very ill during the consulation but serous complications may arise at any stage of illness, particularly in non-immune individuals. They may be some degree of jaundice, as a result of red blood cells haemolysis and cholestasis is common in servere *falciparum* malaria (70) especially in severe infection. Tender hepatomegaly and splenomegaly are common. Recrudescence is common in areas with amount resistant parasites.

5.1.4 Diagnosis

Dianogsis is made by detecting parasites in the blood on thick or thin blood smears.

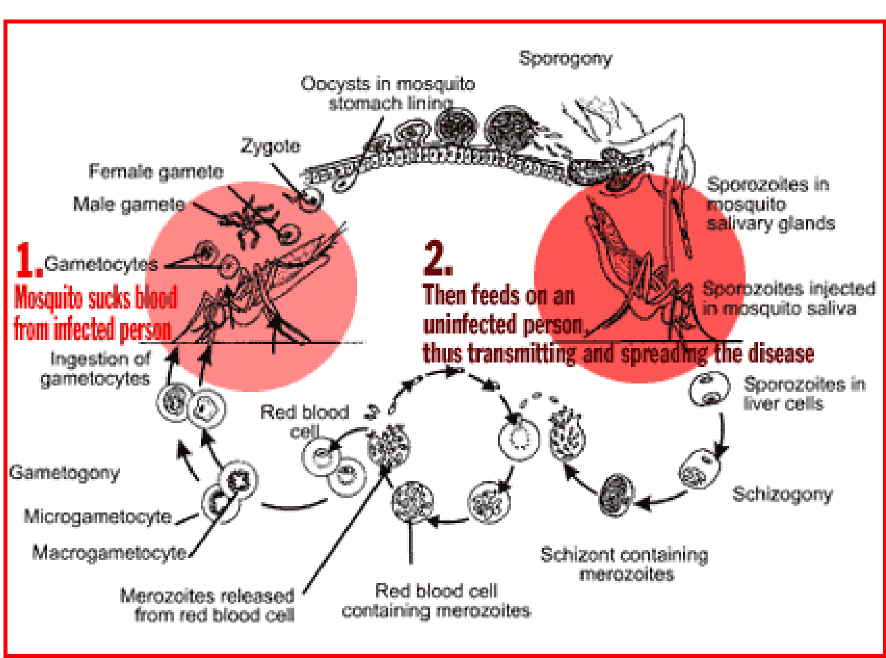


Figure 11 Life Cycle

5.2 Laryngeal Cancer (72)

The larynx is often called the voice box or “Adam’s apple”. It is a short passageway shaped like a triangle that is just below the pharynx in the neck. The pharynx is a hollow tube about five inches long that starts behind the nose and goes

down to the neck to become part of the esophagus. It is divided into three levels; the glottis (or the vocal cards), the supraglottis (the area above the vocal cords including the epiglottis), and the subglottis (the area below the vocal cords). The larynx has several functions. The most obvious is the production of sound for speaking. Another, even more important function is the protection of the airway during swallowing. The vocal cords not only come together to change the sound and pitch of the voice, but they also close tightly when your swallow. This keeps food and saliva from entering your and causing pneumonia or blockage of breathing tubes. The vocal-cords naturally open when you breathe so that air can get in and out of your lungs.

5.2.1 The Risk Factors For Laryngeal

Scientists have found several risk factors that make a person more likely to develop laryngeal or hypopharyngeal cancer:

Tobacco use : Tobacco use is the most important risk factor for cancers of the larynx and hypopharynx. The more one smokes, the greater the risk. The risk of developing cancer in this area is 5 to 35 times greater in smokers than in nonsmokers, depending on how much they smoke.

Alcohol abuse : Heavy drinkers have a risk of laryngeal cancer 2 to 5 times that of nondrinkers. Using both tobacco and alcohol doesn't just add both risks together. The combined risks of tobacco and alcohol are almost multiplied.

Poor nutrition : Nutritional deficiencies often occur with alcohol abuse and may be partly responsible for alcohol's increased risk. Not eating enough food with B vitamins, vitamin A, and retinol may play a role.

Gastroesophageal reflux : Gastroesophageal reflux occurs when acid from the stomach escapes back up the esophagus (food pipe), and cause "heart burn". This acid can irritate the lining of the esophagus. Studies have shown that this irritation may extend to the hypopharynx and can increase the risk for hypopharyngeal cancer.

Microbes that cause cancer: More than 100 years ago researchers began considering the possibility that cancerous tumors were caused by viruses and other infectious agents. Over the past 20 years, however, investigators have not only proved that many different types of cancer indeed stem from viruses, bacteria or parasites, they have also learned that perhaps as many as 15 percent of the world's

cancer deaths can be traced to them. The vast majority of these cases occur in developing counties, where communicable diseases are much more prevalent.

The most common cancer-causing pathogens are the DNA viruses, which propagate by invading the living cells of a host and using the cells' DNA synthesizing and protein-making machinery to generate copies of themselves. Of these carcinogenic agents, the two most important are the human papilomaviruses types 16 and 18, which are sexually transmitted, and the hepatitis B virus. The papillomavirus can lead to cancer of the cervix, among other types of cancer, and the hepatitis B virus can causes liver cancer.

Although papillomavirus type 16 and 18 are responsible for 70 to 80 percent of the world's cases of cancer of the gentals and anus, as many as 30 other papillomaviruses types may be involved in these cancers, which affect women for more often than men. And in certain places not ably Japan the hepatitis C virus causes almost as many cases of liver cancer hepatitis B dose. All told, viral infections,mainly hepatitis, causes as many as 80 percent of liver cancer cases around the globe. Several other viruses have also been found to cause various kinds of cancer. It is believed the contribute world wide to approximately half the cancer of the upper pharynx (4). Differences in cancer rates among socioeconomic groups can usually be attributed to differences in lifestyle. Under privileged people have higher rates of cancers of the mouth, stomach, lung, cervix and liver and of a type of esophageal cancer (squamous cell cancer). Poverty may be thought of as the underlying cause, because it is almost universally associated with higher rates of tobacco smoking, alcohol consumption, poor nutrition and exposure to certain infectious agents.

Human papilloma virus : Human papiloma viruses (HPVs) are a group of about 100 related viruses. Most of these viruses cause warts on the hands, feet, and other skin surfaces that are bother some but not very serious. Several types cause genital warts that sometime can develop into cancer of the cervix, vagina, vulva, or penis. Rarely, this virus can spread during birth from a mother's cervix or vagina to the baby's larynx. This can cause the baby to develop benign non cancerous growths called laryngeal papillomas. This papillomas can cause hoarseness or grow large enough to cause trouble with breathing. Children with these benign growths may have a slightly increased risk of developing laryngeal cancer many years later.

Weakened immune system : Head and neck cancers are more common in people with a weak immune system. Immune system problems may be clue to certain congenital (present at birth) diseases, the acquired immune deficiency syndrome (AIDs), and certain medications given to patients who have received organ transplants.

Occupational exposures : Other risk factors include heavy exposures to wood dust and paint fumes, and to certain chemicals used in the metalworking, petroleum, plastics, and textile industries. Asbestos is a mineral fiber that, in the past, was often used for insulation. It is an important risk factor for two types of lung cancer called mesothelioma and bronchogenic carcinoma. Asbestos may also increase laryngeal cancer risk.

Age : Because cancers of the larynx and hypopharynx usually take many years to form, they are not common in young people. Most patients with these cancers are in their sixties when the cancers are first found.

Race : Cancers of the larynx are about 50 percent more common among African American than among whites.

5.2.2 Cancer of the Pharynx

Cancer of the pharynx (also called pharyngeal cancer) is a malignant tumor that attacks the pharynx, the tube like passage connecting the back of the nose, mouth, and esophagus. The cancer can spread throughout the throat, as well as to other parts of the body and become life threatening.

5.2.3 Occurrence

The cause of pharyngeal cancer is most common among heavy smokers and people who drink large amounts of alcohol. It is seven times more common in men than women and generally occur after age 50.

The following symptoms may occur as the cancer worsens : sligh sore throat that lasts for over 2 week shoarseness trouble with swallowing or feeling of incomplete swallowing earache and/or blocked ear bloody phlegm coughed up swollen lymph node in the neck hard lump in the throat or in the neck lymph node. A large tumor can block the throat, cutting off the air supply possibly causing you to pass out or suffocate. The specialist will determine the treatment for cancer of the pharynx based on the following factor : stage of tumor location of tumor extent of malignancy.

The effects of cancer of the pharynx will vary depending upon the stage in which the tumor is detected and when treatment begins.

6. Quantitative HPLC analysis of the main cucurbitacin in *T. cucumerina*

Phytopharmaceutical (phytomedicine, phytotherapy) is regarded as allopathoic or special or alternative therapy (German Law 1976). The principle of phytomedicine involves the evidence-based herbal medicine, whereas the traditional medicine involves the holistic approach. The plant extract is usually incorporated in phytopharmaceutical product as an active component. The incorporated plant extract must thus contain constant phytochemical composition (active compounds + coexisting compounds (which affect the absorbtion and the excretion of the active compounds) + inert compounds), which is regarded as one active component. The plant extract must be characterized by the following ways (73, 74)

(1) the botanical description of the plant and the phytochemical character of the crude drug
(2) the extraction solvents and method
(3) the ratio of crude drug to the extract (g/g)
(4) the amount of the phytochemical compound, which is responsible for the therapeutic activity (case I: the phytochemical compound = the lead compound). Sometimes the active compound of the plant is partly known and/ or under the discussion (case II: the phytochemical compound = the lead or marker compound). Sometimes the active compound is not known (case III: the phytochemical compound = the marker compound).

In our study the isolated cucurbitacin B, which was the chemotaxonomic character of the cucurbitaceous plants and possesed several pharmacological activities, was used as the lead compound.

6.1 Sample preparation for HPLC analysis

6.1.1 Solid-phase extraction

Solid-phase extraction is necessary for the pretreatment of sample. The plant extract before injecting it into the HPLC apparatus. SPE can prevents the column obstruction and the reversed solid phase is preferred. Only a small amount of

organic solvent is required for the elution, thus a high concentration of the analyte is maintained.

6.1.2 SPE and HPLC (75)

The simplest form of SPE employs a small plastic disposable column or cartridge, often the barrel of medical syringe packed with 0.1 to 0.5 g of sorbent. The sorbent is commonly a reversed-phase material (e.g., C18-silica). The particle size (>40 μm) is larger than that for HPLC column which usually ranges from 3 to 10 μm. Because of the shorter bed length, packing larger particles, and less-well-packed beds, SPE cartridges are much less efficient (N<100) than the HPLC column. One major difference between SPE and HPLC is that the SPE cartridge generally is used once and discarded, since potential interferences may remain on the cartridge.

In SPE, a liquid sample is added to the cartridge and a wash solvent is selected so that the analyte is either strongly retained (k>> 1) or unretained (k=0). When the analyte is strongly retained, interferences are eluted or "washed" from the cartridge so as to minimize their presence in the final analyte fraction. The analyte is then eluted in a small volume with a strong elution solvent, collected, and either (1) injected directly or (2) evaporated to dryness followed by dissolution in the HPLC mobile phase.

6.1.3 The application

SPE is used in sample preparation for six main purposes: (i) removal of interferences and column killers (Fig. 12, 13) (ii) concentration or trace enrichment of the analyte, (iii) desalting, (iv) solvent exchange *In situ*, (v) derivatization sample, (vi) storage and transport interferences that overlap analyte bands in the HPLC separation complicate method development and can adversely effect assay results. Sometimes, a large number of interferences in the original sample may make it impossible to separate them from one or more analyte bands with a single HPLC separation. SPE can be used to reduce or eliminate those interferences. Column killers such as hydrophobic substances (e.g., fats, oils, greases), polymeric material, and particulates, which can either plug or deactivate the HPLC column, can often be removed by RP-SPE. SPE can be used to increase the concentration of a trace component. If SPE cartridge can be selected so that k>> 1 for the analyte, a relatively large volume of sample can be applied before the analyte saturates the cartridge and

begins to elute from the cartridge. A strong solvent (e.g., CAW or MeOH) elutes these analytes from the cartridge in a small concentrated volume, and saves the evaporation time. RP-SPE can be used to desalt samples, especially prior to ion-exchange HPLC the condition of pH and % organic acid are selected to retain the analyte initially, which allows inorganic salts to be washed from the cartridge with water. The analyte can be eluted with organic solvent.

6.1.4 SPE devices

Several devices are used for SPE:(1) cartridge, (2)disk, and (3) coated fiber. The most popular configuration for SPE device is the cartridge. A typical SPE disposable cartridge (syringe-barrel format) is depicted (Fig.13). The syringe barrel is usually medical-grade polypropylene, chosen for its purity. The outlet of the syringe barrel normally has a Luer tip so that a needle can be affixed to direct effluent to a small container or vial. The frits maintaining the partial bed in the cartridge are made of PTFE, polypropylene, or stainless-steel construction with a porosity of 10 to 20 μm to offer little flow resistance. SPE cartridges may vary in design to fit an automated instrument or robotics systems. Cartridges of glass or virgin PTFE are available for ultratrace analyses when the standard syringe-barrel plastics produce unacceptable concentrations of extractable interferences.

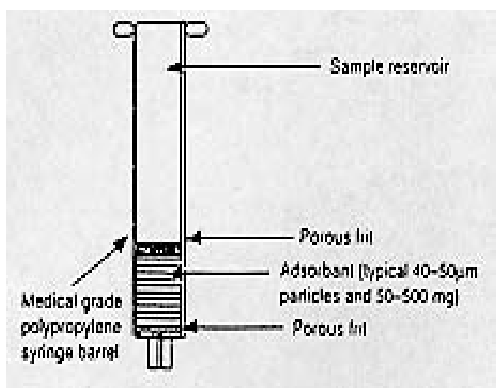


Figure 12 SPE cartridge

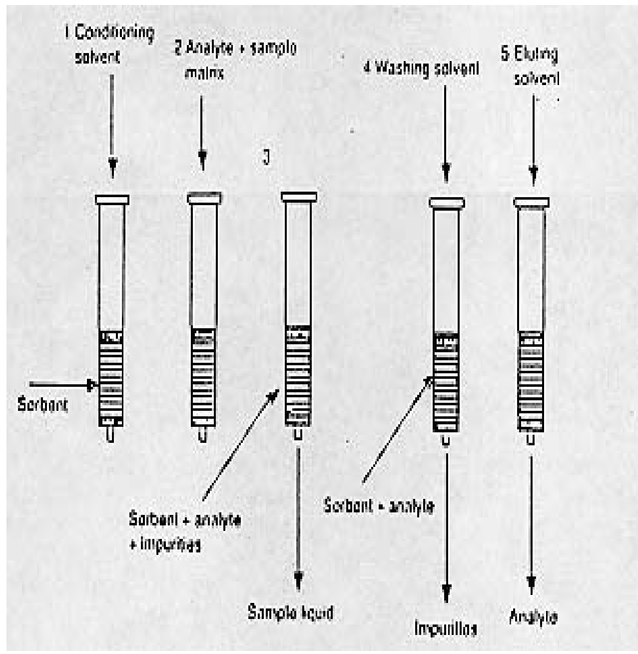


Figure 13 A typical procedure for SPE

6.2 Reversed phase high-performance liquid chromatography (76)

The mobile-phase optimization is based on isocratic runs. The isocratic method development approach a base on the first running of the sample, at the flow rate of 1 ml/min and the temperature around 35-40 °C in the mobile phase of 100% acetonitrile to as certain that all components elute and that the separation is amirable to reversedphase HPLC. Subsequently, the percentage acetonitrile is reduced in 20% increments, using water as another mobile phase component, until the k′ range of the solutes is approximately 0.5-2.0. For the solutes with $0.5 < k' < 20$, the percentage of acetonitrile, methanol, and THF in water are evaluated to determine selectively differences between solvents. Corresponding strengths of various mixtures of acetonitrile, methanol, and THF in water are given in Fig. 14. The numerical points in the figure correspond to the following (1) methanol/water in the proportions required for a k′ range at 0.5-20 (2) acetonitrile/water in the proportions required for a k′ range of 0.5-20; (3) THF/water in the proportions required for a k′ range at 0.5-20; (4) a 50/50 mixture of the solvent system used in (1) and (2); (5) a 50/50 mixture of the

solvent system used in (2) and (3); (6) a 50/50 mixture of the solvent systems used in (1) and (3); and (7) a 33/33/33 mixture of the solvent systems used in (1), (2) and (3). The selectivity, as well as k′, may also by influenced by percentage of water in the mobile phase. Because it is generally desirable to use acetonitrile or methanol-based mobile phases, it is, therefore, also useful to vary the percentage of water in acetonitrile and methanol systems while maintaining the requisite k′ range.

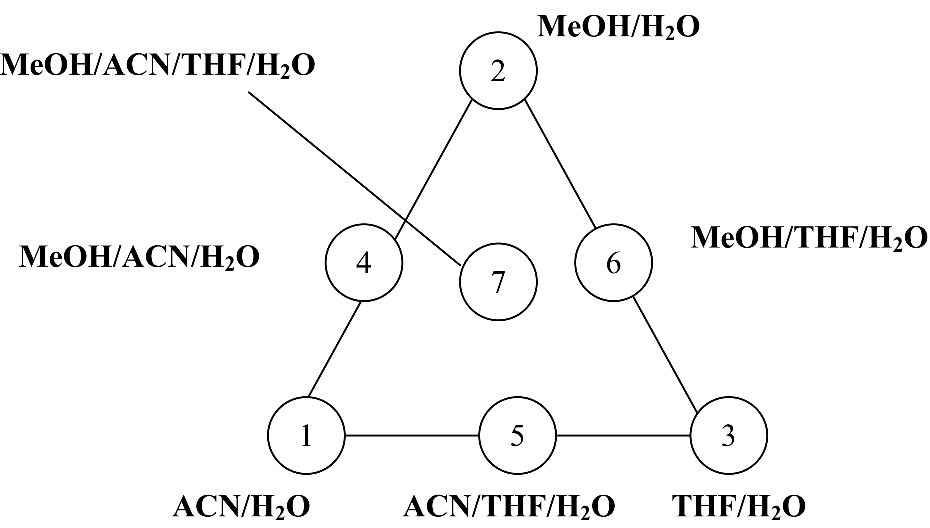


Figure 14 Experiments for optimization of selectivity in reversed-phase HPLC

6.3 Method of validation (76,77, 78)

Validation of analytical methodologies is widely recognized as an important aspect of the utilization of analytical procedures and is widely required in support of product registration applications. Chromatographic methods are commonly used for the qualitative and quantitative analysis of samples. The objective of the analysis is to generate reliable, accurate and interpretable evidences of samples. The method is focused on the followings;

Part I ; defining validation, establishing the need for validation, and identifying significant validation parameters.

Part II ; defining, identifying procedures for and summarizing acceptance criteria, for specific significant validation parameters.

Part III ; defining, identifying procedures for and summarizing acceptance criteria for secondary validation parameters and related topics (e.g., re-validation and system suitability).

6.3.1 Characteristics of an effective validation parameters

Accuracy:The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Precision:The precision of an analytical procedure express the closeness of agreement between a series of measurement from multiple sampling of the same homogeneous sample under prescribed conditions.

Linearity:The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of the analyte in the sample.

Range:The range of an analytical procedure is the the interval between the upper and lower concentration (amounts) of the analyte in the sample for which it has been shown that the analytical procedure has a suitable level of precision, accuracy and linearity.

Robustness: The robustness of an analytical procedure is the measure of its capacity to remain unaffected by small, but deliberate, variations in method parameters and provides an indication of its reliability during normal usage.

Ruggedness : The United States Pharmacopoeia (USP)(79) defines ruggedness as the degree of reproducibility of test results obtained by the analysis of the same samples under a variety of normal test conditions, such as different labs, different analysts, different lost of reagents. Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory and from analyst to analyst.

Sensitivity : The sensitivity of an analytical method is equal to the slope of the calibration line in a linear system.

6.3.2 System suitability testing (80)

System suitability testing ensures that the total system is functioning at any given time. System suitability testing, coupled with previous instrument qualification, periodic calibration, and method validation, provides assurance that the test method will provide accurate and precise data for its intended use. Table 7 review some system suitability tests and the associated validation parameters they monitor run to run.

Table 7 System suitability parameters and recommendations (81)

Parameter	Recommendation
Column capacity (*k'*)	The peak of interest should be well resolved from other peaks and the void volume; generally k should be greater than 2.0
Repeatability	An RSD of less than or equal to 2% for N greater than or equal to 5 is desirable
Relative retention	Not essential if the resolution is stated
Resolution (R_s)	*Rs* should be greater than 2 between the peak of interest and the closest eluted potential interferent (impurity, excipient, degradation product, or internal standard)
Tailing factor (*T*)	T should be less than or equal to 2
Theoretical plate (*N*)	In general should be greater than 2,000

CHAPTER IV
MATERIALS AND METHODS

Part I Phytochemistry

1. Meterials

1.1 Plant materials

Thirty four kg of *T. cucumerina* root and twenty-one kg of fruit were collected in September 2000 from Pichit Horticultural Research Center, Department of Agriculture, Ministry of Agriculture and Cooperative, Pichit Province. The plant name has been identified by Leena Phupat-thanapong , Royal Forest Department , Bangkok, Thailand.

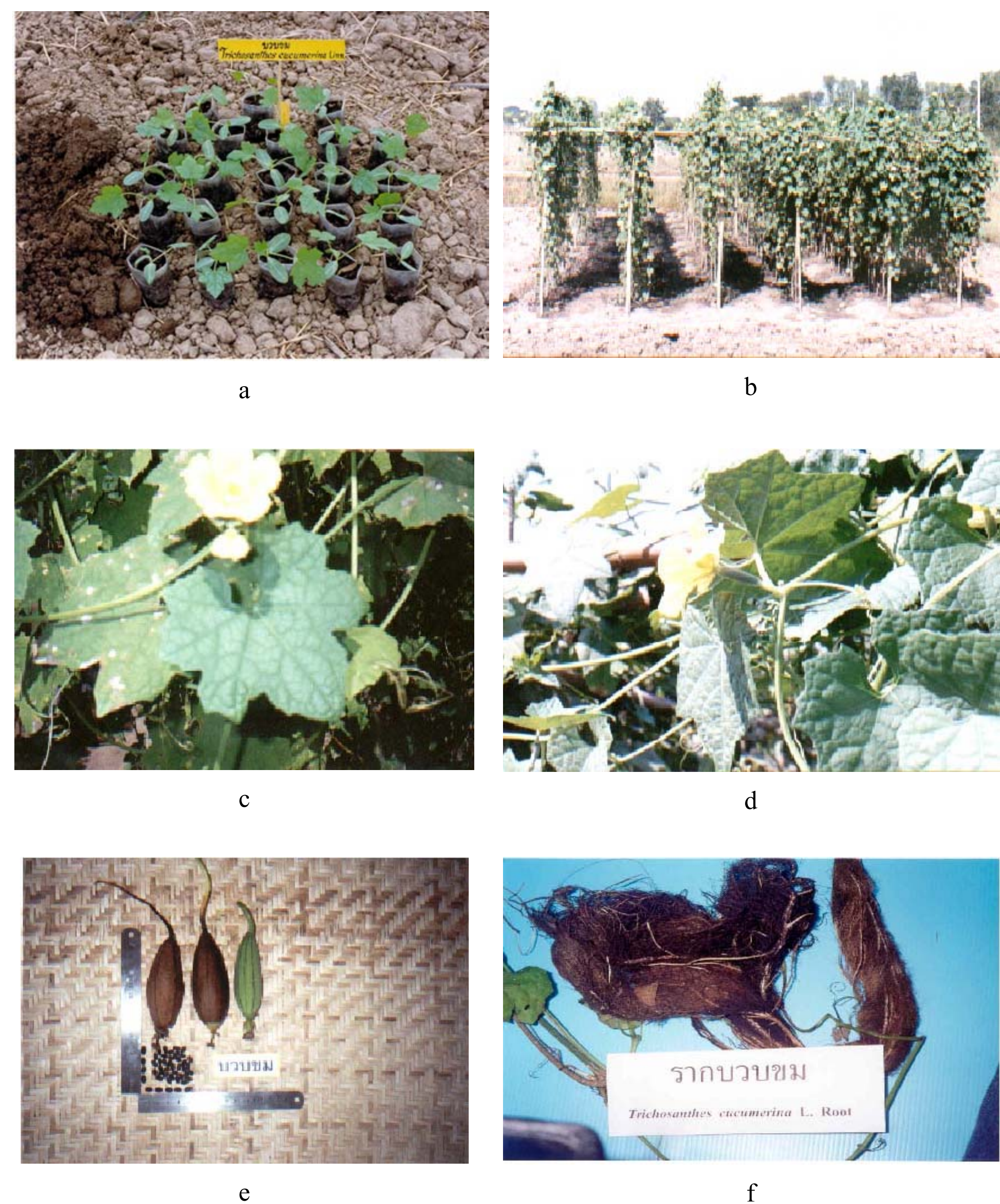


Figure 15 *T. cucumerina* plant a. 20 days after cultivated b. maturity phase (3.5 months) c. and d. leaves, flowers and tendril e. fruits f. root

The root was cleaned, cut and dried at 60 °C for 5 days and pulverized. The powdered root weighed 2 kg.

The fruits were cleaned and pressed. The fruit juice weighed 21 kg, which was approximately 2 l.

1.2 Chemicals

- Acetonitrile AR grade	Merck
- Acetonitrile HPLC grade	Merck
- Dichloromethane AR grade	J.T. Baker
- Ethanol AR grade	J.T. Baker
- Ethylacetate AR grade	J.T. Baker
- Haxane AR grade	J.T. Baker
- Methanol AR grade	J.T. Baker
- Methanol HPLC grade	Merck
- Sulfuric and AR grade	Merck
- TLC precoated plate Silica gel 60 F_{254}	Merck
- TLC precoated plate RP-18 F_{254}	Merck
- Whatman No.1 paper	Whatman

1.3 Chromatographic materials

1.3.1 Silica gel 60 for column chromatography, 63-200μm, Merck, Germany, for the separation of less polar substance i.e., phytosterols and phytosteryl glucosides, ceramides and glycoglycerolipids

1.3.2 Thin-layer chromatography (TLC)

Normal phase : Silica gel 60 F_{254}, pre-coated on TLC aluminium sheets 20x20 cm, layer thickness 0.25cm, Merck, Germany

Reversed phase : RP-8, RP-18 F_{254} pre-coated on TLC glass plate, 5x20 cm, layer thickness 0.25 cm, Merck, Germany

1.3.3 Lobar® RP-8, Lobar RP-18, size A glass column with inner diameter 10 mm, and 240 mm long, Merck, Germany

1.4 Reference substances

1.4.1 SC1, the previously isolated substance by Chanchai Sadsaengchan, was structurally revised to bryonolic acid.

1.4.2 TC1, the previously isolated substance by Weena Jiratchariyakul, was identified as a mixture of cucurbitacin B and dihydrocucur - bitacin B.

1.4.3 GC, a gift from Siripen Jarikasem, was identified as a mixture of β-sitosterol and stigmasterol.

1.5 Chromatographic instruments

1.5.1 Low-pressure liquid chromatography (LPLC)

Peristaltic pump (WIZ), Isco

Fraction collector (Jr foxy), Isco

UV detector (UA-6), Isco

6-Ported valve for sample injection

1.5.2 High-pressure liquid chromatography (HPLC) Waters Model 510

High pump Water Model 510

Automated gradient controller

Detector water 484 UV detector

Automatic injector WISP 710 B

Column chromatography (400 ml, 5 cm, i.d.× 100 cm length)

Column chromatography (100 ml, 2 cm, i.d.× 100 cm length)

1.6 Instrument for physical and structure identification

1.6.1 Melting point apparatus (Electrothermal 9100, Eng. Ltd.)

1.6.2 UV spectrophotometer (Hitachi, U-3200 spectrophotometer)

1.6.3 Magna-IR spectrophotometer (550 Nicolet)

1.6.4 NMR spectrometer, (Avance 400), Chulabhorn Research Institute

1.6.5 Mass Spectrometer,(Hitachi M-60 and with a Fison VG trio 2000 mass spectrometer) Chulalongkorn Institute Research

1.7 Other instruments

Soxhlet apparatus	
Rotary evaporator	Eyela, Japan
Ultrasonic bath	Sonorex, Germany

Refrigerated centrifuge	Sorvall, USA
Fraction collector Retriever II	Isco, USA
Laboratory mill,	Thomas Wiley, USA

2. Method

2.1 Extraction

The powdered root was successively extracted in a Soxhlet apparatus with petroleum ehther (40-60 °C), dichloromethane and ethanol, respectively (Fig.16). The extracts were concentrated under reduced pressure. Solvents were removed at 40 - 50 °C by rotary evaporator. The yields of each extract was recorded.

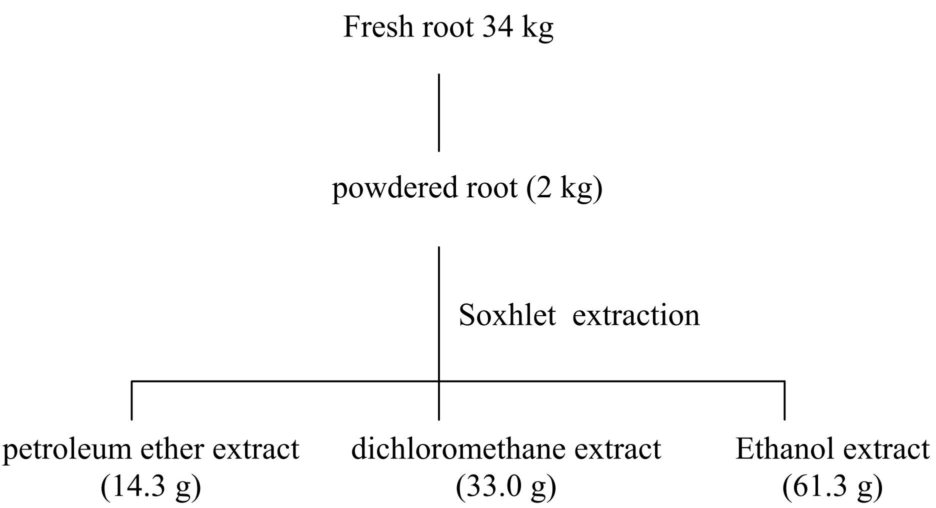


Figure 16 Soxhlet extraction of *T. cucumerina* root

The fruit juice 2 l from fresh fruit 21 kg was partitoned with diethyl ether, ratio 1:1. The organic phase was concentrated under reduced pressure. Solvent was removed at 40-50 °C by rotary evaporator (Fig.17). The yields of the extract were recorded.

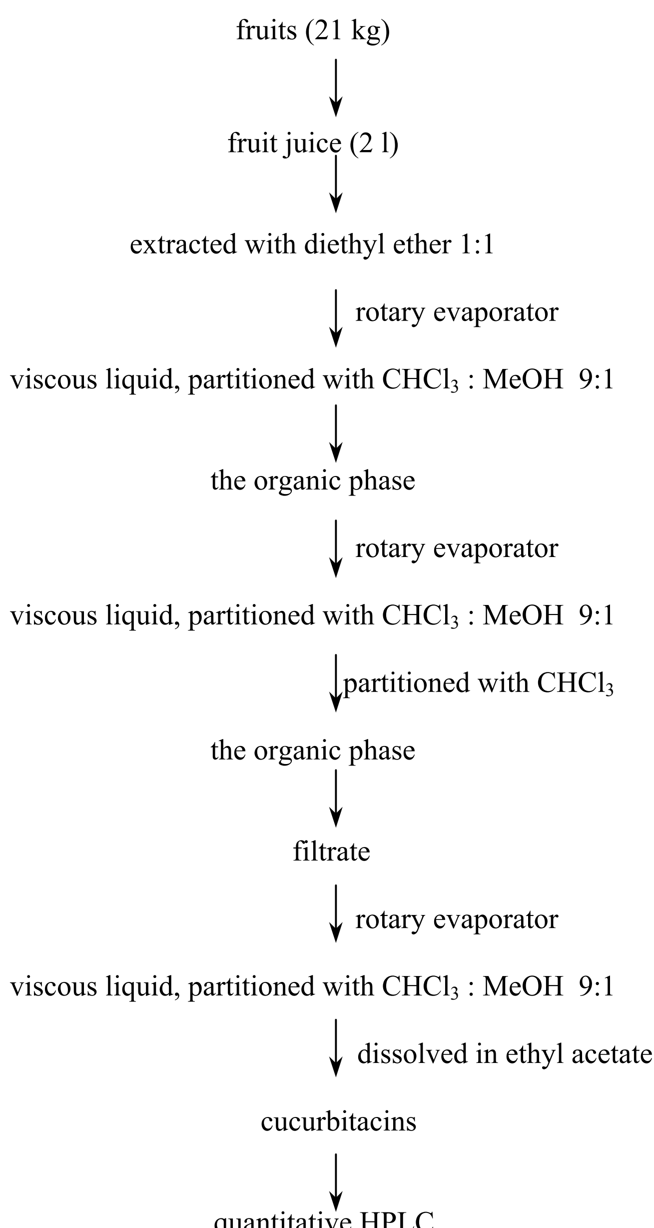


Figure 17 The extraction of *T. cucurmerina* fruit juice

2.2 The Isolation

2.2.1 Column chromatography

The dichloromethane extract (33 g) produced the crystalline substance during the concentration under the reduced pressure. After the filtration, SK1 was obtained, 1.23 g. The filtrate was further evaporated until dryness. The viscous extract weighed 20.04 g. It was mixed with silica gel (silica gel 60, 230-400 mesh ASTM) and stirred on the water bath. The extract granules were chromatographed on the silica gel column, which was prepared by wet method.

The isolation of substances from dichloromethane extract was achieved using column chromatography. The column was eluted with dichloromethane : methanol (100%→95%), gradiently (Fig. 19).

Fr. 1-7 0.002 g was chromatographed on silica gel column, which was eluted with dichloromethane-methanol (100:0→ 100:2→ 100:4→100:5), gradiently. Fraction of 10 ml were collected and combined 50 ml each. Four combined fractions, Fr.2 (5-8), Fr.5-3 (11-16), Fr.5.5 (35) and Fr.6 (24-25) were obtained.

Fr.2 (5-8) 0.23 g was rechromatographed on a silica gel G 60 column, with hexane-ethyl acetate (90:20) gradiently. Fractions of 50 ml were collected and the fraction combinations were monitored on TLC. Four combined fractions were obtained i.e., Fr.2.1 (1-6), Fr.2.2 (7-9), Fr.2.3 (10-17), Fr.2.4 (18-19), Fr.2-5 (20).

2.2.1.1 Isolation of SK1

SK1 was obtained from both dichloromethane and ethanolic extracts. After the filtration, the crystalline of compounds (1.40 g) was obtained. It was further purified by recrystallizing in methanol and SK1 (1.23 g) was formed (Fig.18).

2.2.1.2 Isolation of SK2 and SK3 (Fig. 23)

Fr.5 (0.9720 g) was rechromatographed on a silica gel G60, with hexane-ethyl acetate (90:10), gradiently. Fraction of 10 ml were collected and combined, i.e., Fr. 5.1 (1-5), Fr. 5.2 (6-10), Fr. 5.3 (11-16), Fr. 5.4 (17-34) and Fr. 5.5 (35).

Fr. 5.3 was concentrated and recrystallized in methanol. It produced SK2 (50.1 mg), which gave the conspicuous pink spot with sulfuric acid spray reagent, on TLC.

Fr. 5.5 was chromatographed on silica gel G 60 column (75 ml, 1.5 cm, i.d. x 30 cm, length), and eluted with hexane-ethyl acetate (90:10), gradiently. Fractions of 10 ml were collected and combined. The fraction combinations were examined on the TLC. It gave the eminent pink spot with sulfuric spray reagent. One well separated component, SK3 (6.8 mg) was obtained.

2.2.1.3 Isolation of SK4 and SK5 (Fig. 19)

Fr.6 (24-25), 0.11 g was rechromatographed twice on silica gelG 60 columns (100 ml, 20 cm, i.d. x 30 cm, length), and eluted with dichloro methane -methanol, (100:0→ 100:2→100:4→100:5) gradiently. Fractions of 10 ml were collected and combined. One well separated component, SK4 (50 mg), was obtained. Compound SK4 was washed with hot methanol and further purified using lobar RP-18 column (LPLC) and eluted with acetonitrile:water (55:45).

The isolation of two related compound mixture was achieved using column chromatography with lobar columns (RP-8, RP-18 and Si-60).

The compound mixture appeared as two blue spots with sulfuric acid spray reagent on RP-18 TLC, using methanol-water (9:1) as a solvent system. Further purification was carried out using low pressure liquid chromatography (RP-18 lobar column). The solvent system of acetonitrile-water (55:45) was used as an eluent, at the flow rate of 1 ml/min and equipped with 280 nm UV detector. The SK5 (2 mg) was eluted in 75-84 min.

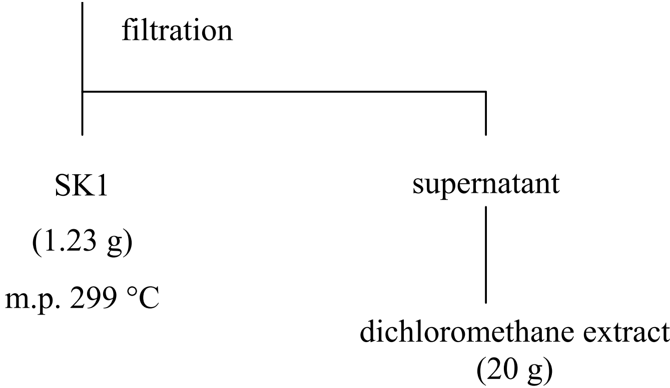


Figure 18 Isolation of SK1

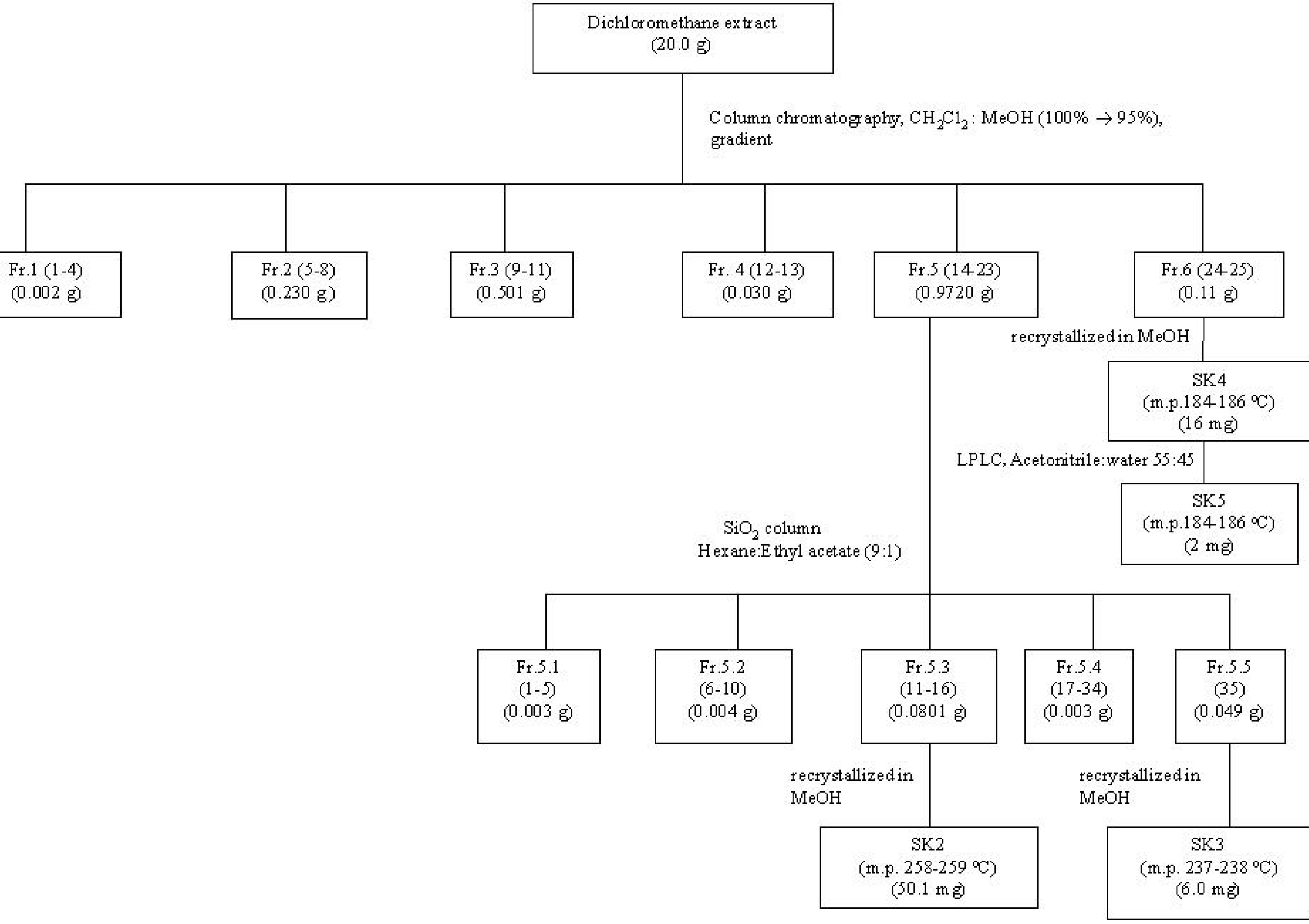


Figure 19 Isolation of SK2, SK3,SK4, and SK5 from dichloromethane root extract

3. Identification

3.1 Melting points

The melting points of the isolated compounds were determined by the Electrothermal 9100 (ENG., LTD), Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University

3.2 Ultraviolet spectra

The ultraviolet spectra of compounds SK1-SK6 were measured with a UV spectrophotometer (U-3200 spectrophotometer, Hitachi) at the Central laboratory, Faculty of Pharmacy, Mahidol University.

3.3 Infrared spectra

The IR-spectra in KBr disc of the isolated compounds were measured with a FT-IR spectrometer (Magna-IR^{TM} spectrometer 550 Nicolet) at the central laboratory of the Faculty of Pharmacy, Mahidol University.

3.4 Nuclear magnetic resonance spectra

The 1D- and 2D-NMR spectra were measured on Chulabhorn Research Institute, Thailand, using following NMR solvents, $CDCl_3$ (for SK4 and SK5), $CDCl_3$: CD_3OD, 1:1 (SK1, SK2 and SK3) and the tetramethylsalisilane (TMS) as internal standard.

3.5 Mass spectra

The EIMS were determined at Scientific and Technological Research Equipment Center, Chulalongkorn University, Thailand.

Part II Bioactivity

The *in vitro* antimalarial activity assessment of of dichloromethane root extract, SK1 and fruit juice of *T. cucumerina*, was conducted at the Department of Microbiology, Faculty of Science, Mahidol University, under the supervision of Professor Dr. Peerapan Tan-ariya.

1. Antimalarial Activity

1.1 Malaria parasites

The strain of *P. falciparum* namely K1 was used in this study. The K1 strain, which is highly resistant to both chloroquine and pyrimethamine but sensitive to mefloquine and artemisinin derivatives, was originally isolated from a malarial patient who was a resident of Kanchanaburi Province, Western of Thailand in 1979 by Professor Sodsri Thaithong at Chulalongkorn University. K1 strain was continuously cultured at Department of Microbiology, Faculty of Science, Mahidol University by using a candle-jar technique according to the method of Trager and Jensen (81) with slight modification.

1.2 Culture medium

The RPMI medium was routinely used for *in vitro* culture of *P. falciparum* throughout this study. It was prepared by dissolving 10.4 g of RPMI 1640 powder containing *L*-glutamine but without sodium bicarbonate (GIBCO) and 5.94 g of HEPES buffer (SIGMA) in 960 ml of double distilled water. Two milliliters of gentamicin sulfate (40 mg/ml) was added to the medium solution before sterilization by filtration through a Millipore filter of 0.45 μm porosity. One hundred milliliters of the sterile medium was transferred into sterile glass bottle as a stock medium, which was stored for a period up to one month at 4°C.

Each stock medium (100 ml) was added with 4.2 ml of 5% (w/v) $NaHCO_3$ (SIGMA) to give a pH of 7.4, 1 ml of 10 mM *D*-glucose (SIGMA), and 1 ml of 2 mM *L*-glutamine (SIGMA). This medium was referred to as complete medium without serum (C-RPMI without serum). The complete RPMI medium was supplemented with 10% (v/v) heat inactivated human serum (groups A, B and O) before being used

for cultivation and this medium was referred to as complete medium with serum (C-RPMI with serum). The medium was stored at 4°C and used within one month.

1.3 Normal human serum

Normal human serum of all blood groups was collected from healthy donors who had no history of malarial infection and had never been to malarial endemic areas. Moreover, donor did not receive any drug within two weeks proir to blood collection. Sera groups A, B and O were used for routine culture of *P. falciparum*, whereas group AB serum was used for assessment of antimalarial activity.

For blood collection, before using the sterilized double blood bag (Terumo®, Japan) the citrate phosphate dextrose (CPD) solution in the bag was discarded. Fresh blood was collected intravenously through a bag and allowed to clot at room temperature for 30 min. The bag was stored at 4°C overnight to allow complete clotting. The clotted blood was centrifuged at 750 *g* for 30 min (4°C) to separate the fresh serum and the clotted blood. This fresh serum was transferred aseptically to a reservoir bag and inactivated at 56°C for 30 min in a waterbath. Approximately 10-12 ml of fresh serum was distributed aseptically into screwed-cap sterile tubes, stored at -20°C and used within 3 months.

1.4 Non-infected erythrocytes

Human erythrocytes were collected from healthy donors with type "O" blood group who had no experience of malaria infection and had never been to malaria endemic areas. Approximately 300 ml of blood was collected in a sterile bag (Terumo®, Japan) containing CPD solution as an anticoagulant. After thoroughly mixing, blood suspension was aseptically dispensed as 50 ml aliquots into sterile glass bottles, stored at 4°C and used within one month.

For cultivation, 50% non-infected erythrocytes suspension was prepared as follows. Ten milliliters of whole erythrocytes was transferred aseptically to a 15 ml centrifuge tube and centrifuged at 750 *g* for 15 min (4°C).Supernatant and buffy coat were removed carefully, and then the packed erythrocytes were washed twice with C-RPMI without 10% normal human serum. For use in the cultivation, the washed

packed erythrocytes was resuspended with an equal volume of C-RPMI with 10% normal human serum to make 50% (v/v) non-infected erythrocytes.

1.5 Cultivation of *P. falciparum* and maintenance of continuous culture

According to the continuous culturing technique described by Trager and Jensen (81), the parasites were routinely maintained as continuous culture in 60×15 mm disposable plastic petri dishes (Nuncon®, Denmark) containing C-RPMI medium with 10% normal human serum. The initial parasitemia was 0.5-1.0% with 3% cell suspension in a total volume of 5 ml. The inoculated petri dishes were placed in a candle jar (a glass desiccator equipped with a stop-cock and a white candle). When the candle went out, the stop-cock was immediately closed. By this method, an atmosphere with low oxygen and high carbon dioxide content, i.e., 17% O_2, 3% CO_2 and 80% N_2 was produced. The candle jar was incubated at 37°C in an incubator.

The medium was changed daily, old medium being removed with a pasture pipette. The thin blood smear was made and stained with Giemsa stain (Appendix). Percentage of parasitemia was estimated by counting the infected erythrocytes in a total of 10,000 erythrocytes. Subculturing was performed when parasitemia is higher than 5-6%. Briefly, the infected erythrocytes were resuspended to make 50% (v/v) suspension in C-RPMI with 10% normal human serum. The parasitemia was reduced to 0.5-1% by diluting with 50% non-infected erythrocytes. Before transferring the suspension into the petri dishes, percent cell suspension were reduced from 50% to 3% with C-RPMI medium with 10% normal human serum. Approximately 5 ml of this suspension was then placed into a petri dish and cultured as described above.

1.6 Synchronization of culture

Generally, continuous culture of *P. falciparum* results in a loss of synchronicity characteristic, which can complicate the evaluation of experimental results. Thus, it is important to start all experiments with a synchronous ring. Briefly, asynchronous cultures of K1 strain was collected into centrifuge tube and centrifuged at 500 g for 10 min (4°C). After the removal of supernatant, 5% (w/v) sterile *D*-sorbital was added to the packed erythrocytes at the ratio 4:1 and gently mixed. The mixture was incubated at 37°C for 20 min in the waterbath. During the period of incubation, only erythrocytes harboring late trophozoites and schizonts were selectively lysed, leaving only the erythrocytes infected with ring stage among non-

infected erythrocytes. Sorbital was removed by centrifugation and the packed erythrocytes was then washed twice with C-RPMI without 10% normal human serum. After the final washed, the packed erythrocytes was adjusted to 3% cell suspension and cultured in a candle jar as described above.

1.7 Freezing and storaging parasites

P. falciparum parasites in continuous culture were harvested whenever at least 5% ring stage was obtained. The culture suspension was collected into a centrifuge tube and centrifuged at 500 *g* for 7 min (4°C). After the removal of supernatant, the packed erythrocytes was resuspended in an equal volume of freezing solution (sorbital, 0.9% NaCl and 99% glycerin) (Appendix). An aliquot of 1-1.5 ml of the suspension was carefully pipetted into a cryopreserved tube and frozen at –196 °C in a liquid nitrogen tank. The frozen parasites were stored as stock culture.

1.8 Thawing parasites

Frozen cryopreservative tubes were removed from liquid nitrogen tank and thawed at 37°C in the waterbath. The suspension was transferred to a sterile centrifuge tube, to which an equal volume of sterile 3.5% sodium chloride solution was added, and then centrifuged at 500 *g* for 7 min (4°C). After the removal of supernatant, the packed erythrocytes were washed three times with C-RPMI without 10% normal human serum. After the final wash, the packed erythrocytes was resuspended in C-RPMI with 10% normal human serum to make 3% cell suspension, and transferred into a sterile petri dish and cultured in a candle jar as described above.

1.9 Preparation of parasites inoculum

Up to 5% ring stage *P. falciparum* obtained by several rounds of sorbital-lysis were pooled aseptically to a 15 ml centrifuge tube and centrifuge at 500 *g* for 7 min (4°C). After the removal of supernatant, the packed erythrocytes were resuspended in an equal volume of C-RPMI medium without 10% normal human AB serum to make a 50% (v/v) cell suspension. The 50% non-infected erythrocytes was added to adjust percentage of parasitemia approximately 0.5%. The erythrocytes suspension was diluted to 3% cell susension with C-RPMI medium with 20% normal human AB serum. This mixture of suspension is referred to as parasite inoculum.

1.10 Antimalarial activities of extract solutions and pure compounds

1.10.1 Assessment of antimalarial activity of *T. cucumerina* dichloromethane root extract SK1 and fruit juice against K1

Fifty microliters of each working solution of extract samples was inoculated into each of the duplicate wells of row A and B of the microtiter plate (Nuncon®, Denmark). Fifty microliters of RPMI medium was added in each well of row B to row H. The working solution of root extract, fruit juice and SK4 in DMSO at concentration of 80 μg/ml was 2-fold serially diluted with RPMI medium to the dilution of 1:2, 1:4, 1:8, 1:16, 1:32 and 1:48 (from wells B-H). The inoculum of K1 strain of *P. falciparum* was prepared as 3% cell suspension with 0.5% parasitemia in C-RPMI with 20% human AB serum as described above. Fifty microliters of each parasite inoculum was added into each well containing 50 μl of working solution (well A) and diluted extract samples (wells B-H). The final volume in each well of the microtitation plate was 100 μl. For control wells, 50 μl of the dissolving solvent and 50 μl of RPMI were pipetted (1/100 DMSO) in drupicate wells of microtriter plate placed in candle jar with an atmosphere of 17% O_2, and 80% N_2, and incubated at 37° C for 48 h.

1.10.2 Evaluation of results

After the 48 h of incubation period, the red cell pellet at the bottom of each well was collected and smeared onto a microscopic slide to make a thin blood film. The slide was then fixed with absolute methanol for 1 min and stained with Giemsa stain for 30 min (Appendix). The slides were washed and allowed to dry at room temperature. The thin blood films were inspected under the light microscope. The number of infected cells with normal parasites on each slide was counted per 10,000 red cells. The EC_{50} (50% effective concentrations) of the testing compounds was determined by plotting the log of the concentrations used of the test solution versus the percentage of the numbers of infected erythrocytes in experimental wells comparing to the control wells (parasite without drug).

2. Anti-Papilloma Virus Activity

2.1. Cell culture (82)

The tissue sections of normal and papilloma laryngeal were washed at least 3 times in Minimum Essential Medium (MEM) with Earle's salts supplemented with antibiotics. The tissues were cultured in Dulbecco's Modified Earle's Medium (DMEM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) in a humidified 5% CO_2 incubator at 37 °C. The confluent primary cells were passages every 5-7 days with 0.1% trypsin-EDTA solution. Only cells in exponential phase of growth were used in the experiments.

2.1 Determination of viable cell number

The cell cultures were trypsinized with 0.1% trypsin-EDTA solution for 1-3 min. after removal of the old medium. After observation unattached cell, fresh medium was added to stop reaction of trypsin. Cell suspension was mixed gently by pipetting and transferred to eppendorf tube. The cell number was mixed equal volume with 0.4% trypan blue and transferred to haemacytometer. All viable cells located in four squares in chamber of haemacytometer (Brightline Improved Neubauer 0.1 mm deep, BOECO, Germany) were counted as the number of cell below.

Then, the number of cell = $X/4 \times 2 \times 10^4$

where $X = X_1 + X_2 + X_3 + X_4 \ / \ 4$

$X_1 + X_2 + X_3 + X_4$ = total cell counts obtained from column number 1, 2, 3, 4 respectively

2 = dilution factor

2.3 Sulphorhodamine B (SRB) assay

In the experiments, the viable cells were determined using the sulphorhodamine B (SRB) assay as previously described (83).

2.3.1 Cell fixation

This assay was used for determining the number of cells which grown in a 96-well tissue microtiter plate. Following removal of the growth medium from a 96-well microtiter plate, cultured cells were fixed with 100 µl/well of 20% cold trichloroacetic acid (TCA) to produce a final TCA concentration of approximately 12%. Then, the cultures were incubated at 4 °C for 30 minutes and washed five times

with distilled water to remove TCA, growth medium, and serum protein. Plates were air dried and stored at room temperature or 4 °C until use. Background optical densities were measured in wells incubated with growth medium without cells.

2.3.2 SRB assay

In the experiments, the variable cells were determined using the sulphorhodamine B (SRB) assay as previously described below.

TCA-fixed cells were subsequently stained for 30 minutes at room temperature with 100 µl/well of 0.4% (w/v) SRB (Sigma chemical Co.,Ltd.) in 1% acetic acid. Excess SRB was removed and the cultures were quickly rinsed five times with 1% acetic acid which was poured directly into a culture well from a beaker to remove unbound dye. This procedure permitting rinsing to be performed quickly so that aborption of protein-bound dye did not occur. Residual wash solution was removed by sharply flicking plates over a sink. After being rinsed, the cultures were air dried. Following that, bound dye was solubilized with 200 µl/well of 10 mM unbuffered Tris base (pH 10) for 5 min. on a gyratory shaker. The amount of bound SRB released was determined by measuring the optical density (OD) at 550 nm using Microplate Reader Model Anthos II.

2.3.3 To evaluate the growth characteristics of cell cultures

The study was undertaken in order to see the growth characteristic of cell culture used in the experiment. The growth of cell was performed on 96-well microtiter plate for 5 consecutive day. To prove that the SRB assay was a suitable assay for quantitative of cell numbers in a 96-well plate in growth curve and cytotoxicity assays in this study, the SRB calibration curve was established. This assay was performed to see the correlation of absorbance (OD) for the SRB assay and cell numbers by plating of cell culture in a 96-well microtiter plate. Briefly, cells were seeded at concentrations ranging from 1.0 x 10^5 cells/ 190 µl/well. A standard curve was plotted the number of cell against the corresponding absorbance at 550 nm.

2.3.4 Cytotoxicity assay

The growth inhibitory activity of SK1 on papilloma primary cells was conducted in a 96-well microtiter plate and the number of viable cells was determined after treatment using the SRB assay based on the standard procedure. Briefly, cells were seeded at a density of 5.0 x 10^4 cells/ 190 µl/well from row A to

row H. Then 10 µl of various concentrations of the test substances or DMSO at concentration 10% were added and cells were cultured for a further 4 days. Eight wells were prepared for each sample concentration. Then, the number of viable cells was determined by cellular protein content with sulphorhodamine B. Six wells were kept as control cultures, i.e., cells were not exposed to test compound. To test the effect of solvent (DMSO) on cell growth, 2 wells of untreated control was prepared in the same way as the treated cells, but not contained any test substances. The degree of inhibition of growth provides an indication of toxicity. The 50% of viable cell was calculated for ED_{50}.

Part III HPLC Quantitative Analysis

1. Chemicals and Materials

1.1 Chemical

Acetonitrile	HPLC grade	Merck, Germany
Methanol	HPLC grade	Merck, Germany
Water	HPLC grade	Merck, Germany

1.2 Materials

5µm Hypersil® BDS C18, 250 x 4.6 mm i.d

Javelin® BDS guards column

HyperFil membrane filter (0.45 µm)

Chromabond C18

Pipet tips (25, 200, 1000 µl)

1.3 Instruments

High-Pressure Liquid Chromatography (HPLC)

- Water Model 510 pump
- Water 484 UV detector
- Automatic controller WISP 710B
- Water 745B Data Module

Pipetman 25, 200, 1000 µl (Gibson Medical Electronics, France)

Ultrasonic bath (Sonorex)

2. Methods

2.1 Preparation of the stock and standard solutions

2.1.1 Stock solution

The cucurbitacin B (SK5), 5 mg, was accurately weighed into 5 ml volumetric flask. Added 2 ml of methanol and sonicated until the clear solution was obtained. Adjusted to volume with methanol. Cucurbitacin B was used as the lead compound.

2.1.2 Standard solution

The stock solution was quantitatively diluted with methanol to concentrations of 20, 50, 75, 100, 125 and 150 µg/ml immediately before use.

2.2 Preparation of sample solutions

2.2.1 The root extract

Accurately weighed 0.2 g of the extract, dissolved with 2 ml 80% ethanol, sonicated in ultrasonic bath for 15 min., and filtered through filter paper. The clear solution was cleaned up by Chromabond® which had been activated immediately before use. Eluted with 100% distilled water (6 ml), distilled water-methanol, 1:1 (6 ml) and absolute methanol (6ml). The methanol eluted fraction was added into 10 ml volumetric flask and the volume was adjusted with methanol.

2.2.2 The fruit juice

Pipet 2 ml of fruit juice into 10 ml volumetric flask. Add 2 ml of methanol, sonicate for 15 min. and adjust to volume with methanol.

2.3 HPLC procedure

The standard and sample solutions were injected into HPLC column using the following chromatographic conditions:

Column :	5µm HYPERSIL® BDS C18
Solvent system :	acetonitrile-water (40:60)
Flow rate :	1 ml/ min
Detection :	210 nm
Volume :	20 µl

The chromatograms and retention times were recorded.

2.4 Validation of HPLC quantitative method

2.4.1 System suitability test

The suitability test for isocratic system was determined by using four parameters:- precision (%CV), number of theoretical plates (N), tailing factor (TF), and resolution factor (R_s)

2.4.1.1 Precision

Three replicate injections were performed using isocratic system. The standard solutions were prepared as described in section 2.1. The chromatographic conditions were proceeded as in section 2.3.

The precision was determined in term of percentage of coefficient of variation (%CV) or relative standard deviation (RSD), which was calculated using the following equation:

$$\%CV = [SD/\overline{X}] \times 100 \qquad [1]$$

where

$\overline{X}$ = mean value

SD = standard deviation

2.4.1.2 The column coefficiency (number of theoretical plates, N)

The number of theoretical plate (N) was calculated from the following equation:

$$N = 5.554 \times [t_R / W_{1/2}\ h]^2 \qquad [2]$$

where

t_R = the retention time of peak

$W_{1/2}$ = the peak width at the half height.

2.4.1.3 Tailing factor (TF)

Tailing factor (TF) was calculated using the following equation:

$$TF = W_{0.05}/2 \times A_{0.05} \qquad [3]$$

where

$W_{0.05}$ = the width of peak at 5% of the peak height

$A_{0.05}$ = the distance from peak maximum to the leading edge of the peak, the distance being measured at a point 5% of the peak height from baseline.

2.4.1.4 The resolution factor (R_s)

The resolution factor (R_s) was calculated by using the following eqution :

$$R_s = 2(t_2-t_1)/(W_2+W_1)$$

where t_1 = the retention time of the first peak

t_2 = the retention time of the second peak

W_1 = the peak width of the first peak

W_2 = the peak width of the second peak

2.4.2 Method validation

In this study, typical parameters to be considered in the method validation study included accuracy (recoveries), precision (%CV) and linearity (r^2).

2.4.2.1 Accuracy

The accuracy of the proposed method was evaluated as the percentage of recovery by using the standard addition technique. The percentage of recovery was calculated as follows :

$$\% \text{ Recovery} = \left[\frac{C_S - C_A}{C_A^*} \right] \times 100$$

where C_S = measured concentration of spiked sample

C_A = measured concentration of unspiked sample

C_A^* = concentration of spiking solution

2.4.2.2 Precision

Precision was evaluated by intra-day and inter-day precision. The sample solutions of the homogeneous sample were prepared by the consecutive injections of five sample solutions on the same day. Inter-day precision was performed on the same way of intra-day test, but on three different days. The relative standard deviation (RSD) or percentage of coefficient of variation (%CV) was calculated.

2.4.2.3 Linearity

Five final concentrations of standard solution were used for evaluation of linearity. The standard calibration plot was constructed by least-square linear regression of the peak area of the mixture of cucurbitacin B (SK5) versus concentration.

2.4.2.4 Limit of detection and quantitation

The calculation is based on the standard deviation of the response (σ) and the slope of the calibration curve (S) at levels approaching the limit according to equations 4 and 5:

$$LOD = 3.3 \, (\sigma/S) \qquad [4]$$

$$LOQ = 10 \, (\sigma/S) \qquad [5]$$

The standard deviation of the response can be determined based on the standard deviation of the blank, on the residual standard deviation of the

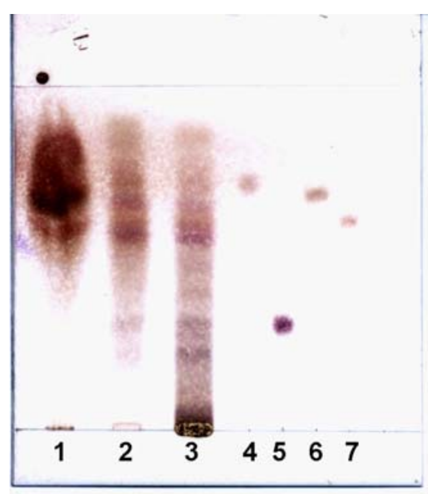
regression line, or the standard deviation of y intercepts of regression lines. The ICH calculation method can reduce the bias that sometimes occurs when determining the S/N. This bias can result because of differences in opinion about how to determine and measure the noise.

CHAPTER V
RESULTS

Part I Phytochemistry

1. The extraction

The powdered drug (2.0 kg) from the root (34 kg fresh) was successively extracted in a Soxhlet apparatus using petroleum ether (40-60°C), dichloromethane and 95% ethanol, respectively (Fig. 20). This yielded 14.3 g of petroleum ether extract, 33.0 g of dichloromethane extract and 61.3 g of ethanolic extract, corresponding to 0.72, 1.65 and 3.07 %yields of extractives (based on powdered drug) (Table 8), respectively. The fractionation data as well as the constituents of the isolated compounds were shown in Table 9. The thin-layer chromatograms were shown in Fig.21.



Solvent system : Chloroform : methanol, 9:1

Absorbent : silica gel GF_{254} aluminum sheet

Detection : spray with 10 % ethanolic sulfuric acid and heated on the hotplate until the colors develop

1 = petroleum ether extract

2 = dichloromethane extract

3 = ethanol extract

4 = β-sitosterol

5 = β-sitosteryl glucoside

6 = TC1(a mixture of cucurbitacin B and dihydrocucurbitacin B)

7 = SC1(bryonolic acid)

Figure 20 Thin-layer chromatogram of *T. cucumerina* root extract

2. The isolation

The dichloromethane root extract, a dark-brown viscous liquid, was fractionated by column chromatographic technique (Fig. 22). The isolation was performed repeatedly by conventional and low-pressure column chromatography. Compounds SK1, SK2, SK3, SK4, and SK5 were isolated (Table 9).

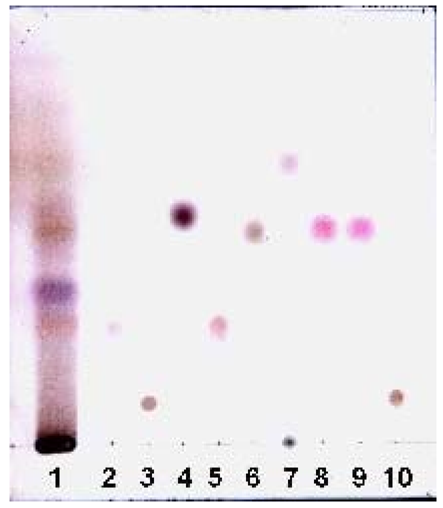
Table 8 Substances isolated from dichloromethane root extract

Compound	Weight (g)	% yield (w/w) (based on powdered drug)
SK1	1.230	$6.2x10^{-2}$
SK2	0.0501	$2.5x10^{-3}$
SK3	0.0068	3.4×10^{-4}
SK4	0.016	$8.0x10^{-4}$
SK5	0.002	$1.0x10^{-4}$

Table 9 Adsorption chromatography of the isolated compounds

Compound	solvent system	Compound type
SK1	Hexane:EtOAc 6:4 (Fig. 24)	pentacyclic triterpene
SK2	Hexane:EtOAc 6:4	phytosteryl glucoside
SK3	Hexane:EtOAc 6:4	pentacyclic triterpene
SK4*	$CHCl_3$:MeOH 9:1 (Fig. 3)	cucurbitacin (triterpene)
SK5	$CHCl_3$:MeOH 9:1	cucurbitacin (triterpene)

* The separation was achieved on the reversed phase using acetronitrile:water (6/4) as a mobile phase.



Solvent system : Hexane : Ethyl acetate, 6:4

Adsorbent : Silica gel GF_{254}

Detection : spray with 10 % ethanolic sulfuric acid and heated on the hotplate until the colors develop

1 = dichloromethane extract

2 = SC1

3 = TC1 (a mixture of cucurbitacin B and dihydrocucurbitacin B)

4 = GC (a mixture of β-sitosterol and stigmasterol)

5 = SK1

6 = Fr.1

7 = Fr.2

8 = SK2

9 = SK3

10 = mixture of SK4 and SK5

Figure 21 Thin-layer chromatogram of the isolated compounds from dichloromethane root extract

3. The identification

The structure elucidation of the isolated substances from *T. cucumerina* root were accomplished using NMR spectroscopy. The isolated substances included the followings :

SK1 : D:C-friedoolean-8-en-3β-ol-29-oic acid (bryonolic acid)

SK2 : 5α-stigmasta-7,22-dien-3β-*O*-glucopyranoside (chondrillasteryl glucoside)

SK3 : D:C-friedoolean-8-en-3-en-29-oic acid (bryononic acid)

SK4 : 25-(acetoxy)-2β, 16α, 20β-trihydroxy-9β-methyl-19-nor-10α-lanosta-5, 23-dien-3,11,22-trione (cucurbitacin B)

SK5 : 25-(acetoxy)-2β, 16α, 20β-trihydroxy-9β-methyl-19-nor-10α-lanosta-5-en-3, 11, 22-trione (dihydrocucurbitacin B)

3.1 SK1

Bryonolic acid $C_{30}H_{48}O_3$ $M_R = 456$

D : C-friedoolean-8-en-3β-ol-29-oic acid

The column chromatography of the dichloromethane root extract resulted the isolated compound SK1 in forms of the floating flakes (1.40 g) and the amorphous solid, which was further recrystallized in methanol and yielded the white crystalline needles (1.23 g). SK1 had the melting point of 299-300 °C, and produced the purple color with 10% ethanolic sulfuric acid spray reagent. The UV spectrum of SK1 showed the maximum absorption in methanol at 207 nm. The EIMS (Fig. 22) showed the molecular ion peak at 457.2 amu, which was resulted from one ^{13}C in SK1, i.e. $^{12}C_{29}$ ^{13}C $H_{48}O_3$. SK1 possessed the molecular weight of 456, $C_{30}H_{48}O_3$. The base peak at m/z 439.2 amu, the ion peaks at m/z 411.4 and 409.4 amu were fragmented by the splitting of the chemical bonds as shown in Fig. 23.

The IR spectrum (Fig. 24) confirmed the presences of the carboxylic acid (-COOH) by ν(C= O) 1691cm^{-1}, accompanying with ν(O-H) around 3000cm^{-1}, the hydroxy group (-OH) by ν (O-H) 3467 cm^{-1}, the symmetrical and asymmetrical stretchings of CH_3 - and CH_2 - groups around 3000 cm^{-1} and the double bond by δas (CH_3), δs (CH_2), 1467 cm^{-1}.

The ^{13}C {^{1}H} spectrum (Fig. 25) together with the DEPT spectrum (Fig. 26) revealed the amount and types of carbons in SK1. The compound contained

seven methyls (7×Q), eleven methylenes (11×T) three methines (3×D) and nine quarternary carbons (9×S). The assignment of ^{13}C chemical shifts (Table 10) was accomplished by the comparison with 20-epibryonolic acid (84).

The assignment of ^{1}H chemical shifts was performed using the HMQC (Tables 11) and COSY experiments. The positions of the carboxylic acid and the double bond were supported by HMBC correlations (Table 11).

The complete NMR data of SK1 was presented in Table 10. The structure of SK1 belonged to bryonolic acid (D:C-friedoolean-8-en-3β-ol-29-oic acid).

Table 10 ^{13}C NMR data of SK1 compared with the literature values

Signal	δ ^{13}C SK1 in $CDCl_3$: CD_3OD	20 – epibryonolic acid in DMSO-d_6 (84)
1	15.384 Q (C-24)	16.07 (C-24)
2	17.198 Q (C-27)	17.27 (C-27)
3	19.026 T (C-6)	18.89 (C-6)
4	19.649 Q (C-25)	19.73 (C-25)
5	20.482 T (C-11)	20.23 (C-11)
6	21.922 Q (C-26)	21.88 (C-26)
7	24.880 T (C-15)	24.58 (C-15)
8	27.290 T (C-2)	27.17 (C-2)
9	27.427 T (C-7)	27.67 (C-7)
10	27.730 Q (C-23)	28.19 (C-23)
11	29.638 T (C-12)	29.43 (C-12)
12	30.100 T (C-21)	29.85 (C-21)
13	30.440 T (C-19)	30.02 (C-19)
14*	30.755 S (C-17)	30.59 (C-17)
15*	30.983 Q (C-28)	31.06 (C-28)
16*	32.689 Q (C-30)	32.57 (C-30)
17	34.191 T (C-22)	34.02 (C-22)
18	34.865 T (C-1)	34.74 (C-1)
19*	36.860 T (C-16)	36.75 (C-16)
20*	37.018 S (C-13)	36.76 (C-13)
21	37.330 S (C-10)	37.16 (C-10)
22	38.591 S (C-4)	38.50 (C-4)
23	40.071 S (C-20)	39.44 (C-20)
24	41.664 S (C-14)	41.42 (C-14)
25	44.514 D (C-18)	44.24 (C-18)
26	50.372 D (C-5)	50.27 (C-5)
27	78.707 D (C-3)	76.89 (C-3)
28	133.767 S (C-8)	133.41 (C-8)
29	133.804 S (C-9)	133.89 (C-9)
30	182.004 S (C-29)	179.80 (C-29)

* The assignments were corrected according to the carbon types and the epimerisation at C-20.

Table 11 HMQC and HMBC correlations of SK1

C	δ ^{13}C SK1 in $CDCl_3$: CD_3OD	HMQC δ ^{1}H	HMBC δ ^{1}H
1	34.87 T	1.76, 1.12	0.95 (H-25), 3.20 (H-3)
2	27.29 T	1.74, 1.10	3.20 (H-3), 0.79 (H-24)
3	78.71 D	3.20	1.76 (H-1), 0.79 (H-24), 0.97 (H-23)
4	38.59 S		0.79 (H-24), 0.97 (H-23)
5	50.37 D	1.35	1.76 (H-1), 0.79 (H-24), 0.95 (H-25), 0.97(H-23)
6	19.03 T	1.92, 1.85	
7	27.43 T	1.54, 1.32	
8	133.77 S		0.96 (H-26)
9	133.80 S		0.95 (H-25)
10	37.33 S		
11	20.48 T	1.52, 1.33	
12	29.64 T	2.10, 0.95	0.890 (H-27)
13	37.02 S		0.890 (H-27), 1.51 (H-18)
14	41.66 S		0.890 (H-27), 0.96 (H-26)
15	24.88 T	1.50, 1.33	0.96 (H-26)
16	36.86 T	1.65, 1.35	1.03 (H-28)
17	30.76 S		1.03 (H-28)
18	44.51 D	1.51	0.890 (H-27), 1.03 (H-28), 1.69 (H-19)
19	30.44 T	2.40	1.51 (H-18)
20	40.07 S		1.51 (H-18), 1.20 (H-30)
21	30.10 T	2.00-2.20, 1.00-1.50	
22	34.19 T	2.00-2.20, 1.00-1.51	1.03 (H-28)
23	27.73 Q	0.98	
24	15.38 Q	0.79	
25	19.65 Q	0.95	
26	21.922 Q	0.96	
27	17.20 Q	0.90	1.51 (H-18)
28	30.98 Q	1.03	
29	182.00 S		1.20 (H-30), 2.00-2.20 (H-21), 2.40 (H-19)
30	32.69 Q	1.20	

Table 12 400 MHz ^{1}H-NMR data of SK1 in $CDCl_3$:CD_3OD (1:1)

H	δ ^{1}H ($CD_3OD/CDCl_3$)
1a	1.76 (1H) *m*
1b	1.12 (1H) *ddd* (17, 13, 5)
2a	1.74 (1H) *m*
2b	1.10 (1H) *m*
3	3.2 (1H) *dd* (11, 5)
5	1.35 (1H) *m*
6a	1.92 (1H) *m*
6b	1.75 (1H) *m*
7a	1.54 (1H) *m*
7b	1.32 (1H) *m*
11a	1.52 (1H) *m*
11b	1.33 (1H) *m*
12a	2.10 (1H) *m*
12b	0.95 (1H) *m*
15a	1.50 (1H) *m*
15b	1.36 (1H) *m*
16a	1.65 (1H) *m*
16b	1.35 (1H) *m*
18	1.511H) *dd* (16,4)
19	2.40 (2H) *d* (16)
21a, 22a	2.00-2.20 (2H) *m*
21b, 22b	1.00-1.45(1.00-1.50) 2H *m*
23	0.98 (3H) *s*
24	0.79 (3H) *s*
25	0.95 (3H) *s*
26	0.96 (3H) *s*
27	0.90 (3H) *s*
28	1.03 (3H) *s*
30	1.20 (3H) *s*

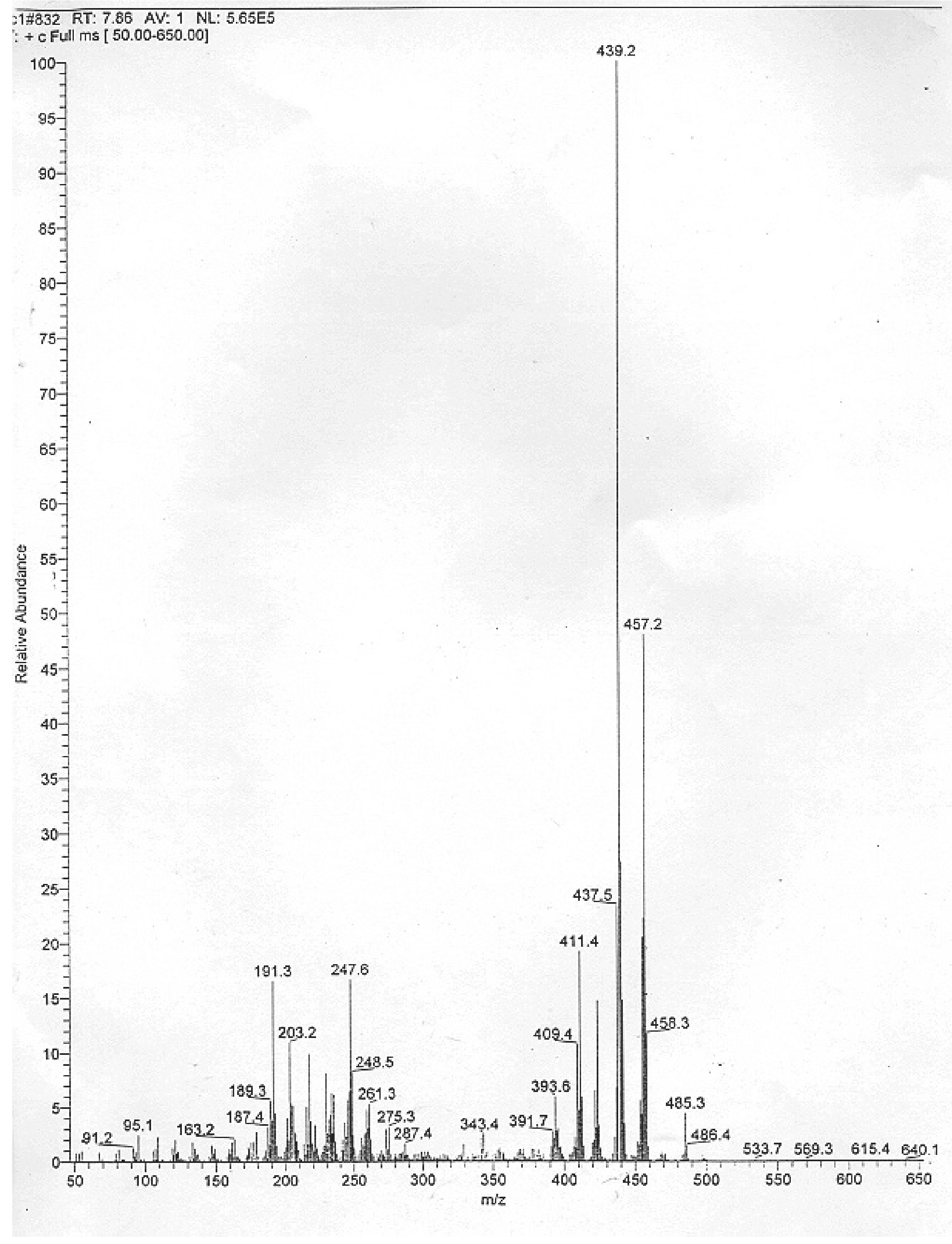


Figure 22 EIMS spectrum of SK1

COOH

HO

$C_{30}H_{48}O_3$

70 eV

COOH

HO

$C_{30}H_{48}O_3^{+\cdot}$ m/z 456

COOH

HO

$C_{30}H_{48}O_3^{+\cdot}$ m/z 456

-COOH$^{\cdot}$

$C_{29}H_{47}O^{+}$

m/z 411

RDA

-OH$^{\cdot}$ (α-split)

$C_{30}H_{47}O_2^{+}$

m/z 439

-CH_2O

(β-split)

$C_{29}H_{45}O^{+}$

m/z 409

$C_{15}H_{24}O^{+\cdot}$

m/z 220

-OH$^{\cdot}$

(α-split)

-CH_2O

(β-split)

$C_{15}H_{13}^{+}$

m/z 203

$C_{14}H_{22}^{+\cdot}$

m/z 190

Figure 23 EIMS fragmentation of SK1

Figure 24 IR spectrum of SK1

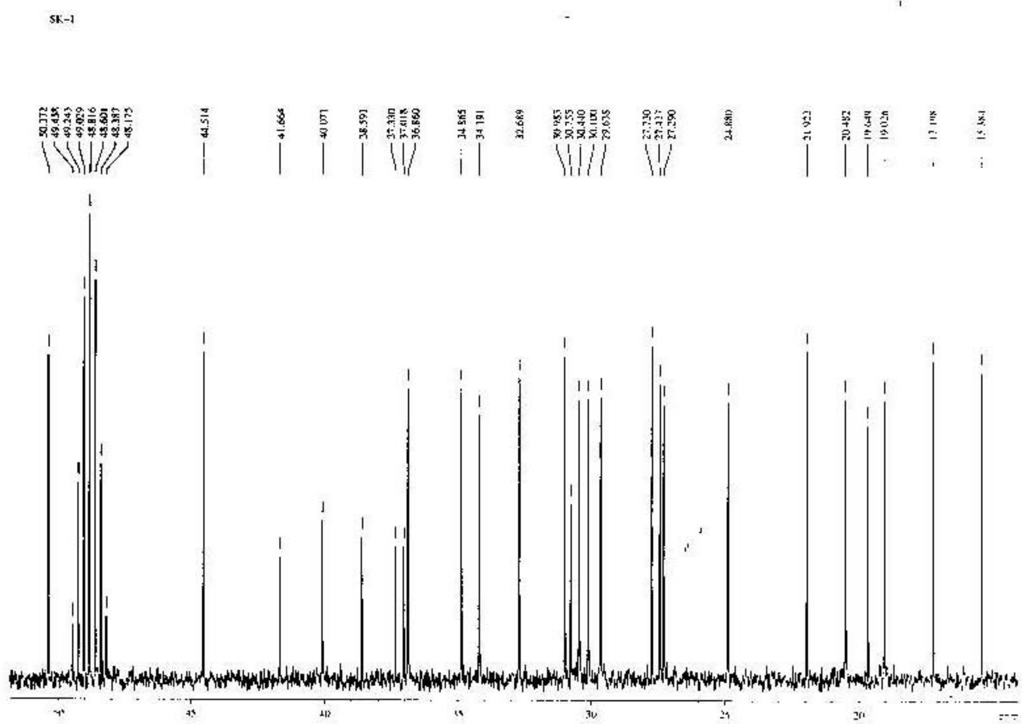


Figure 25 $^{13}C\{^{1}H\}$ broadband decoupled spectrum of SK1

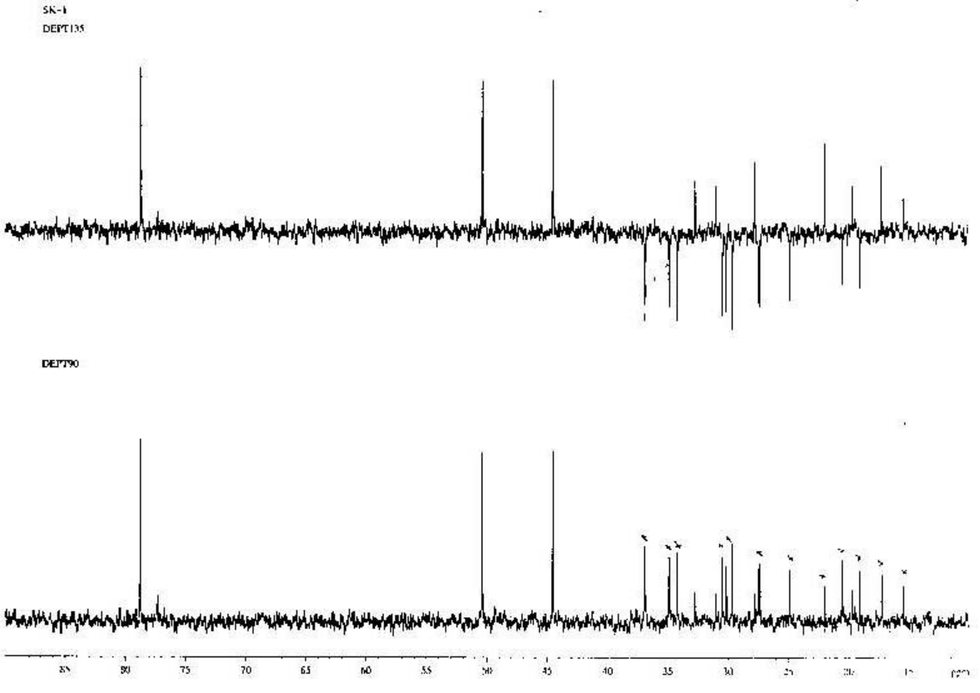


Figure 26 DEPT spectrum of SK1

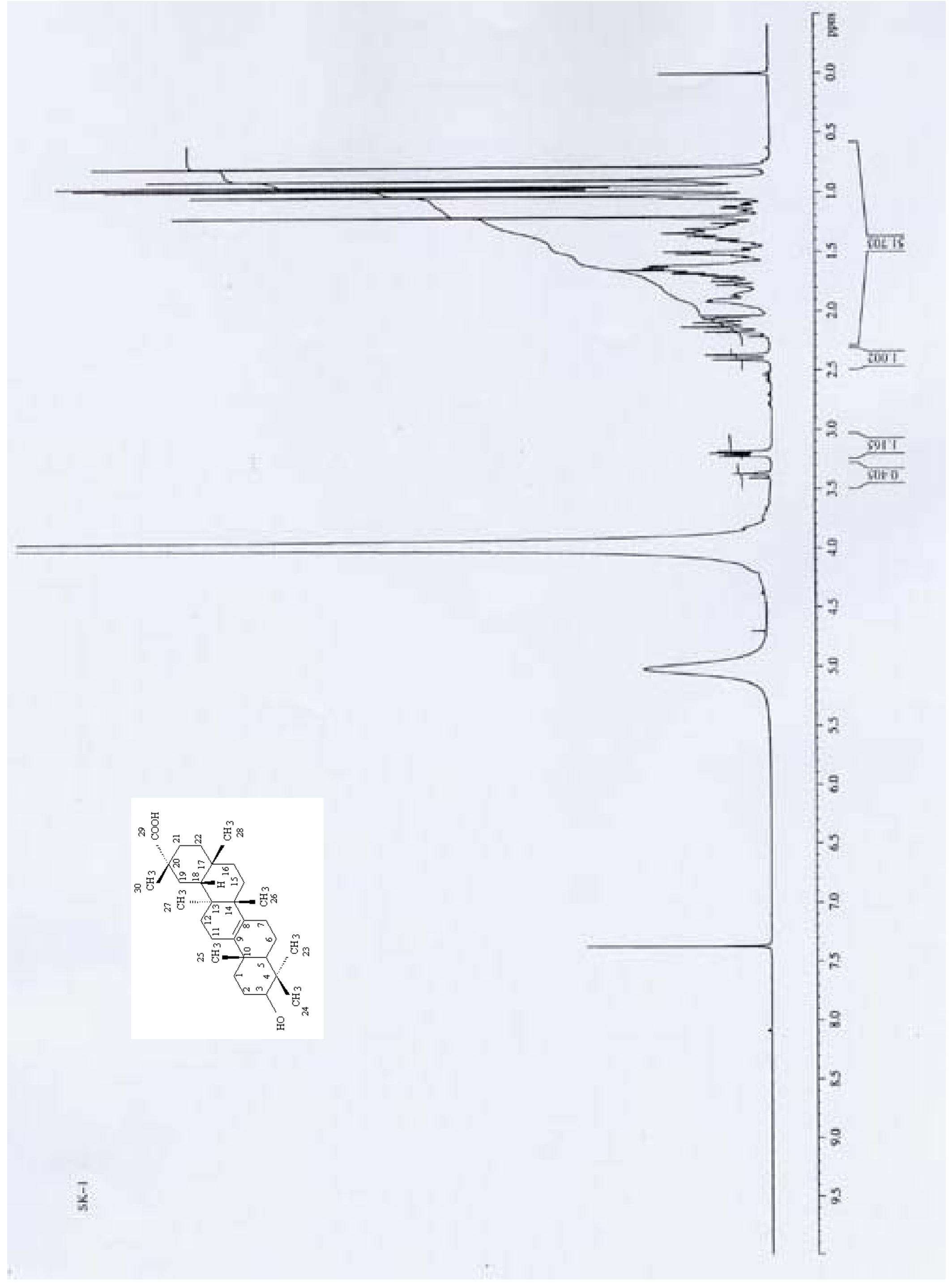


Figure 27 400 MHz ^{1}H-NMR spectrum of SK1 in $CDCl_3$:CD_3OD

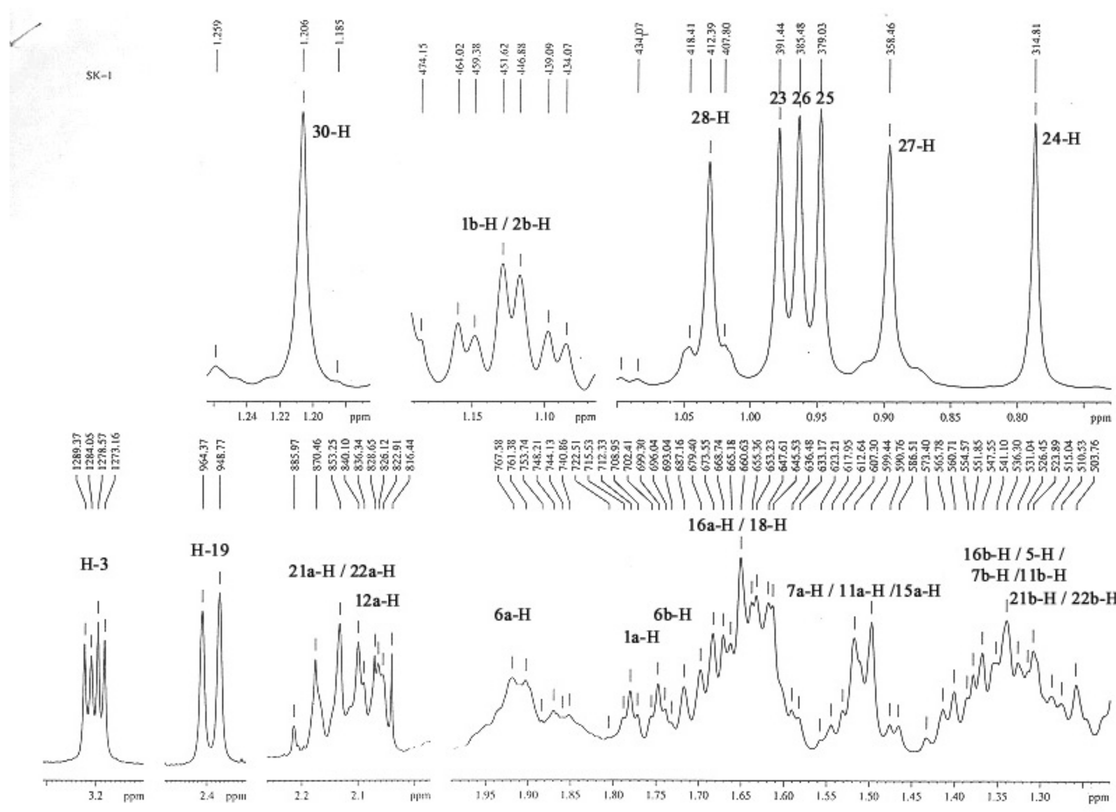


Figure 28 400 MHz ^{1}H-NMR expanded spectrum of SK1 in $CDCl_3$:CD_3OD (δ 0-3.3)

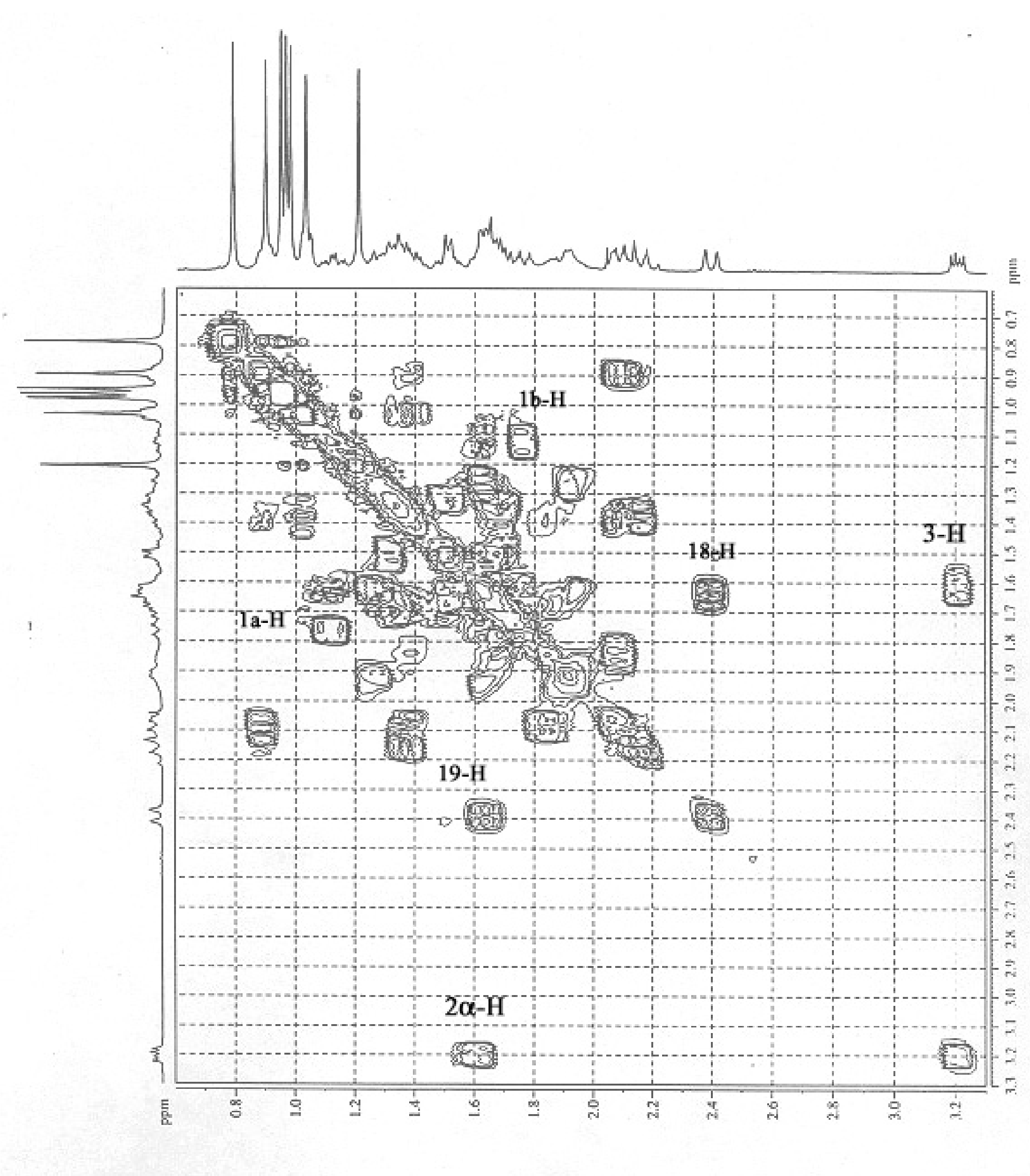


Figure 29 H-H COSY spectrum of SK1 in $CDCl_3$:CD_3OD

3.2 SK2

chondrillasteryl -3-*O*-glucopyranoside $C_{35}H_{58}O_6$ M_R 574

(5α-stimasta-7, 22-dien-3β-*O*-glucopyranoside)

SK2, $C_{35}H_{55}O_6$, was isolated from the dichloromethane root extract. It appeared as white needles with the melting point of 258-259°C. The UV spectrum of SK2 showed the maximum absorption in methanol at 209 nm. The EIMS of SK2 (Fig.30) showed the aglycone ion peak at m/z 412 amu. The ion peak at m/z 273 amu and the base peak at m/z 271 amu were caused by the clevage at the side chain as shown in Fig.31.

The IR spectrum of SK2 (Fig. 32) confirmed the presences of hydroxy group by ν(O-H) 3437.9 cm^{-1}, and the double bond by ν (C=C) 1637.8 cm^{-1}.

The $^{13}C\{^1H\}$ spectrum of SK2 and the DEPT spectrum determined the amount and types of carbons in SK2. There were six methyls (6×Q), ten methylenes (10×T), sixteen methines (16×D) and three quarternary carbons (3×S). The assignment of ^{13}C chemical shifts was coincided with chondrillasteryl-3β-*O*-glucopyranoside as shown in Table 13. SK2 thus possessed the molecular weight of 574.

The assignment of ^1H chemical shifts was accomplished by the HMQC correlation of SK2 (Table 14).

Table 13 ^{13}C NMR data of SK2 compared with the literature

Sig.	C	δ ^{13}C of SK2 in $CD_3OD/CDCl_3$	Chondrillasteryl-3-*O*-glucopyranoside in CD_3OD (85)
1	19	12.81 Q	12.4
2	29	13.13 Q	12.7
3	18	13.65 Q	13.2
4	27	19.65 Q	19.3
5	21	21.62 Q	21.0
6	26	22.07 Q	21.0
7	11	22.33 T	21.7
8	15	24.79 T	23.4
9	28	26.19 T	25.8
10	16	29.19 T	29.0
11	6	30.12 T	30.1
12	2	30.47 T	32.3
13	10	32.64 D	34.6
14	4	34.98 T	34.7
15	25	35.16 D	37.1
16	1	37.96 T	37.5
17	12	40.27 T	39.7
18	5	40.99 D	40.2
19	20	41.57 D	41.2
20	13	44.10 S	43.7
21	9	50.25 D	49.8
22	24	52.08 D	51.6
23	17	55.92 D	55.4
24	14	56.75 D	56.2
25	6′	62.27 T	63.0
26	4′	70.66 D	71.9
27	2′	74.35 D	75.5
28	3	77.27 D	77.2
29	3′	78.34 D	78.7
30	5′	79.49 D	78.8
31	1′	101.87 D	102.4
32	7	118.17 D	118.0
33	23	130.35 D	129.8
34	22	138.96 D	138.8
35	8	140.41 S	139.7

Table 14 HMQC correlation of SK2

C	δ ^{13}C ($CD_3OD/CDCl_3$)	δ ^{1}H ($CD_3OD/CDCl_3$)
1	37.96 T	
2	30.47 T	
3	77.27 D	3.28 *m*
4	34.98 T	
5	40.99 D	
6	30.12 T	
7	118.17 D	5.17 *m*
8	140.41 S	
9	50.25 D	
10	32.64 D	
11	22.33 T	
12	40.27 T	
13	44.10 S	
14	56.75 D	
15	24.79 T	
16	29.19 T	
17	55.92 D	
18	13.65 Q	0.56 *s*
19	12.81 Q	0.84 *s*
20	41.57 D	
21	21.62 Q	1.04 *d* (7)
22	138.96 D	5.17 *m*
23	130.35 D	5.04 *m*
24	52.08 D	
25	35.16 D	
26	22.07 Q	0.81 *s*
27	19.65 Q	0.81 *s*
28	26.19 T	
29	13.13 Q	0.84 *t* (14, 14)
1′	101.87 D	4.0-4.5
2′	74.35 D	3.20 *t* (8, 8)
3′	78.34 D	3.41 *m*
4′	70.66 D	3.43 *m*
5′	79.49 D	3.67 *m*
6′	62.27 T	6′a 3.83 *dd* (12, 2) 6′b 3.76 *dd* (12, 4)

Figure 30 EIMS of SK2

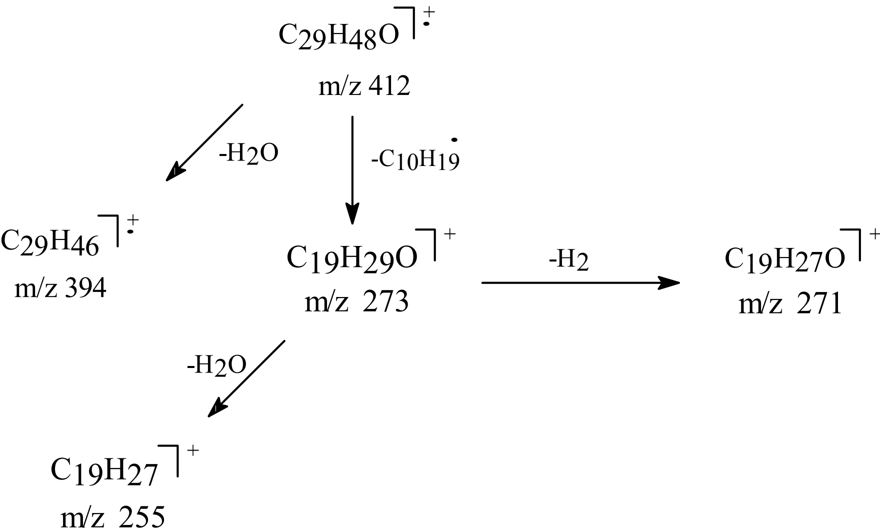


Figure 31 EIMS fragmentation of SK2

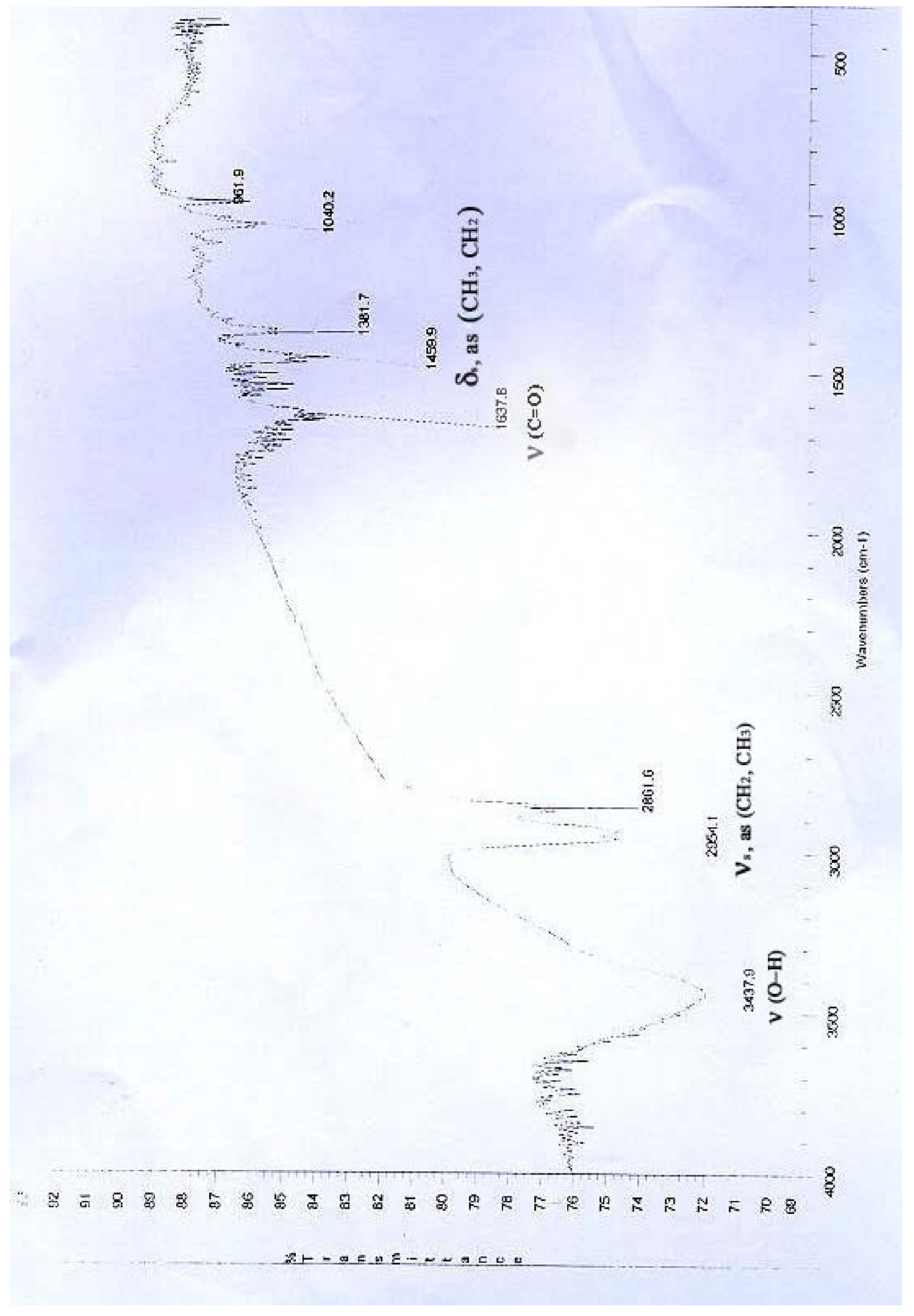


Figure 32 IR spectrum of SK2

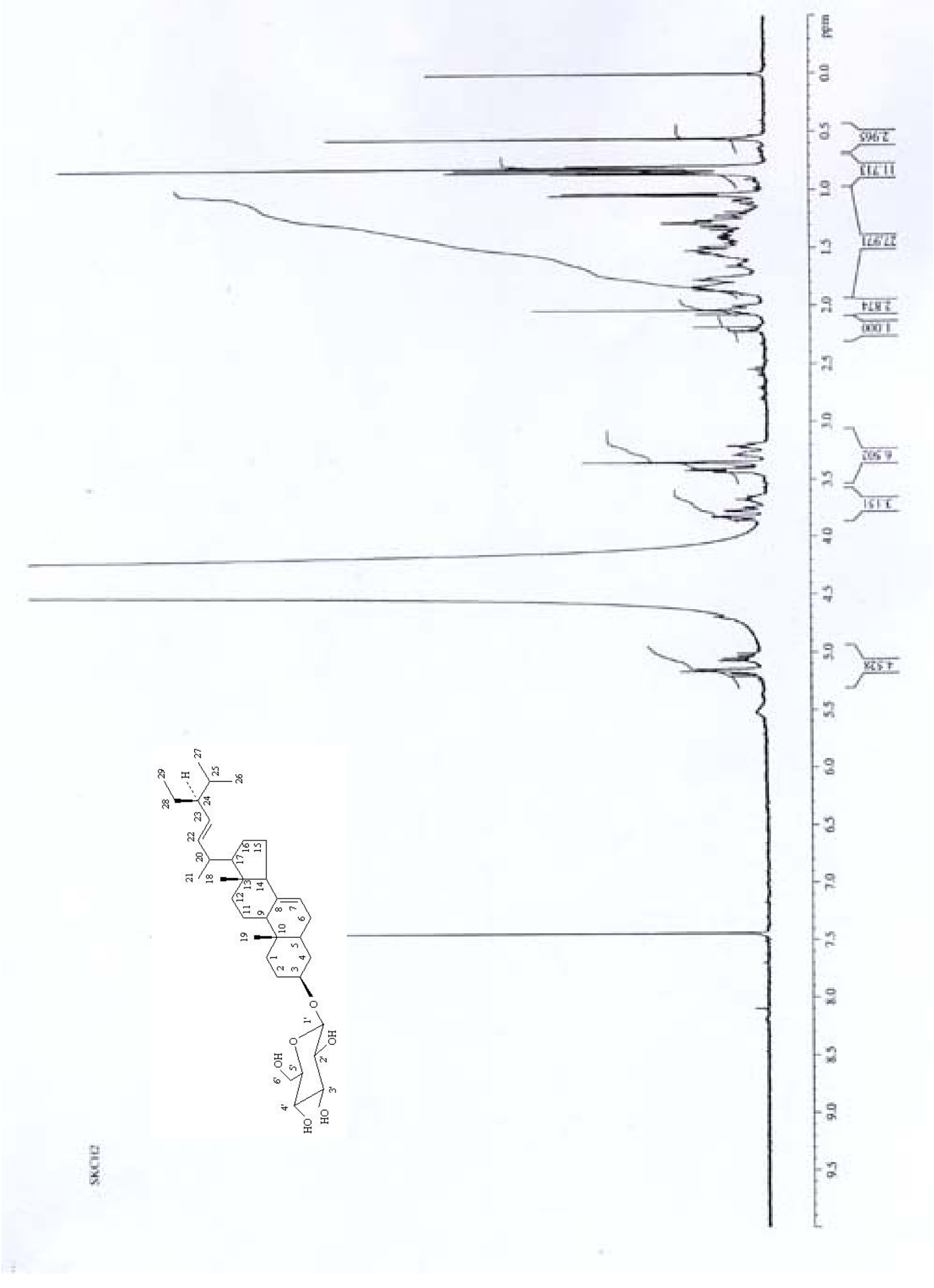
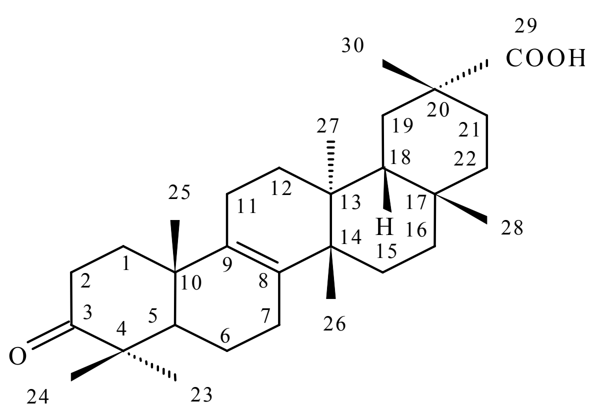


Figure 33 400 MHz ^{1}H-NMR spectrum of SK2 in $CDCl_3$:CD_3OD

Figure 34 400 MHz ^{1}H-NMR expanded spectrum of SK2 in $CDCl_3$:CD_3OD (δ 3.15-5.25)

3.3 SK3



Bryononic acid $C_{30}H_{46}O_3$ M_R = 454

D:C-friedoolean-8-en-3-on-29-oic acid

Compound SK3 was isolated from the silica gel column chromatography of dichloromethane root extract. It appeared as white needles with the melting point of 237-238 °C. The UV spectrum of SK3 showed the maximum absorption in methanol at 205 nm. The molecular ion peaks were seen at m/z 455, 454 amu in EIMS (Fig. 35). The ion peak at m/z 455 amu was resulted from the presence of one ^{13}C atom in the compound molecule, i.e $^{12}C_{29}{}^{13}CH_{46}O_3$. SK3 possessed the molecular weight of 454, $C_{30}H_{46}O_3$. The ion peaks at m/z 439, 394 and 257 amu were fragmented as shown in Fig.36.

The IR spectrum (Fig.37) confirmed the presence of the carboxylic acid (-COOH) by ν (C=O) 1691 cm^{-1} and the deformation vibrations (δ(C-H), δ(O-H) by 1459 cm^{-1}.

The $^{13}C\{^{1}H\}$spectrum together with the DEPT spectrum revealed the amount and types of carbons in SK3. The compound contained ten methyls (7xQ), eleven methylenes (11xT), two methines (2xD) and ten quarternary carbons (10xS). The assignment of ^{13}C chemical shifts (Table 15) was accomplished by the comparison with the literature compound, bryononic acid (85).

The assignment of ^{1}H chemical shifts was performed using the HMQC correlation (Table 15).

Table 15 ^{13}C NMR data of SK3 compared with literature values

Signal	δ ^{13}C SK3 in $CDCl_3$	Bryononic acid in $CDCl_3$ (86)
1	17.879 Q (C-27)	18.1 (C-27)
2	19.377 Q (C-25)	19.4 (C-25)
3	20.464 T (C-6)	20.5[(b)] (C-6)
4	20.545 T (C-11)	20.6 (C-11)
5	21.104 Q (C-23)	21.1 (C-23)
6	21.761 Q (C-26)	21.6 (C-26)
7	25.053 T (C-15)	25.2 (C-15)
8	26.657 Q (C-24)	26.8 (C-24)
9	27.493 T (C-7)	27.7[(b)] (C-7)
10	29.565 T (C-21)	29.5 (C-21)
11	29.639 T (C-12)	29.9 (C-12)
12	30.489 T (C-19)	30.4 (C-19)
13	30.828 S (C-10)	30.8 (C-10)
14	31.133 Q (C-28)	31.2 (C-28)
15	32.643 Q (C-30)	32.7 (C-30)
16	34.205 T (C-1)	34.2 (C-1)
17	34.425 T (C-16)	34.4[(a)] (C-16)
18	35.339 T (C-2)	35.4[(a)] (C-2)
19	36.828 T (C-22)	36.8[(a)] (C-22)
20	37.136 S (C-14)	37.0 (C-14)
21	37.397 S (C-17)	37.4 (C-17)
22	40.232 S (C-20)	40.0 (C-20)
23	42.037 S (C-13)	42.2 (C-13)
24	44.521 D (C-18)	44.4 (C-18)
25	47.102 S (C-4)	47.1 (C-4)
26	51.113 D (C-5)	51.1 (C-5)
27	132.680 S (C-9)	132.7 (C-9)
28	134.960 S (C-8)	134.9 (C-8)
29	183.602 S (C-29)	185.5 (C-29)
30	218.046 S (C-3)	218.2 (C-3)

a, b = interchangeable

Table 16 HMQC correlation of SK3

C	δ ^{13}C	δ^{1}H
1	34.205 T	1.98-2.07 *m*, 2.32-2.60 *m*
2	35.339 T	1.50-1.55 *m*
3	218.046 S	
4	47.102 S	
5	51.113 D	1.60 *m*
6	20.464 T	0.88-0.93 *m*
7	27.493 T	2.10-2.20 *m*
8	134.960 S	
9	132.680 S	
10	30.828 S	
11	20.545 T	0.88-0.93 *m*
12	29.639 T	1.40-1.70 *m*
13	42.037 S	
14	37.136 S	
15	25.053 T	1.28-1.35 *m*
16	34.425 T	1.98-2.07 *m*, 2.32-2.60 *m*
17	37.397 S	
18	44.521 D	1.52 *m*
19	30.489 T	2.40 *d*(15)
20	40.232 S	
21	29.565 T	1.40-1.70 *m*
22	36.828 T	1.30-1.35 *m*
23	21.104 Q	0.97 *s*
24	26.657 Q	1.09 *s*
25	19.377 Q	1.04 *s*
26	21.761 Q	1.04 *s*
27	17.879 Q	0.87 *s*
28	31.133 Q	1.06 *s*
29	183.602 S	
30	32.643 Q	1.24 *s*

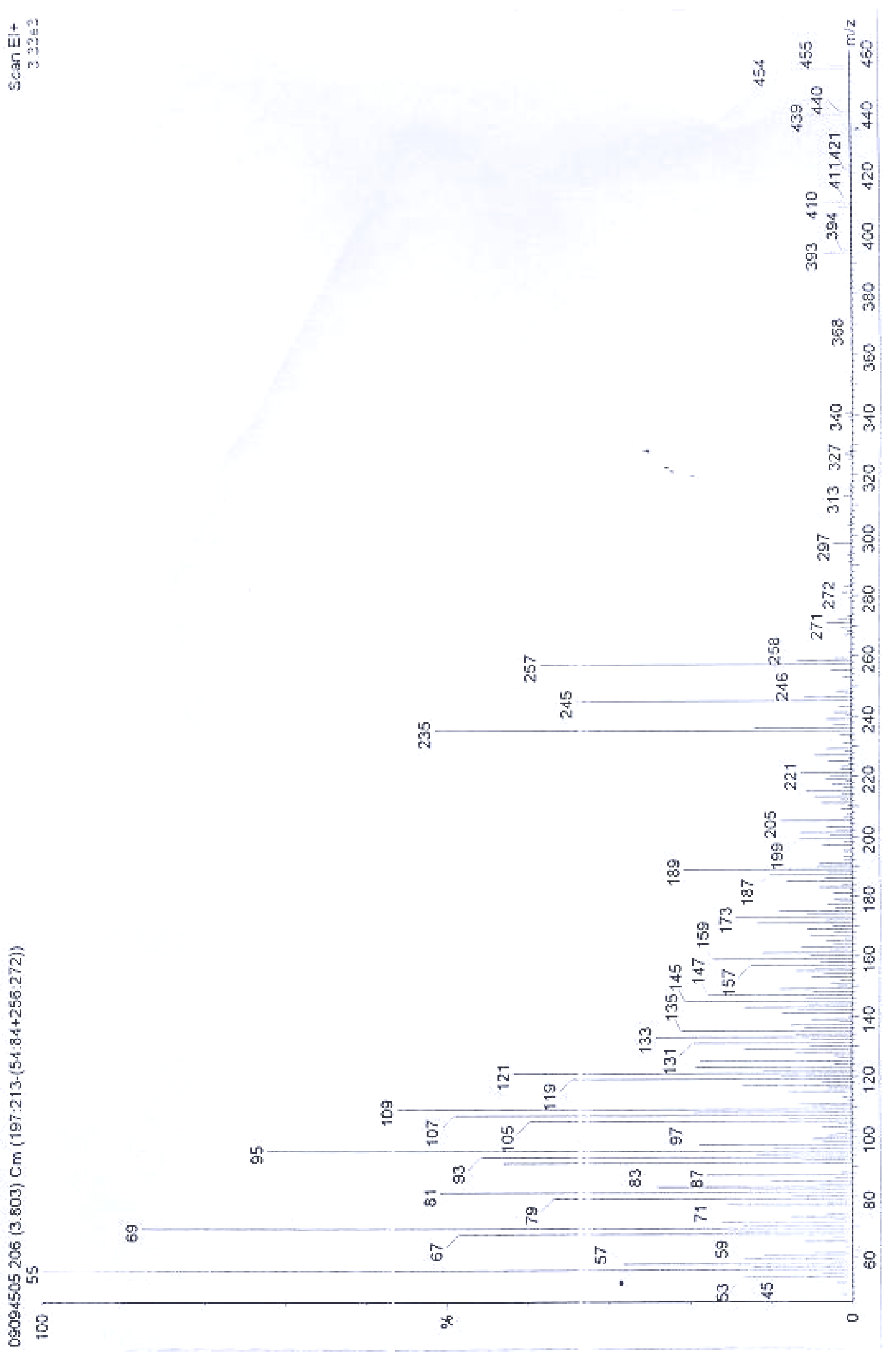


Figure 35 EIMS of SK3

$C_{30}H_{46}O_3$ —70 ev→ $[C_{30}H_{46}O_3]^{+}$

m/z 454

COOH

O

$[C_{30}H_{46}O_3]^{+\cdot}$

m/z 439

$-\ CH_3^{\cdot}$

$[C_{29}H_{43}O_3]^{+}$

m/z 439

$-\ CO_2H$

$[C_{28}H_{42}O_3]^{+}$

m/z 394

RDA

CH_2

CH_2

A

B

O

$[C_{18}H_{25}O]^{+\cdot}$

m/z 257

Figure 36 EIMS fragmentation of SK3

Figure 37 IR spectrum of SK3

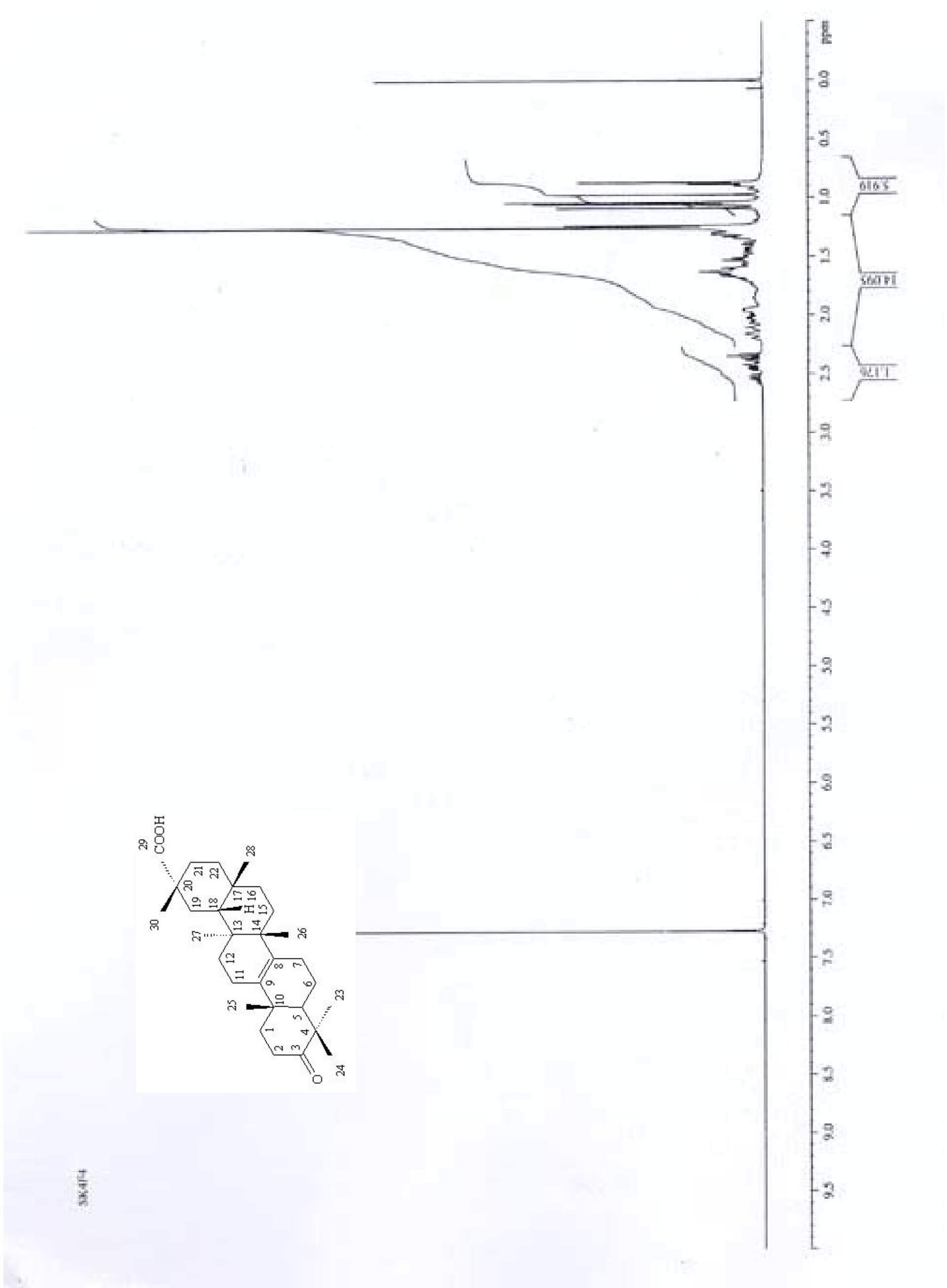


Figure 38 400 MHz ^{1}H-NMR spectrum of SK3 in $CDCl_3$

Figure 39 400 MHz ^{1}H-NMR expanded spectrum of SK3 in $CDCl_3$

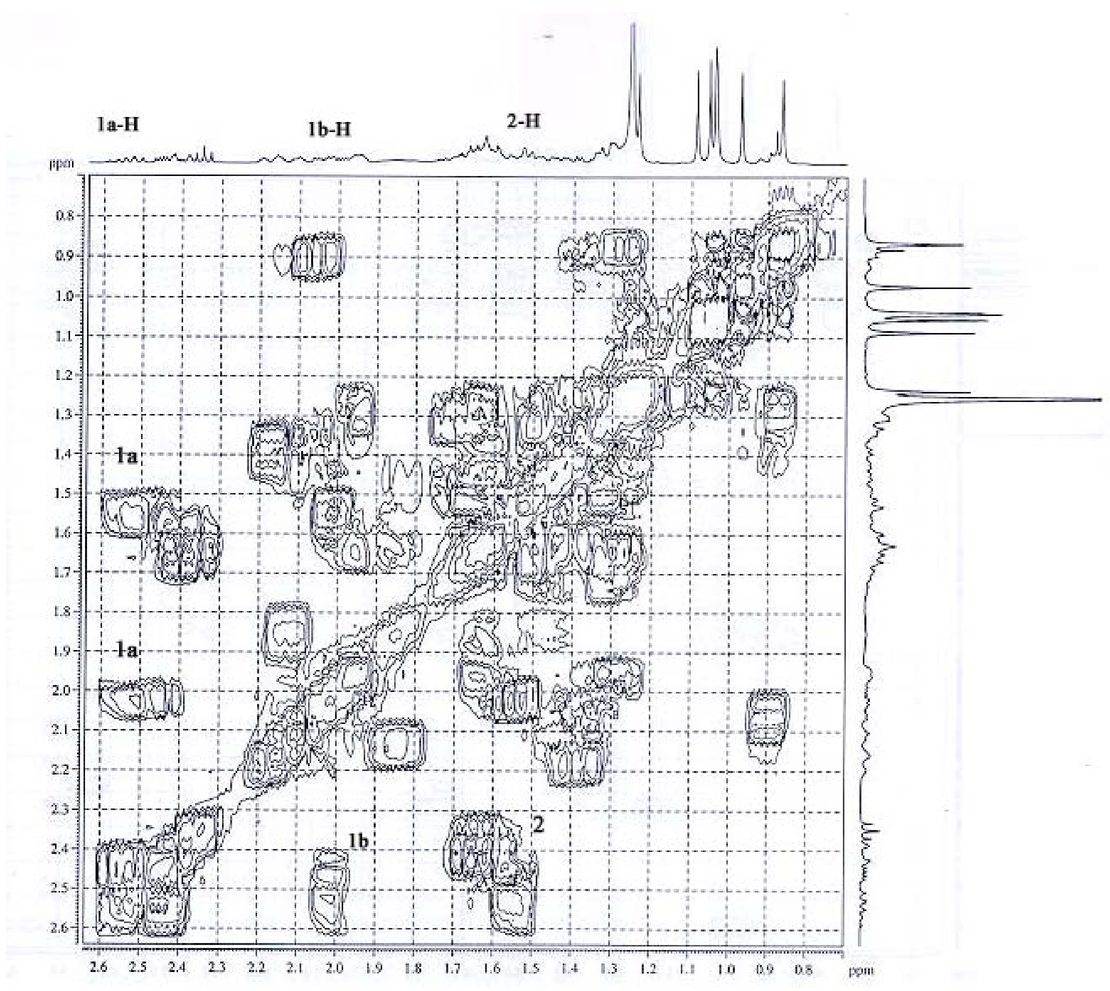


Figure 40 H-H COSY spectrum of SK3 in $CDCl_3$

3.4 SK4

Cucurbitacin B ($C_{32}H_{46}O_8$) M_R = 558

25-(acetoxy)-2β, 16α, 20β-trihydroxy-9β-methyl-19-nor-10α-lanosta-5, 23-diene-3,11,22-trione

Compound SK4, 16 mg, was isolated from the dichloromethane root extract using conventional silica gel column chomatography and low-pressure liquid chromatography (LPLC). The extract yielded SK4, 0.048 % w/w. Compound SK4 appeared as colorless needles. It had the melting point of 185-186°C. The UV spectrum of SK4 showed the maximum absorption in methanol at 210 nm. The EIMS revealed the ion peak at m/z 498 amu, which was resulted from the loss of the acetate moiety (60 amu) from the compound (Fig. 41). Thus, SK4 possessed the molecular weight of 558 amu. The ion peak at m/z 499 amu was caused by one ^{13}C atom in the molecule, $^{12}C_{29}$ $^{13}CH_{42}O_6$. The ion peaks at m/z 403, 385, 111 and 96 amu were fragmented as shown in Fig 42.

The IR spectrum of SK4 (Fig. 43) confirmed the presences of the hydroxy group by ν(O-H) 3465.6 cm^{-1}, the carbonyl (at C3 and C31 by ν(C=O) 1700.2, 1720.5 cm^{-1}, respectively the side chain double bond by ν(C=C) 1632.5 cm^{-1} and the unsaturation in the skeleton by δas (CH_3), δs (CH_2) 1470.2 cm^{-1}.

The ^{13}C {H} spectrum together with the DEPT spectrum revealed the amount and types of carbons in SK4. The compound contained nine methyls (9×Q), four methylene (4×T), eight methines (8×D) and eleven quarternary carbons (11×S).

The assignment of ^{13}C chemical shifts (Table 17) was accomplished by the comparison with the literature values (2).

The assignment of ^{1}H chemical shifts (Table 18) was performed using the HMQC and HMBC correlations of SK4 together with the expanded ^{1}H-NMR spectrum (Fig. 45).

SK4 contained the minor coexisting compound, which was later isolated and identified as SK5.

Table 17 The assignment of ^{13}C chemical shifts of SK4

Sig.	C	δ ^{13}C of SK4 in $CDCl_3$	cucurbitacin B (2) in $CDCl_3$
1	19	18.847 Q	18.609
2	18	19.783 Q	19.544
3	30	20.003 Q	19.744
4	28	21.218 Q	20.932
5	CH_3-Ac	21.862 Q	21.640
6	7	23.830 T	23.550
7	21	23.896 Q	23.630
8	27	25.919 Q	25.619*
9	26	26.367 Q	26.100*
10	29	29.323 Q	29.064
11	10	33.734 D	33.417
12	1	35.960 T	35.687
13	8	42.353 D	42.403
14	15	45.308 T	45.021
15	9	48.072 S	47.785
16	13	48.406 S	48.119
17	12	48.618 T	48.333
18	4	50.180 S	49.925
19	14	50.665 S	50.362
20	17	50.195 D	57.867
21	16	71.282 D	70.953
22	2	71.613 D	71.340
23	20	78.203 S	79.936
24	25	79.249 S	79.018
25	23	120.285 D	119.971
26	6	120.395 D	120.131
27	5	140.390 S	140.027
28	24	151.915 D	151.684
29	C=OAc	170.000 S	169.965
30	22	202.415 S	202.159
31	3	211.991 S	211.866
32	11	212.982 S	212.744

* = interchangeable

Table 18 HMQC and HMBC correlations of SK4

C	δ ^{13}C SK5 in $CDCl_3$:	HMQC δ^{1}H	HMBC δ ^{1}H
1	35.960 T	2.32, 1.22	
2	71.613 D	4.42	2.32(H1a)
3	211.991 S		1.28 (H29)
4	50.108 S		1.34 (H28), 1.28 (H29),5.79(H6)
5	140.390 S		1.44(H7a),1.28(H29)
6	120.395 S	5.79	1.97(H8)
7	23.830 T	2.41, 1.98	
8	42.353 D	1.97	5.97 (H6)
9	48.072 S		1.97 (H8), 2.71 (H10)
10	33.734 D	2.75	5.79 (H6)
11	212.982 S		1.36 (H19)
12	48.618 T	3.25, 2.68	
13	48.406 S		4.35(H16),3.25(12a),2.68(12b)
14	50.665 S		3.25 (H12), 0.98 (H18)
15	45.308 T	1.88, 1.44	
16	71.282 D	4.35	2.50(H17)
17	58.195 D	2.50	1.45(H21),0.98(H18)
18	19.783 Q	0.98	2.50 (H17),3.25 (H12a),2.68(H12b)
19	18.847 Q	1.36	3.25(H12a),2.68(H12b),1.92(H8)
20	78.203 S		1.45 (H-21), 6.47(H23),4.35(H16)
21	23.896 Q	1.45	
22	202.415 S		1.45 (H21), 2.50 (H17),7.07(H24)
23	120.285 D	6.47	7.07 (H-24),
24	151.915 D	7.07	1.55 (H26), 1.58 (H27)
25	79.249 S		1.55 (H26),1.58(H27),6.47(H23)
26*	26.367 Q	1.55	7.07(H24)
27*	25.919 Q	1.58	7.07 (H24)
28	21.218 Q	1.34	1.28(H29)
29	29.323 Q	1.28	
30	20.003 Q	1.08	1.97(H8),1.88(H15a),1.44(H15b)
31	170.00 S		2.02 (H32)
32	21.909 Q	2.02	

OH signals approved at δ 1.62,3.65,4.22

* = interchangeable

Table 19 400 MHz ^{1}H-NMR data of SK4 in $CDCl_3$

H	δ ^{1}H(J)
1a	2.32 (1H) *ddd* (12.6, 6, 3.5)
1a	1.22 (1H) *ddd* (13.2)
2	4.42 (1H) *ddd* (12.8, 5.9, 4)
6	5.79 (1H) *dddd* (5.8, 2)
7a	2.41 (1H) *dddd* (19.7, 7.9, 2.4)
7b	1.98 (1H) *m*
8	1.97 (1H) *d* (8)
10	2.75 (1H) *s*
12a	3.65 (1H) *d* (14.4)
12b	2.68 (1H) *s*
15a	1.88 (1H) *dd* (13, 8.8)
15b	1.44 (1H) *s*
16	4.35 (1H) *m*
17	2.50(1H) *d* (7)
18	0.98 (3H) *s*
19	1.36 (3H) *s*
21	1.45 (3H) *s*
23	6.47 (1H) *d* (15.6)
24	7.07 (1H) *d* (15.6)
25-OAc	2.016 *s* (1H)
26	1.55 *s* (3H)
27	1.58 *s* (3H)
28	1.34 *s* (3H)
29	1.28 *s* (3H)
30	1.08 *s* (3H)
32	2.02 *s* (3H)

Figure 41 EIMS of SK4

M^1 —70 eV→ $M - 60]^{+\bullet}$

$^{12}C_{29}{}^{13}CH_{42}O_6]^{+\bullet}$ m/z 499

$^{12}C_{30}H_{42}O_6$ m/z 498

$C_{30}H_{42}O_6]^{+\bullet}$ —α - split at C_{20-22}→ $C_{24}H_{35}O_5]^{+\bullet}$ m/z 430

HO, O, OH, O, H, H

HO, O, OH, H, H, O

$C_{24}H_{35}O_5]^{+}$

m/z 430

$-H_2O$

$C_{24}H_{33}O_4$

m/z 412

21, OH O, 22, R

α - split at C20-22

and 21-CH_3 rearrange to C-22

O, 22

$C_7H_{11}O]^{+}$

m/z 111

O, O, H, R

McLafferty Rearrangement

O

$C_6H_8O]^{+\bullet}$

m/z 96

Figure 42 EIMS fragmentation of SK4

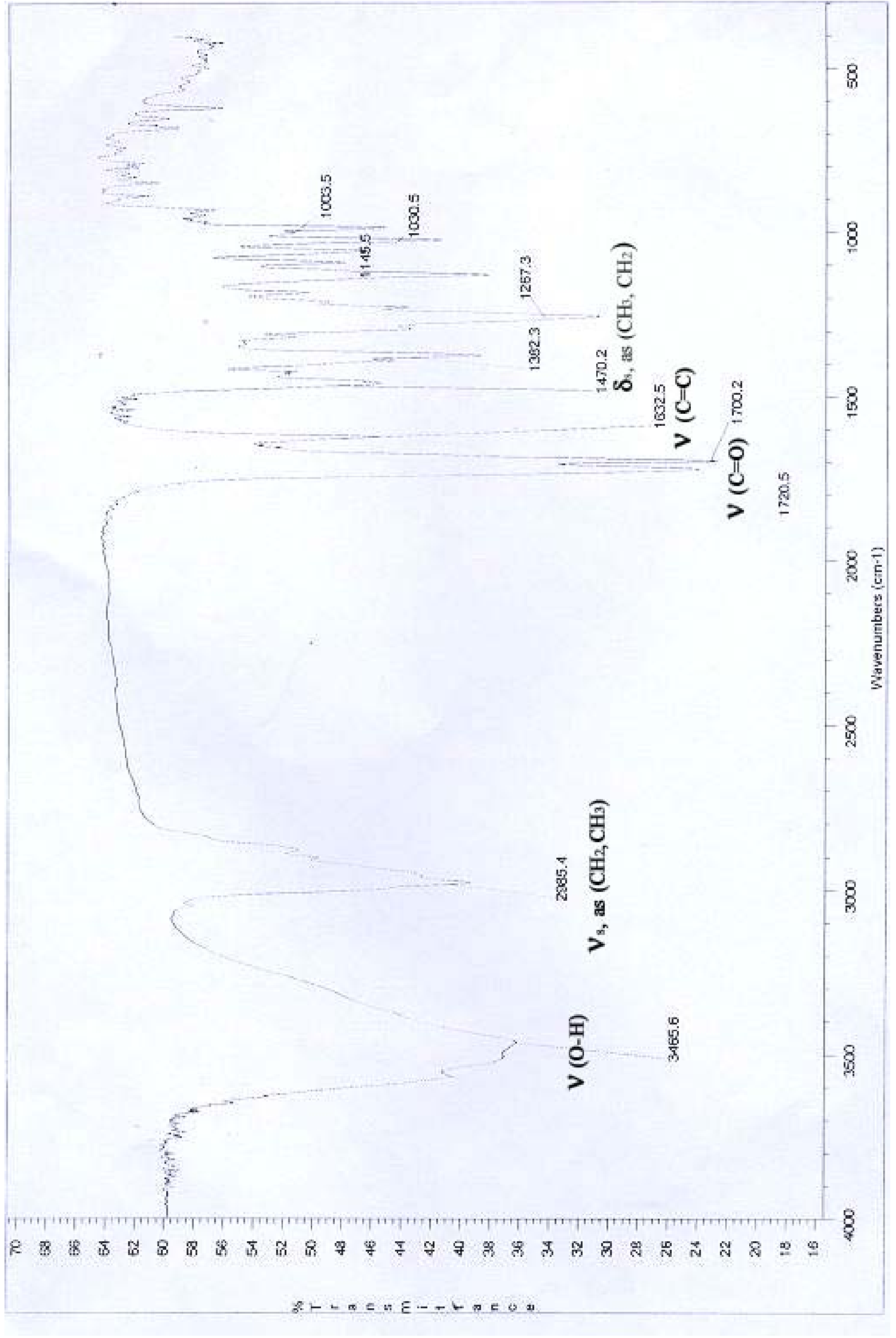


Figure 43 IR spectrum of compound SK4

Figure 44 400 MHz ^{1}H-NMR spectrum of compound SK4 in $CDCl_3$

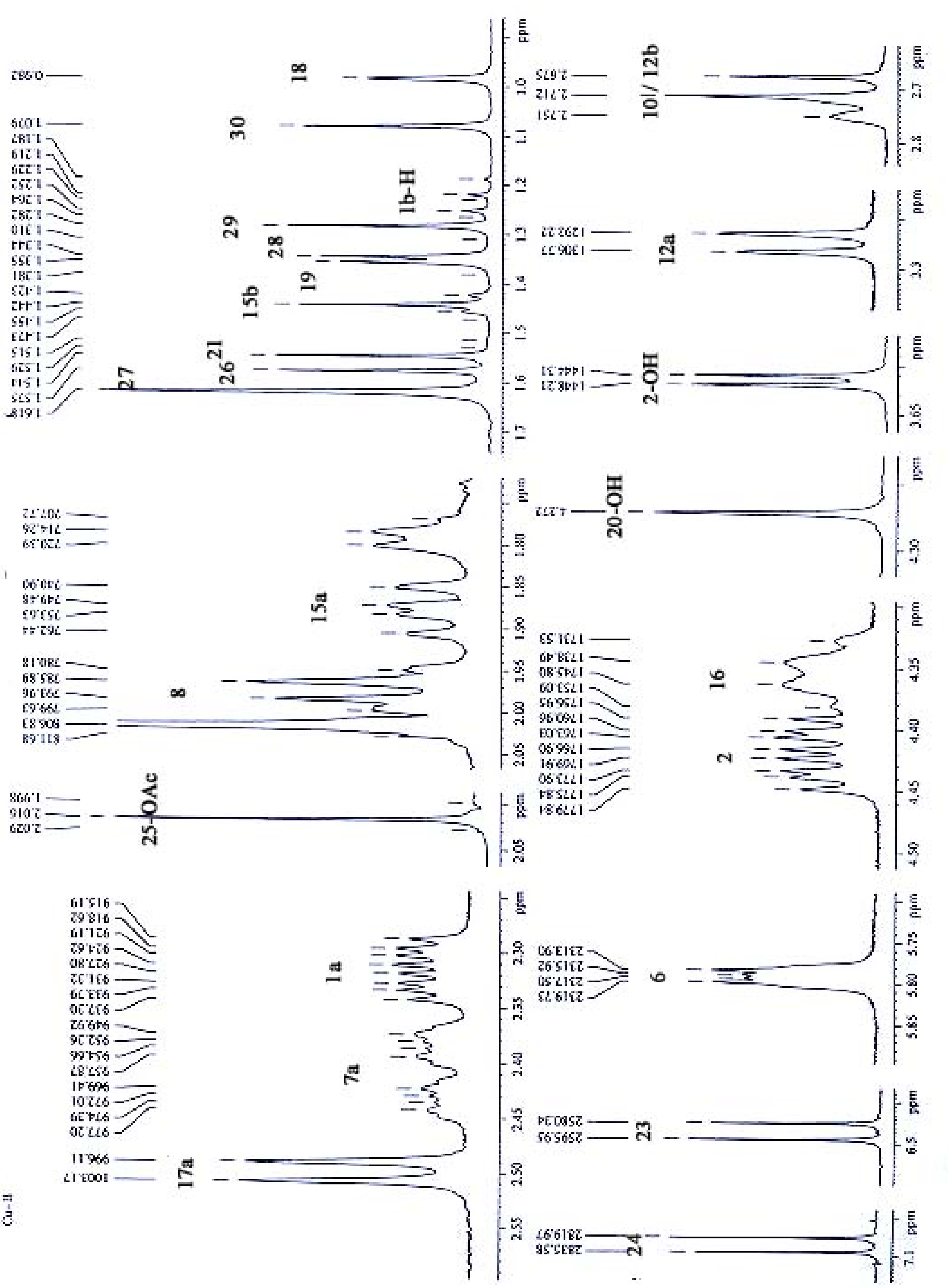


Figure 45 400 MHz ^{1}H-NMR expanded spectrum of compound SK4 in CD_3OD

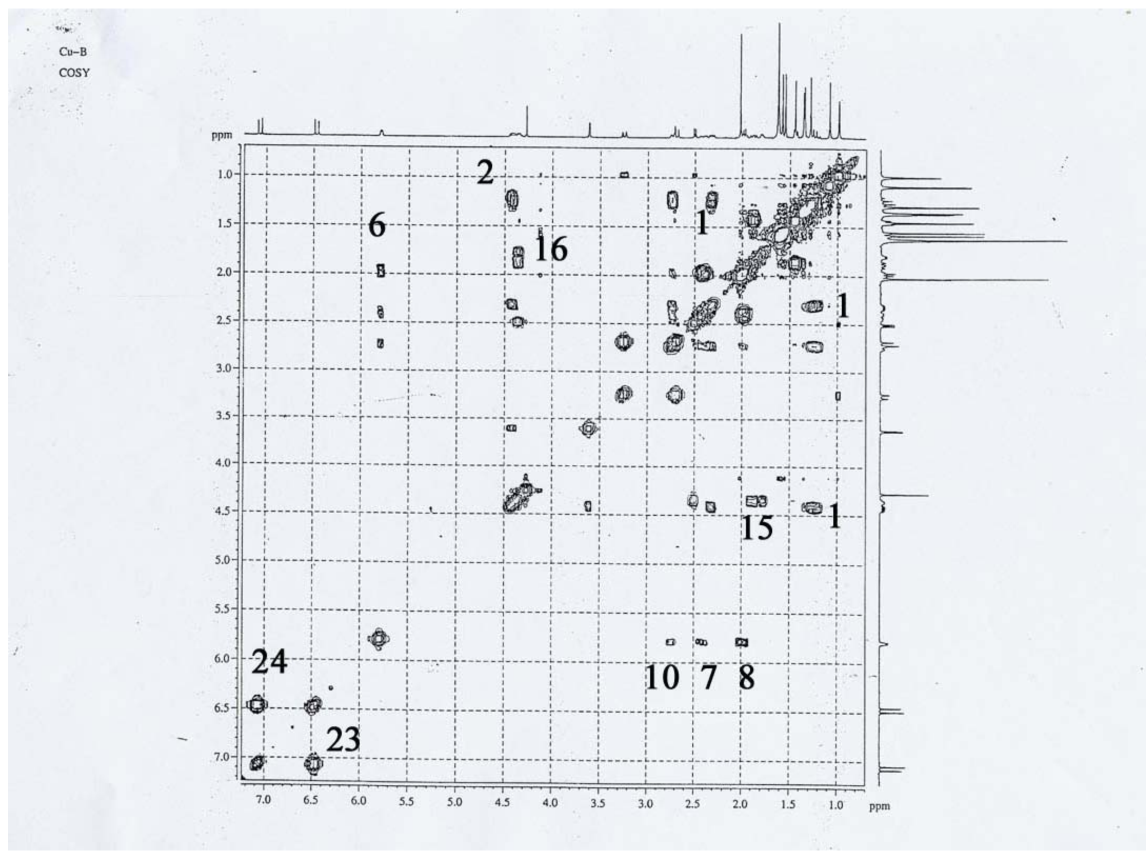
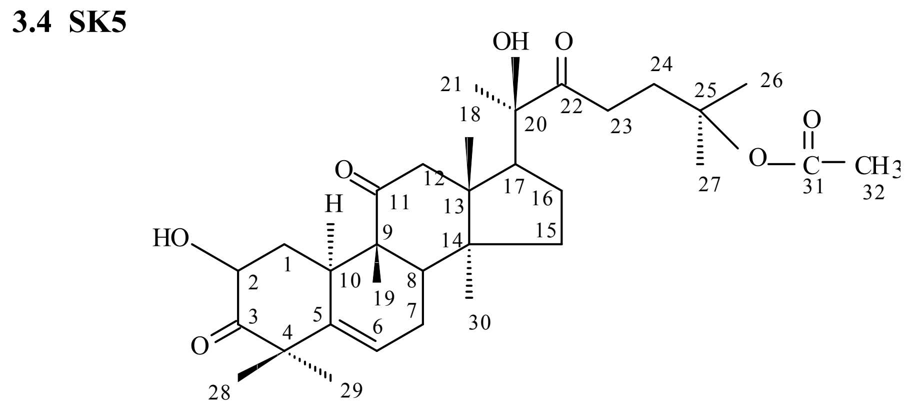


Figure 46 H-H COSY spectrum of compound SK4 in $CDCl_3$

3.4 SK5



Dihydrocucurbitacin B $C_{32}H_{48}O_8$ M_R 560

23, 24-dihydro-25-(acetoxy)-2β, 16α, 20β-trihydroxy-9β-methyl-19-nor-10α-lanosta-5,-ene-3,11,22-trione

Compound SK5, 2 mg, was isolated from SK4 using low-pressure reversed phase column chromatography. It was colorless needles and had the melting point of 199-203 °C. The UV spectrum of SK5 in methanol showed the end absorption. The EIMS produced the characteristic ion peaks similarly to SK4 (Fig. 47).

The IR spectrum (Fig.48) revealed the presences of the hydroxy group by ν (O-H) 3420.8 cm^{-1}, the skeleton strechings by νs,as (CH_3), νs, as (CH_2), ν (CH) 2960.1 cm^{-1}, the carbonyl at C-3 by ν (C=O) 1709.7 $cm^{-1}$and the double bond in the skeleton by δas (CH_3), δs (CH_2) 1470.2 cm^{-1}. The streching, ν (C=C), of the double bond at side chain (Δ^{23}) was disappeared.

The $^{13}C\{^1H\}$ spectrum together with the DEPT spectrum revealed the amount and types of carbons in SK5. The compound contained nine methyls (9×Q), six methylenes (6×T), six methines (6×D) and eleven quarternary carbons (11×S). The assignment of ^{13}C chemical shifts (Table 20) was accomplished by the comparison with the literature values (2).

The assignment of ^1H chemical shifts (Table 21) was performed using the HMQC correlation spectrum.

SK5 still contained several NMR signals of SK4. The identification could be supported by IR spectrum which did not show the double bond streching, ν (C=C) at the side chain.

Table 20 The assignment of ^{13}C-chemical shifts of SK5

Sig.	C	δ ^{13}C of SK5 in $CDCl_3$	dihydrocucurbitacin B (2) in $CDCl_3$
1	19	18.861 Q	18.489 Q
2	18	19.809 Q	19.530 Q
3	30	20.014 Q	19.744 Q
4	28	21.207 Q	20.946 Q
5	CH_3-Ac	21.909 Q	22.134 Q
6	7	23.823 T	23.590 T
7	21	23.878 Q	24.137 Q
8	27	25.862 Q	25.512 Q
9	26	26.401 Q	25.833 Q
10	29	29.317 Q	29.038 Q
11	23	29.317 T	30.346 T
12	10	33.677 D	33.444 D
13	24	34.731 T	34.512 T
14	1	35.962 T	35.687 T
15	8	42.320 D	42.003 D
16	15	45.273 T	45.208 T
17	9	48.392 S	48.039 S
18	13	48.606 S	48.119 S
19	12	48.657 T	48.386 T
20	4	50.202 S	49.948 S
21	14	50.643 S	50.336 S
22	17	58.149 D	57.479 D
23	16	70.978 D	70.752 D
24	2	71.597 D	70.326 D
25	20	78.184 S	78.617 S
26	25	79.407 S	80.967 S
27	6	120.410 D	120.078 D
28	5	140.308 S	140.147 S
29	>COAc	170.000 S	170.024 S
30	22	213.032 S	213.589 S
31	3	212.125 S	211.706 S
32	11	212.982 S	212.707 S

Table 21 HMQC correlation spectrum of SK5

C	$\delta^{13}C$	$\delta^{1}H$ (J, Hz)
1	35.962 T	1.18-1.27 *m*
2	71.597 D	4.42 *m*
3	212.125 S	
4	50.202 S	
5	140.308 S	
6	120.410 D	5.79 *m*
7	23.823 T	2.1-2.0 *m*
8	42.320 D	1.97 *m*
9	48.392 S	
10	33.677 D	2.75 *m*
11	211.982 S	
12	48.657 T	3.25 *m*, 2.68 *m*
13	48.606 S	
14	50.643 S	
15	45.273 T	1.90 *m*, 1.45 *m*
16	70.978 D	4.28 *m*
17	58.149 D	2.50 *d* (7)
18	19.809 Q	0.98 *s*
19	18.861 Q	1.3 *s*
20	78.184 S	
21	23.878 Q	1.44 *s*
22	213.032 S	
23	29.317 D	1.2-1.3 *m*
24	34.731 D	2.0-2.1 *m*
25	79.407 S	
26	26.401 Q	1.54 *s*
27	25.862 Q	1.57 *s*
28	21.207 Q	1.34 *s*
29	29.317 Q	1.28 *s*
30	20.014 Q	1.08 *s*
31	170.00 S	
32	21.909 Q	2.01 *s*

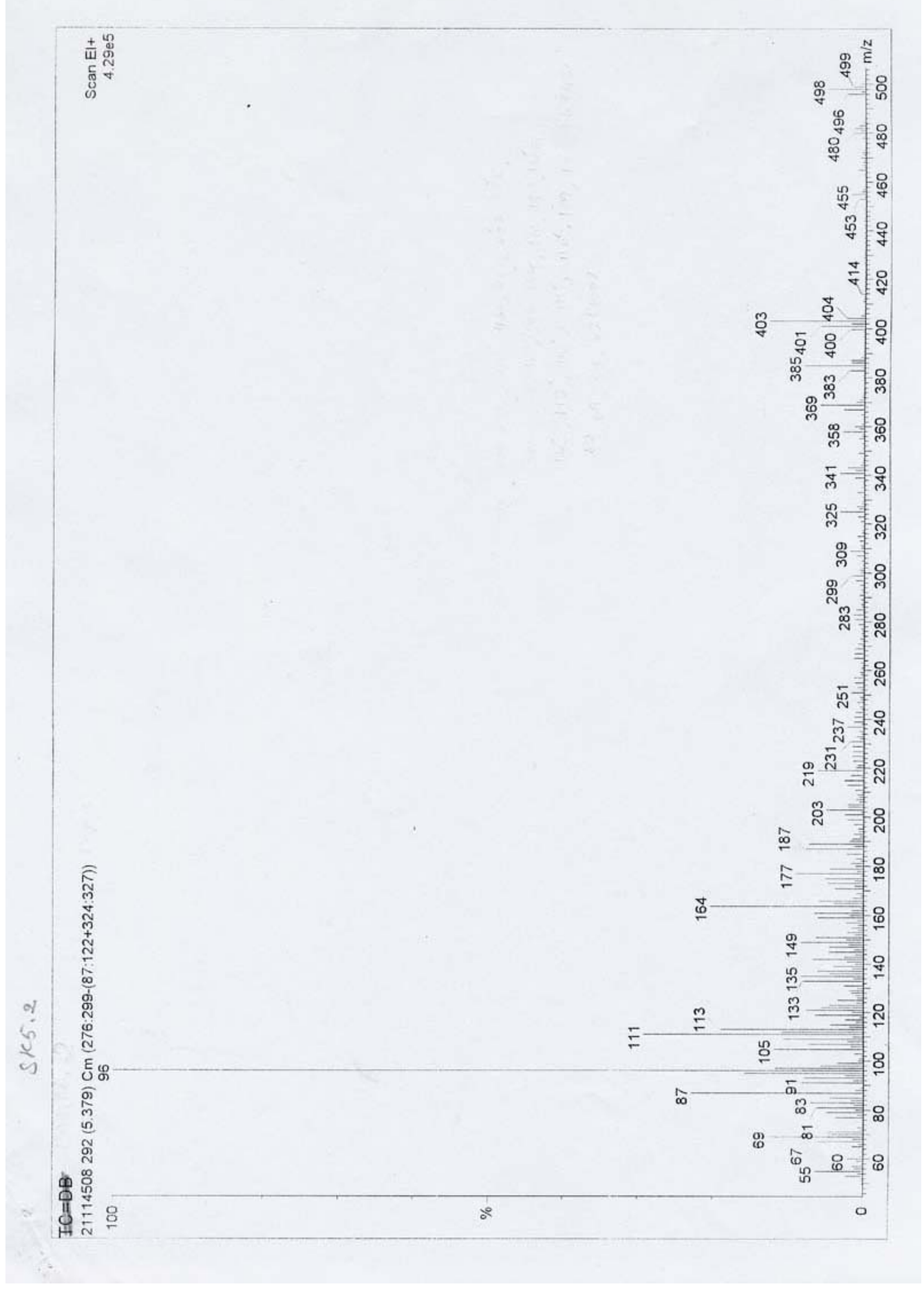


Figure 47 EIMS of compound SK5

Figure 48 IR spectrum of compound SK5

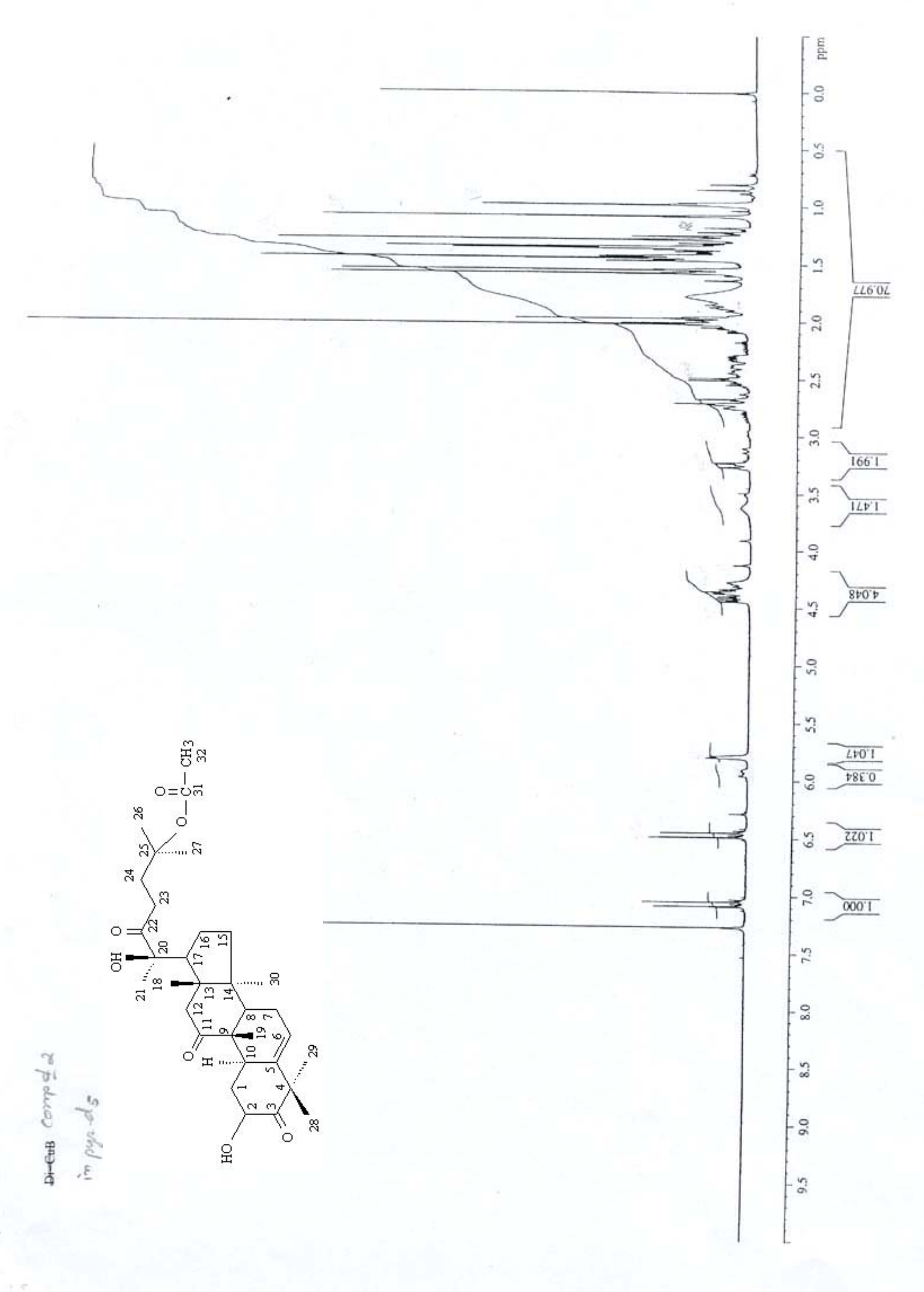


Figure 49 400 MHz ^{1}H-NMR of compound SK5 in $CDCl_3$

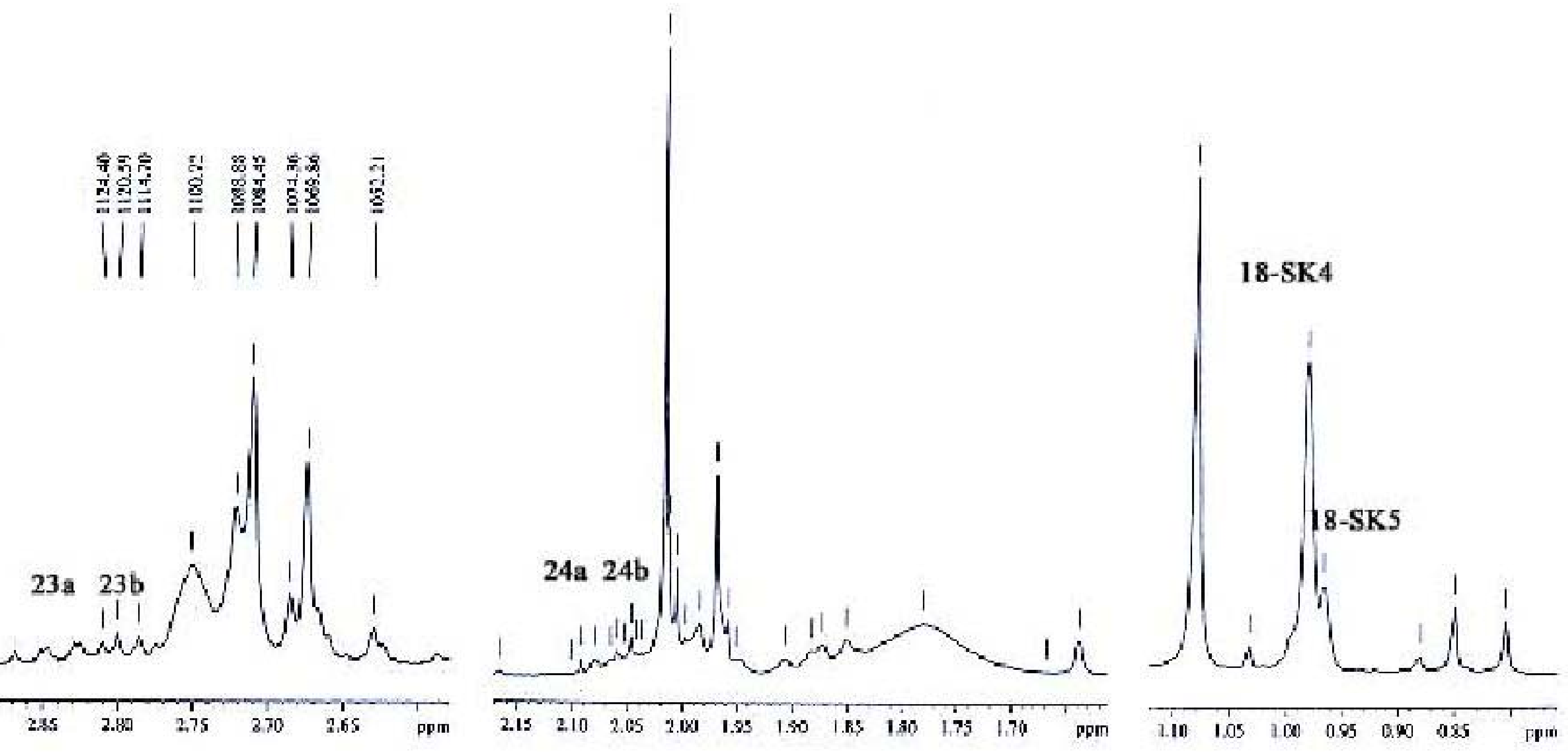


Figure 50 400 MHz ^{1}H-NMR expanded spectrum of compound SK5 in CD_3OD

Part II Bioactivity

1. Antimalarial activity

IC_{50} values of the fruit juice, the root extract and SK4 of *T. cucumerina* against *P. falciparum* K1 strain *in vitro* culture were shown in Table 22. IC_{50} of the root extract (312.50 μg/ml) was much higher than those of SK4 (0.625 μg/ml) and the fruit juice (0.089 μg/ml).

It should be noted that IC_{50} of SK4 (pure compound) was seven times higher that of fruit juice , which was supposed to contain cucurbitacin B and dihydro-cucurbitacin B(89).

Table 22 IC_{50} values of the root extract, the fruit juice and SK4 against asexual stages of *P. falciparum* K1 strain.

Medicinal plant	IC_{50} (μg/ml)
T. cucumerina	
the fruit juice	0.089
the root extract	312.50
SK4	0.625

1.1 Observation on the morphology of normal parasites and abnormal or dead parasites after being exposed to *T. cucumerina* fruit juice, root extract and SK4

The morphology of normal K1 strain of *P. falciparum* in continuous culture observed under the light microscope was shown in Fig. 51.

As shown in Fig. 51a, mixed stages of *P. falciparum*, i.e., ring, trophozoite and schizont stages were normal in appearance. The rings were characterized by violet-stained nuclei and the large food vacuole occupied most areas within the blue cytoplasmic region. The increase in cytoplasm and the increase to 1/3 of erythrocytes size indicated the ring developed into trophozoites. Trophozoites nuclei were characterized by the violet color, and the brown pigment granules were sometimes seen adhering to their cytoplasm. At schizont stage, the blue color of the expanded cytoplasm and violet color of separated nuclei within erythrocyte's cytoplasm were

usually observed together with the brown pigment granules. Complete nuclear division yielded about 16-24 nuclei. Each nucleus migrated to the periphery of cytoplasm and was completely surrounded by a small portion of parasite cytoplasm, forming a merozoite known as a mature schizont stage (Fig. 51b). When the mature schizont ruptures, these merozoites are released (Fig. 51c) and then invaded new erythrocytes to initiate a new erythrocytic schizogony over and over.

Morphological changes of K1 strain of *P. falciparum* after being exposed to the fruit juice, the root extract and SK4 were similar as shown in Fig. 52. Normal ring and schizonts were observed in the control well whereas in the well where the complete inhibition was observed, all parasites were inhibited at the ring stage. Most of the dead parasites showed pyknotic nuclei with no cytoplasm (Fig. 52a), however; some might still be found to have residue of cytoplasm without vacuole. The infected erythrocytes harboring dead ring stage parasites showed no change in size, shape or color; furthermore, the well that showed the incomplete inhibition contained both viable and dead parasites. In these well, only a few normal mature schizonts and ring stage parasites were detected together with some dead trophozoites and dead schizonts. As for dead trophozoites, the contour of their nuclei could not be distinguished from their cytoplasm (Fig. 52b). The cytoplasm was stained a faint pinkish-blue color. Dead immature and mature schizonts had fewer nuclei when compared with the normal ones (Fig. 52c). The nuclei were fragmented into small parasites. The contour of cytoplasm was demonstrated by a faint red color. The number of normal parasites increased reciprocally corresponded with the concentration of drug. In wells that showed no inhibition, only normal parasites were observed and most of them were found predominantly in the ring stage.

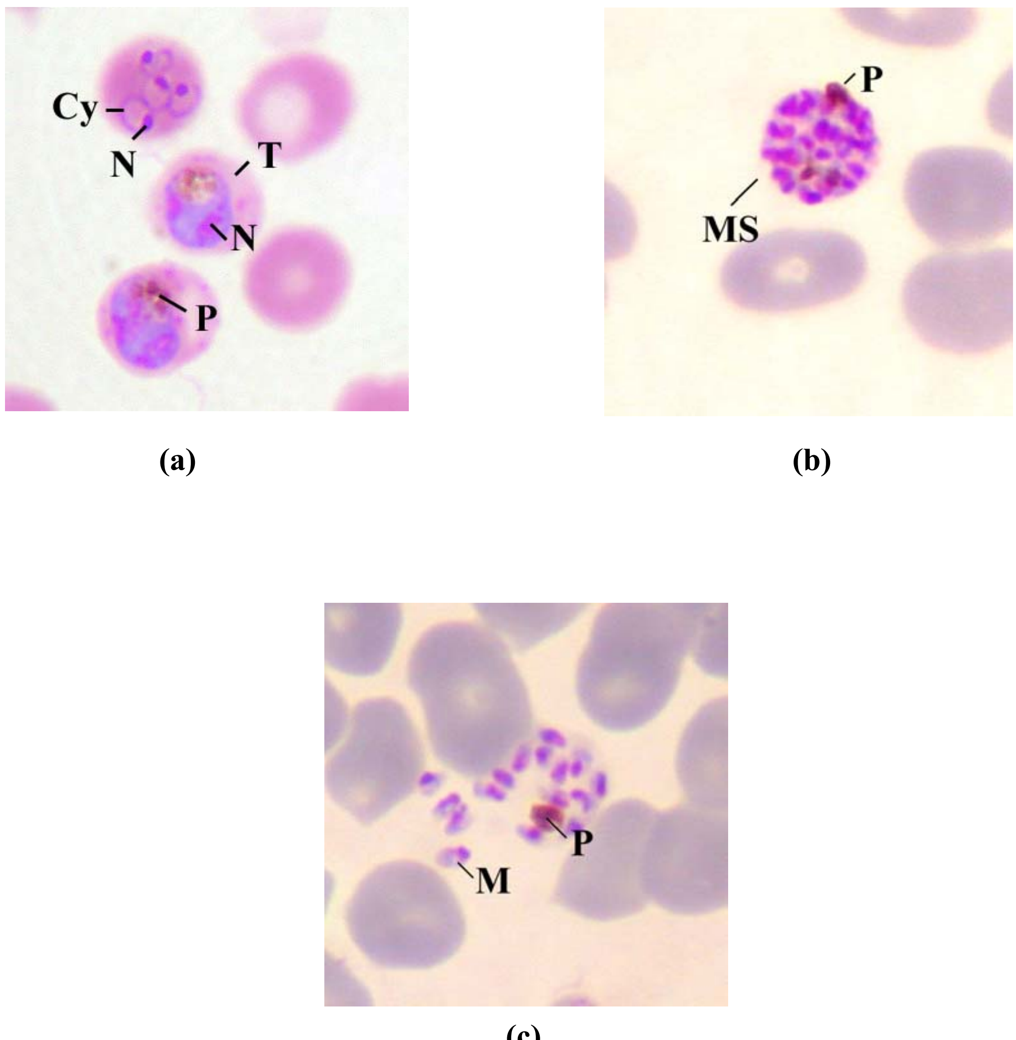
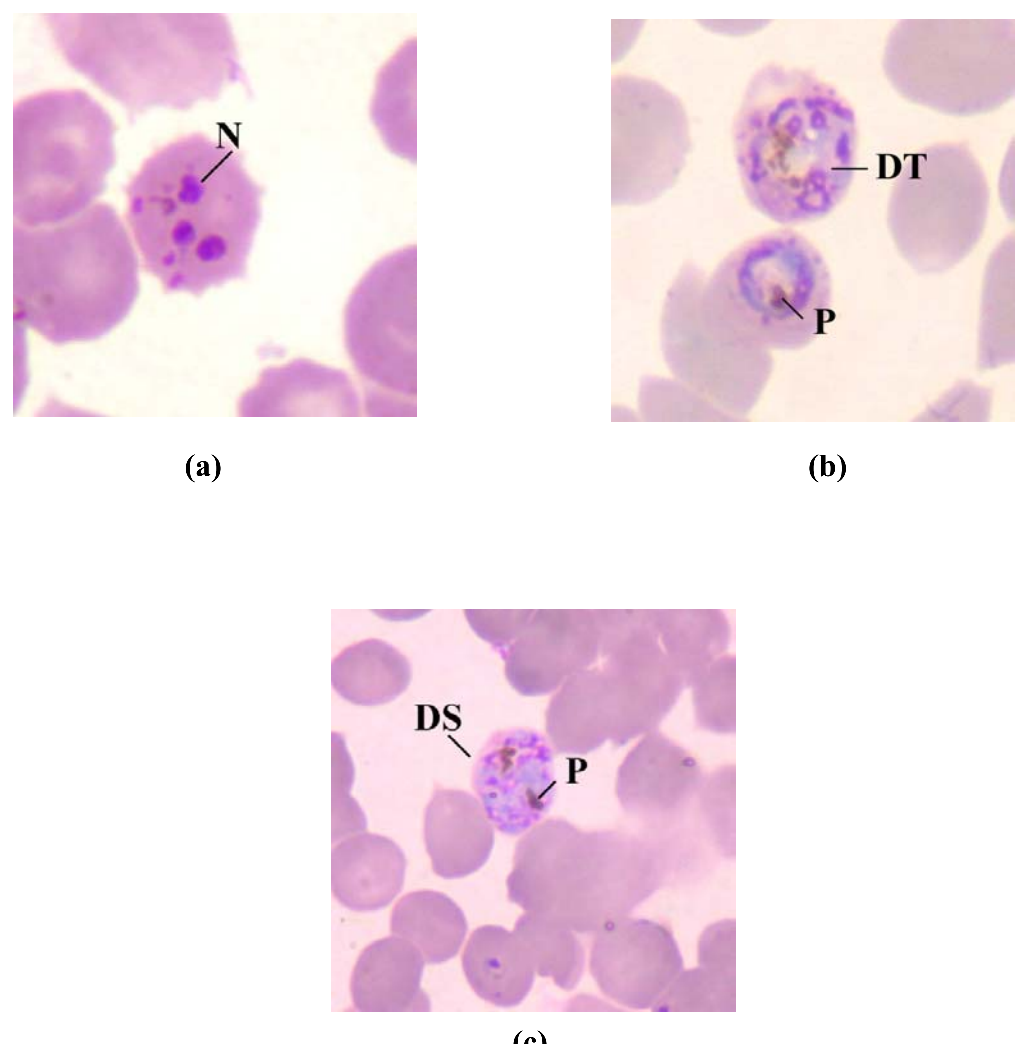


Figure 51 Normal stages of K1 strain of *P. falciparum* in continuous culture; (a) mixed stages , (b) mature schizont and (c) merozoites

Note : N = nucleus, Cy = cytoplasm, P = malarial pigment, T = trophozoite, MS = mature schizont, M = merozoites



(a) (b)

(c)

Figure 52 Abnormal or dead stages of K1 strain of *P. falciparum* after being exposure to *T. cucumerina*; (a) abnormal or dead rings with no cytoplasm, (b) abnormal or dead trophozoites and (c) abnormal or dead schizont

Noted : N = nucleus, DT = dead trophozoite, P = malarial pigment, DS = dead schizont

2. Antipapilloma virus (83)

Cytotoxicity of SK1 against papilloma primary cell culture by sulphorhodamine B assay was carried out in Ramathibodi Hospital under the supervision of Assoc. Prof. Somyos Kunachark, Head of the Department of Otolaryngology.

Stock solution : 10 mg/ml of SK1 in 100% DMSO

Concentration (ng/ml)	O.D 550
250.00	0.395
125.00	0.263
62.50	0.481
31.25	0.466
15.63	0.409
7.81	0.368
3.91	0.331
1.95	0.311
0.98	0.667
0.49	1.246
0	1.12

Min-max (1.246 – 0.263)/2 = 0.4915 ED_{50} = 1.5 ng/ml

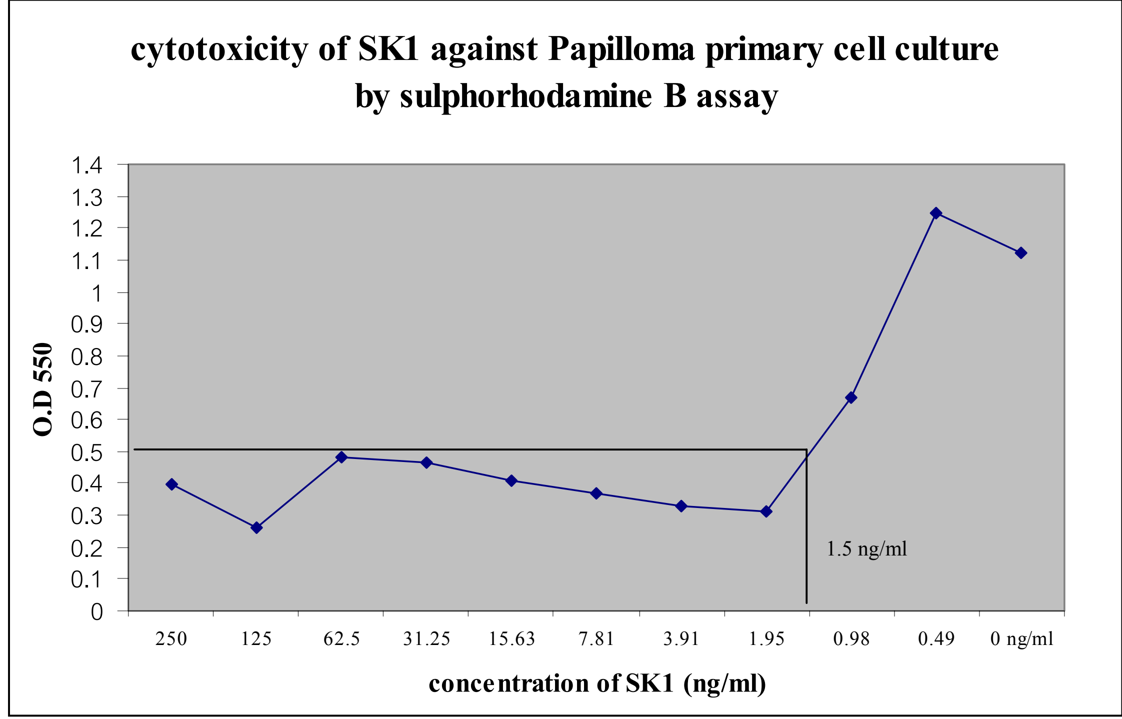


Figure 53 ED_{50} curve of SK1 against papilloma virus

Part III High-performance liquid chromatography (HPLC) quantitative analysis of cucurbitacin B

1. System suitability test

From the preliminary study, the appropriate method for the separation of cucurbitacin was reversed-phase chromatography (C18). Various mobile phase compositions were tested in order to improve the resolution. It was found that the mixture of acetonitrile and water at the ratio of 40:60 yielded the best resolution. Under this condition, cucurbitacin B and dihydrocucurbitacin B were well separated from each other with the retention times of 16.4 and 18.7 min, respectively (Fig.54).

The parameters of the system suitability test at this condition were evaluated.

1.1 Precision

Precision of the method was determinded by replicate injection using the isocratic system of acetonitrile and water (40:60). The replicated injections were performed using isocratic system. The precision was determined either in term of percentage of coefficient variation (%CV) or relative standard deviation (RSD), which was calculated by the following equation:

$$\%CV\ (\%RSD) = [SD/\overline{X}] \times 100$$

$\overline{X}$ = mean value

SD = standard deviation

The data for precision were shown in Table 23.

Table 23 Retention time data of cucurbitacin B (n = 12)

Injection no.	**t_R Day 1 (min)**	**t_R Day 2 (min)**	**t_R Day 3 (min)**
1	19.50	16.56	16.73
2	19.53	16.61	16.71
3	16.55	16.62	16.74
4	16.57	16.64	16.74
5	16.50	16.67	16.74
6	16.59	16.71	16.77
7	16.52	16.70	16.77
8	16.54	16.74	16.81
9	16.55	16.79	16.81
10	16.60	16.78	16.86
11	16.65	16.73	16.89
12	16.59	16.79	16.89
Mean	16.54	16.74	16.78
SD	0.04	0.01	0.07
%RSD	0.25	0.62	0.40

1.2 The column efficiency (number of theoretical plates, N)

The number of theoretical plates (N) was calculated by the following equation

$$N = 5.54 \times [t_R / W_{1/2h}]^2$$

where t_R = the retention time of peak

$W_{1/2h}$ = the peak width at half height

The number of theoretical plate for this study were 46,270 for cucurbitacin B (SK4).

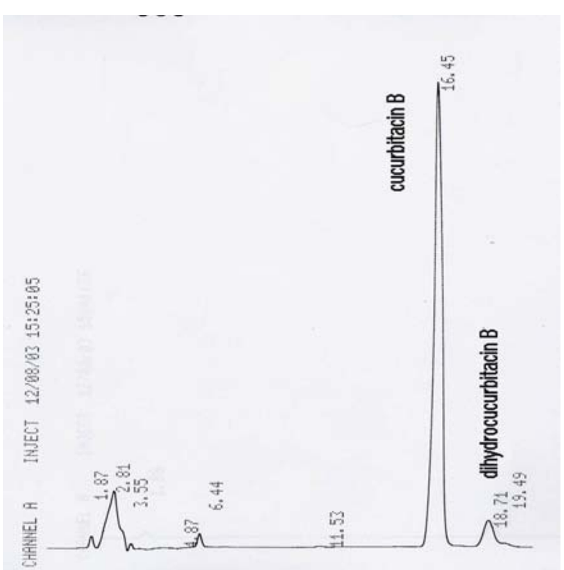


Figure 54 HPLC chromatogram of cucurbitacin B as a lead compound (5µm Hypersil BDS C18 column; mobile phase: acetonitrile-water, 40:60; flow rate:1 ml/min.)

1.3 Tailing factor (TF)

Tailing factor (TF) was calculated by using the following equation :

$TF = W_{0.05}/[2 \times A_{0.05}]$

$W_{0.05}$ = the width of peak at 5% of the peak height

$A_{0.05}$ = the distance from the peak maximum to leading edge of the peak, the distance being measured at a point 5% of the peak height from base line

The tailing factor values of this study were 1.

1.4 The resolution factor (R_s)

The resolution factor (R_s) was calculated by using the following equation

$R_s = 2 \times (t_{R2}-t_{R1})/[W_1+W_2]$

where t_{R1} = the retention time of the first peak

t_{R2} = the retention time of the second peak

W_1 = the peak width of the first peak

W_2 = the peak width of the second peak

The resolution factor of this study was 2.825.

Table 24 The results of system suitability

Parameter	Cucurbitacin B (SK4)
Precision (%RSD)	
- Interday	0.70%
- Intraday	0.25-0.40%
Theoretical plate (N)	46,270
Tailing factor (TF)	1
Resolution factor (Rs)	2.825
Retention time (min)	16.7

2. Method validation

2.1 Accuracy

The accuracy of both methods were determined using the standard addition method. Five different concentrations of the cucurbitacin were added into the extract and the fruit juice. Each concentration was injected three times. The percentage recoveries were evaluated. The results were shown in Table 25 and 26.

Table 25 Percentage recoveries obtained from spiking method of the root extract (n=5)

Sample no.	Day I			Day II		
	Amount add	Amount found	% recovery	Amount add	Amount found	% recovery
1	53.25	54.52	102.38	49.38	50.09	101.43
2	79.88	83.12	104.06	74.07	83.12	112.21
3	106.50	108.46	101.84	98.76	108.46	109.82
4	133.13	141.25	106.10	123.45	141.25	114.42
5	159.76	164.41	102.92	148.14	164.42	110.99
Mean			103.46			109.77
SD			1.69			4.97
%RSD			1.63			4.52

Table 26 Percentage recoveries obtained from spiking method of the fruit juice (n=5)

Sample no.	Day I			Day II		
	Amount added	Amount found	% recovery	Amount add	Amount found	% recovery
1	45.73	43.69	95.55	42.23	44.10	104.43
2	68.59	79.28	115.58	63.35	66.88	105.58
3	91.46	100.57	109.96	84.47	91.53	108.36
4	114.33	113.87	99.61	105.58	98.97	93.74
5	137.19	128.42	93.60	126.70	138.99	107.70
Mean			102.86			103.96
SD			9.51			5.93
%RSD			8.73			5.70

3.2 Precision

The precision of this method was expressed as the percentage of relative standard deviation (%RSD), on the basis of the peak area for three replicate injections of the cucurbitacin B ranging from 20 – 150 μg/ml (0.02-0.15 mg/ml). The %RSD were shown in Table 27.

Table 27 Precision measurement of cucurbitacin B (n=3)

Conc. (μg/ml)	**Area under cucurbitacin B peak**			**mean**	**SD**	**% RSD**
20	768507	784099	776500	776368	7796.83	1.00
50	2045894	2030594	2064100	2057880	24768.41	1.20
75	3188574	3244599	3206245	3213139	28641.74	1.89
100	3901042	3391254	3987251	3933611	46807.99	1.11
125	4752163	4918171	4872012	4847449	85686.53	1.77
150	5645952	5614855	5782654	5681154	89266.42	1.57

2.3 Linearity

The linearity of the peak-area (y) versus concentration (x, μg/ml) curve of cucurbitacin B (SK4) was investigated in the range of 20-150 μg/ml. The values of slope and y-intercept of three day operation of cucurbitacin B (SK4) were shown in Table 28.

Table 28 The slope, y-intercept and r^2 value of cucurbitacin B calibration curve

Day	Slope	y-intercept	r^2
1	36871.54	193664.1	0.9971
2	37271.01	187322.1	0.9962
3	37071.27	190493.1	0.9969
Mean	37071.27	190493.1	0.9967
SD	199.735	3171	0.0005

2.4 Limit of Measurement

The calculation was based on the standard deviation of y-intercepts of regression line (σ) and the slope of the calibration curve (S):

$$LOD = 3.3 (\sigma/S)$$

$$LOQ = 10 (\sigma/S)$$

which $\sigma = 3171$ and $S = 36871.54$

LOD and LOQ for this method were 0.28 μg/ml and 0.86 μg/ml, respectively.

3. Determination of the cucurbitacin B in *T. cucumerina* root extract and fruit juice.

The determination of cucurbitacin B were performed on the root extract and the fruit juice of *T. cucumerina* using the established and validated method. The optimum condition used was a mixture of acetonitrile-water (40:60) as mobile phase, a 5μm Hypersil BDS C18 column as a stationary phase, flow rate at 1 ml/min, and detected by UV detector 210 nm. The chromatogram showed the t_R of cucurbitacin B obtained from the root extract and the fruit juice was 16.57 and 16.71 min , respectively. A

typical chromatogram of the root extract and the fruit juice of was shown in Fig.55 and 56.

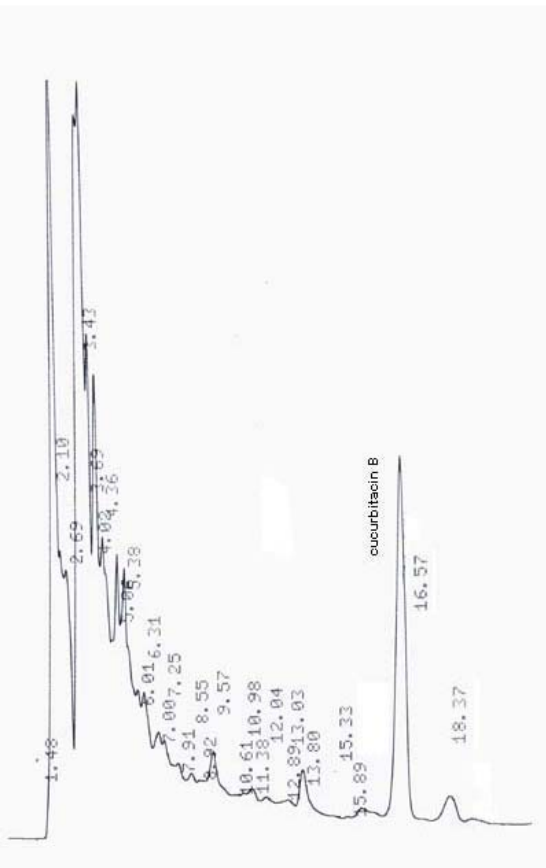


Figure 55 HPLC chromatogram of the root extract (5µm Hypersil BDS C18 column; mobile phase: acetonitrile-water, 40:60; flowrate: 1 ml/min.)

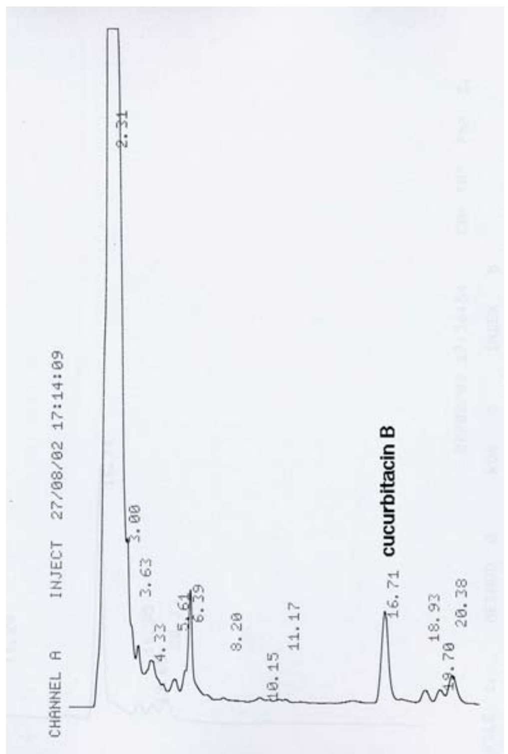


Figure 56 HPLC chromatogram of the fruit juice
(5µm Hypersil BDS C18 column; mobile phase: acetonitrile-water, 40:60; flowrate: 1 ml/min.)

CHAPTER VI
DISCUSSION

The root extract and the fruit juice of *T. cucumerina* were tested for their anti-malarial activity. Both of them were active against *P. falciparum* K1 strains with IC_{50} 312.50 μg/ml and 0.089 μg/ml, respectively. The antimalarial activity of the extract was rather weak, possibly because of the presence of inorganic salts and sugars in the extract, which masked the activity of the active components.

The root extract and the fruit juice were subjected for the phytochemical study in this research. The powdered root was successively extracted in a Soxhlet apparatus using petroleum ether, dichloromethane and 95% ethanol. The dichloromethane extract, which contained the rich isoprenoid derivatives were isolated using chromatographic technique (Fig.23). Five isoprenoid derivatives (SK1,SK2, SK3, SK4, and SK5) were isolated from the extract. The previous isolated substances by Chanchai Sardseangjun (20) included SC1, was identified as (3β-hydroxy-olean-13(18)-en-28-oic, and SC2 as 24β-ethyl-cholest-7,22-dien-3β-ol. Our study could isolated the same compounds they were SK1 (SC1) and SK3 (SC2). But both structures have been revised to bryonolic acid (D:C-friedoolean-8-en-3β-ol-29-oic acid, SK1) and bryononic acid (D:C-friedoolean-8-en-3-en-29-oic acid, SK3). One phytosteryl glucoside was also isolated from the root extract and identified as chondrillasteryl glucopyranoside (24β-ethyl-cholest-7,22-dien-3β-ol or 5α-stigmasta-7,22-dien-3β-*O*-glucopyranoside). The double bond position at C7 of phytosterols has been characteristic to the cucurbitaceous plants (69).

The fruit juice of *T. cucumerina* was extracted by partitioning with diethyl ether. The ethereal extract was further isolated on chromatographic column using silica gel 60. Compound SK4, which was later identified as cucurbitacin B, was obtained accompanying with the minor coexisting substance (SK5) from this step. The mixture

was subjected to lobar RP-18 column using acetonitrile-water (55:45) as a mobile phase. The sepation process was monitored by HPLC.

The isolatied compounds from the root extract and the fruit juice were shown in Table 29. Bryonolic acid (SK1) was the main component of the root extract. The fruit juice contained cucurbitacin B (SK4) 5 μg/ml and dihydrocucurbitacin B (SK5) 4.2 μg/ml. SK1,SK2 and SK3 were absent in the fruit juice.

Table 29 Percentage yields of the isolated compounds from *T. cucumerina*

Compound	**%yield (w/w)***
	the root extract
SK1	6.2×10^{-2}
SK2	2.5×10^{-3}
SK3	3.4×10^{-4}
SK4	8.0×10^{-4}
SK5	1.0×10^{-4}

* = gravimetric method

The structure elucidation of the isolated compounds was achieved using spectroscopic methods, especially NMR spectroscopy. All isolated compounds could be identified as;

SK1 : bryonolic acid

SK2 : chondrillasteryl glucoside

SK3 : bryononic acid

SK4 : cucurbitacin B

SK5 : dihydrocucurbitacin B

SK1, the isolated compound with the highest yield, was tested for the activity against papillomavirus. SK1 possesed a very potent activity with ED_{50} (1.5 ng/ml) . The root extract and the compound SK1 itself have been thus very interesting active compounds for the development of phytomedicine. SK4 and SK5 are cucurbitacins, which have been previously about the cyto-toxicity against nasopharynx carcinoma (KB cell) with the ED_{50} value of < 0.1 μg/ml(2).

SK4 could be isolated in a sufficient amount, and the antimalarial activity against K1 strain was carried out. SK4 possesed the IC_{50} of 0.625μg/ml. The antimalarial activity of SK4 in K1 strain (IC_{50} 0.625 μg/ml). The presence of SK4 in the root extract and the fruit juice together with its interesting activity, SK4 was thus selected as the lead compound for HPLC analysis.

The optimum condition for HPLC analysis was tested on reversed phase TLC and in this study the HPLC condition;

mobile phase : acetonitrile:water (40:60)
column : 250 x 4.6 mm 5 μm Hypersil® BDS C18
flow rate : 1 ml/min
detection : 210 nm
injection volume : 20μl

The HPLC method should be validated to assure the reproducible result. To validate the method, the system suitability test and the method validation were necessarily performed.

1. System suitability

The results of the system suitability test (Table 31), which was isocratically operated with the above condition, the obtained %RSD was less than 2.0%, the number of theoretical plates (N) was more than 2,000, the tailing factor (TF) was less than 2, and the resolution factor was more than 1.5. These were complied with the requirement of USP. Therefore, the system was suitable for the analysis of this compound.

Table 30 System suitability

Parameter	Cucurbitacin B (SK4)	Acceptable criteria (USP)
Precision (%RSD)		< 2%
- Interday	0.70%	
- Intraday	0.25-0.40%	
Theoretical plate (N)	46,270	> 2,000
Tailing factor (TF)	1	< 2
Resolution factor (Rs)	2.825	> 1.5
Retention time (min)	16.7	

2. Method validation

2.1 Accuracy

The results of accuracy (Table 25 and 26) revealed the percentage recoveries of the root extract and the fruit juice between 101-114% and 94-108% with 109.72% and 103.96% mean recovery and 4.97 and 5.93% standard deviation, respectively. The accuracy was complied with the USP requirements (80-120%). Therefore, this method was accurate for the analyse of the root extract and the fruit juice.

2.2 Precision

The results of precision of this method (Table 27) revealed the percentage of relative standard deviation (%RSD). The result was complied with the USP requirement (< 2.0%). The method was precise.

2.3 Linearity

Acceptability of linearity data was judged by examining the correlation coefficient of the linear regression line for the response versus concentration plot. According to USP, a correlation coefficient of >0.999 is generally considered as evidence of acceptable fit of the regression line. In this study, the r^2 = 0.9971 was the result of this study (Fig. 57).

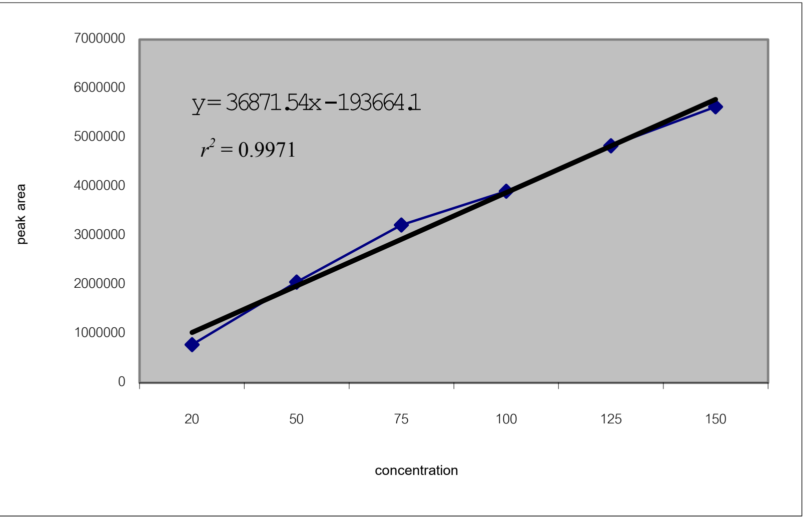


Figure 57 Calibration curve of cucurbitacin B

2.3 Limits of detection and measurement

The calculation is based on the standard deviation of y-intercepts of regression line (σ) and the slope of the calibration curve (S):

$$LOD = 3.3\ (\sigma/S)$$

$$LOQ = 10\ (\sigma/S)$$

which σ = 3171 and S = 36871.54

LOD and LOQ for this method were 0.28 ppm and 0.86 ppm, respectively.

The detection limit of the method is the lowest amount of analyte in a sample, which can be detected but not nessesarily quantitated as an exact value. The LOD is generally determined where the signal to noise ratio equals 3. The LOQ, which can be quantitatively determined with suitable precision and accuracy is normally determined where the signal to noise ratio equals 10.

Table 31 Method validation

Parameter	Validated value	Acceptable criteria (USP)
Accuracy (% recovery)	106-103 %	80-120%
Precision (%RSD)	1.0-1.89 %	>2%
Linear equation	y = 1615x + 2827.24	
Correlation coefficient (r^2)	0.9971	>0.99
LOD (ppm)	0.28	
LOQ (ppm)	0.86	

The content of cucurbitacin B was calculated using the linear regression obtained from the calibration curve of cucurbitacin B (Fig. 57); y = 36871.54x + 193664.1 (r^2 = 0.9971). The calculated content of cucurbitacin B was 1.03% w/w in the root extract and 0.0057% w/v in the fruit juice.

CHAPTER VII
CONCLUSION

Trichosanthes cucumerina belonging to the family Cucurbitaceae, has been used as remedy for purgative, fever and bronchitis. The cytotoxicity of the bitter compounds named cucurbitacin of the plants in this family have been reported. With the traditional used for fever treatment and the bitter taste of this compound, the hypothesis for anti-malarial activity of *T. cucumerina* was established. The phytochemical study in this plant performed in both root extract and fruit juice. Both parts were active in anti-malarial activity at the different level (IC_{50} 312.50 μg/ml for the root extract and 0.089 μg/ml for the fruit juice (based on cucurbitacin B content)). This result revealed that there were the active compounds containing in both root extract and fruit juice.

The dichloromethane extract was fractionated by Silica gel 60 column chromatography with dichloromethane-methanol, gradiently, as the mobile phase. The compound SK1, SK2, SK3 and SK4 were isolated and purified by various chromatographic techniques. The identification of the isolated compounds was achieved using modern NMR technique.

The fruit juice was extracted by partition with diethyl ether. The extract was isolated by using chromatographic technique and finally obtained the compound SK4 as same as in the dichloromethane root extract.

With the modern NMR technique, SK4 was identified as the mixture compound of cucurbitacin B and dihydrocucurbitacin B. They were separated by LPLC RP-18 column using acetonitrile-water (55:45) as mobile phase, with HPLC monitoring. Cucurbitacin B (SK5) was selected as the lead compound in the HPLC quantitative analysis of root extract and fruit juice.

The quantitative analysis of the cucurbitacin B (SK5) using high-performance liquid chromatography (HPLC) was developed. An appropriate isocratic condition was performed using 5μm Hypersil®BDS C18 column (250 x 4.6 mm i.d.), the solvent

system of acetonitrile-water (40:60) and UV detector at 210 nm. The cucurbitacin peak appeared at the retention time of 16.7 minute. The calibration curve of cucurbitacin B was linear in the range 20-150 ppm, with the correlation coefficient 0.9971 (n=15), and the detection limit (3.3[σ/s])was 0.28 ppm. The recoveries of cucurbitacin B spiked into the extract and the fruit juice were above 90%, complied with the requirement of the USP. The content of the cucurbitacin B in root extract and fruit juice were 1.03% w/w and 0.0057%w/v, respectively.

Compound SK1 was tested for antipapilloma viral activity. The active compound would be potential as a medicine for laryngeal cancer. SK1 produced a potent ED_{50} of 1.5 ng/ml.

The root extract contained a high yield of SK1 (620 mg/g). The quantitative HPLC analysis for SK1 has been planned for the future study.

Table 32 The isolated compounds from *T. cucumerina* root extract

Code	Name	Structure	%yield	Activity	
				Anti-malarial	**Anti-papilloma viral**
SK1	bryonolic acid		6.2×10^{-2}	ND	Active (LD_{50} 1.5 ng/ml)
SK2	chondrillasteryl glucoside		2.5×10^{-3}	ND	ND
SK3	bryononic acid		3.4×10^{-4}	ND	ND
SK4	cucurbitacin B		8.0×10^{-4}	Active asextual stage (IC_{50} 0.625 μg/ml)	ND
SK5	dihydrocucurbitacin B		1.0×10^{-4}	ND	ND

ND = Not determined

APPENDIX

APPENDIX

1. Complete RPMI 1640 medium

1.1 RPMI medium

RPMI 1640 powder with L-glutamine and without sodium bicarbonate (GIBCO BRL)	10.4 g
HEPES (SIGMA)	5.94 g
Gentamicin sulfate (80 mg/2ml)	2 ml
Double distilled water	960 ml

Dissolve 10.4 g of RPMI 1640 powder and 5.94 g of HEPES in 960 ml of double distilled water. Add 2 ml of gentamicin sulfate and sterile by filtration through 0.45 μm filter membrane. Store as stock medium of 100 ml at 4°C.

1.2 D-glucose (20% w/v $C_6H_{12}O_6$)

D-glucose (SIGMA)	20 g
Double distilled water	100 ml

Dissolve 20 g of D-glucose in 100 ml of double distilled water and sterile by filtration. Aliquot into sterillized tube as 10 ml and store at -20°C.

1.3 L-glutamine (200 mM $C_5H_{10}N_2O_3$)

L-glutamine (SIGMA)	2.922 g
Double distilled water	100 ml

Dissolve 2.922 g of L-glutamine in 100 ml of double distilled water and sterile by filtration. Aliquot into sterillized tube as 10 ml and store at -20°C.

1.4 Sodium bicarbonate solution (5% w/v $NaHCO_3$)

$NaHCO_3$ (MERCK)	5 g
Double distilled water	100 ml

Dissolve 5 g of $NaHCO_3$ in 100 ml of double distilled water and sterile by filtration through 0.45 µm filter membrane. Aliquot into sterilized tube as 10 ml and store at room temperature.

Each stock RPMI medium (100ml) was added with 5% (w/v) of $NaHCO_3$ 4.2 ml, 20% D-glucose 1 ml, 200 mM L-glutamine 1 ml and 10% (v/v) heat inactivated human serum (group A, B, and/or O). This medium referred to as complete RPMI medium with serum.

2. Sodium chloride solution (3.5% NaCl)

NaCl (MERCK)	3.5 g
Double distilled water	100 ml

Dissolve 3.5 g of NaCl in 100 ml of double distilled water and sterile by filtration. Aliquot into sterillized tube as 10 ml and store at room temperature.

3. Freezing solution

Sorbital (BDH)	7.56 g
0.9% NaCl solution (MERCK)	180 ml
99% Glycerine (RPE)	70 ml

Mix these solutions and filter with 0.45 µm filter membrane.

4. Phosphate buffer solution (PBS) pH 7.0

Solution A = 1/15 M Na_2HPO_4 (MERCK)

Na_2HPO_4	9.5 g
Distilled water	1000 ml

Solution B = 1/15 M $NaH_2PO_4 \bullet 2H_2O$ (MERCK)

$NaH_2PO_4 \bullet 2H_2O$	9.2
Distilled water	1000 ml

To prepare phosphate buffer solution pH 7.0, a volume of 60 ml of solution A and 40 ml of solution B are diluted with 900 ml distilled water.

5. Phosphate buffer saline pH 7.2

Solution A	72 ml
Solution B	28 ml
Distilled water	900 ml

A volume of 72 ml of solution A and 28 ml of solution B are diluted with 900 ml distilled water and sterile by autoclave.

6. Method for staining thin blood smears

- Fix the thin blood smears in absolute methanol for 1 min.
- Dilute the stock solution of Geimsa stain to 1:4 with phosphate buffer pH 7.0
- Stain thin blood smears with Giemsa stain solution for 15 min.
- Rinse with running tap water.
- Drain and dry at room termperature.

BIOGRAPHY

NAME	Mrs. Sumonthip Kongtun
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