การวิเคราะห์หาสารออกฤทธิ์ต้านออกซิเดชันในสมุนไพรเพชรสังฆาต

นางสาว จุฑามาส เที่ยงธรรม

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
สาขาวิชาเภสัชเคมี ภาควิชาเภสัชเคมี
คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2546
ISBN 974-17-3918-4
ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

DETERMINATION OF ANTIOXIDANT CONSTITUENTS IN THE MEDICINAL PLANT *CISSUS QUADRANGULARIS* LINN.

Miss Juthamas Thiangtham

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy in Pharmaceutical Chemistry

Department of Pharmaceutical Chemistry
Faculty of Pharmaceutical Sciences
Chulalongkorn University

Academic Year 2003
ISBN 974-17-3918-4

Thesis Title	DETERMINATION OF ANTIOXIDANT CONSTITUENTS IN THE MEDICINAL PLANT *CISSUS QUADRANGULARIS* LINN.
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จุฑามาส เที่ยงธรรม : การวิเคราะห์หาสารออกฤทธิ์ต้านออกซิเดชันในสมุนไพรเพชรสังฆาต (DETERMINATION OF ANTIOXIDANT CONSTITUENTS IN MEDICINAL PLANT *CISSUS QUADRANGULARIS* LINN.) อาจารย์ที่ปรึกษา : ผศ.ดร. ชำนาญ ภัตรพานิช, อาจารย์ที่ปรึกษาร่วม : รศ.ดร. เอกรินทร์ สายฟ้า, 74 หน้า ISBN : 974-17-3918-4

เพชรสังฆาต (*Cissus quadrangularis* Linn.) เป็นสมุนไพรที่ใช้กันอย่างแพร่หลายในการบำบัดอาการริดสีดวงทวาร โดยที่ยังไม่มีรายงานการศึกษาหาปริมาณสารออกฤทธิ์ เพื่อการควบคุมคุณภาพวัตถุดิบ และผลิตภัณฑ์ยาสำเร็จรูปของเพชรสังฆาต วิทยานิพนธ์นี้ได้ศึกษาองค์ประกอบทางเคมีสารสกัดชั้น ethyl acetate ในสารสกัด ethanol ของเพชรสังฆาตซึ่งสามารถแยกสารในกลุ่ม flavonoid และได้ทำการพิสูจน์ว่าเป็น quercetin โดยเปรียบเทียบกับสารมาตรฐาน quercetin และสารนี้เป็นสารที่มีฤทธิ์ DPPH radical scavenging activity และ antioxidant capacity

ได้ศึกษาพัฒนาและประเมิน (Validate) วิธีวิเคราะห์ โดยใช้เทคนิค High Performance Liquid Chromatography (HPLC) พบว่าสภาวะที่เหมาะสมที่ใช้ในการวิเคราะห์ ประกอบด้วย Column C-18 Aquasil (5 um, 250 × 4.6 mm i.d.), ตัวทำละลายเคลื่อนที่คือ 0.05 % ortho-phosphoric acid และ acetonitrile อัตราส่วน (65 : 35), UV detector ที่ 260 nm พบ peak ของสาร quercetin จะปรากฏที่ 10.68 นาที และสาร ethyl paraben ซึ่งถูกใช้เป็น Internal standard จะปรากฏที่ 13.68 นาที ได้ประเมินวิธีวิเคราะห์ในหัวข้อ ความจำเพาะเจาะจง ภาวะเชิงเส้น ความแม่นยำ ความถูกต้อง และความเหมาะสมของระบบ พบว่าภาวะเชิงเส้นของสาร quercetin ในระหว่างค่าความเข้มข้น 0.3 – 1.5 ไมโครกรัมต่อมิลลิลิตร มีค่า R^2 = 0.9996 ความแม่นยำของการวิเคราะห์ภายในวันเดียวกัน การวิเคราะห์ต่างวัน ต่างนักวิเคราะห์ และต่างเครื่องมือของสาร quercetin ที่คำนวณได้จากค่าสัมพัทธ์เบี่ยงเบนมาตรฐาน มีค่าน้อยกว่า 2 ความถูกต้องของการวิเคราะห์สาร quercetin ที่คำนวณได้จากค่าเปอร์เซ็นต์คืนกลับเฉลี่ยเท่ากับ 97.53 (% RSD = 1.31)

ได้ใช้วิธีวิเคราะห์ที่ผ่านการประเมินแล้วหาปริมาณสาร quercetin จากวัตถุดิบเพชรสังฆาต ที่เก็บในช่วงเวลาต่าง ๆ (กุมภาพันธ์ เมษายน มิถุนายน สิงหาคม ตุลาคม และธันวาคม) จากแหล่งปลูกจังหวัดพิษณุโลก ตรวจพบ quercetin ได้ คือ 10.15, 1.54, 2.54, 3.25, 7.88 และ 13.59 mg % ตามลำดับ อีกทั้งยังได้ทำการวิเคราะห์ หาปริมาณสาร quercetin จากวัตถุดิบเพชรสังฆาต จากแหล่งปลูกจังหวัดพิษณุโลก จังหวัดปราจีนบุรี และจังหวัดประจวบคีรีขันธ์ ในเดือนเมษายน พบปริมาณสาร quercetin ดังนี้ คือ 1.54, 1.88 และ 0.83 mg % การวิเคราะห์หาปริมาณสาร quercetin จากผลิตภัณฑ์ยาสำเร็จรูปขององค์การเภสัชกรรม จำนวน 2 ตัวอย่าง พบปริมาณสาร quercetin 13.45 และ 9.95 mg % ตามลำดับ

ภาควิชา	เภสัชเคมี	ลายมือชื่อนิสิต .....
สาขาวิชา	เภสัชเคมี	ลายมือชื่ออาจารย์ที่ปรึกษา .....
ปีการศึกษา	2546	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4276558833 : MAJOR PHARMACEUTICAL CHEMISTRY
KEY WORD: HAEMORRHOIDS / DPPH RADICAL SCAVENGING ACTIVITY / ANTIOXIDANT CAPACITY / QUERCETIN / HPLC / *CISSUS QUADRANGULARIS* LINN.

MISS JUTHAMAS THIANGTHAM : THESIS TITLE. (DETERMINATION OF ANTIOXIDANT CONSTITUENTS IN MEDICINAL PLANT *CISSUS QUADRANGULARIS* LINN.) THESIS ADVISOR : ASST. PROF. CHAMNAN PATARAPANICH, Ph.D., THESIS COADVISOR : ASSOC. PROF. EKARIN SAIFAH, Ph.D., 74 pp. ISBN : 974-17-3918-4

Cissus quadrangularis Linn. (*C.quadrangularis*), or "Phet-Sang–Khat", a climbing herb of the Vitaceae family that has been widely used in Thai traditional medicine as remedy for haemorrhoid. The identification and quality control of active principle in raw materials and finished product using as a marker to ensure consistent therapeutic efficacy of the product has never been reported. In this study, the activities (DPPH radical scavenging activity and antioxidant capacity) guided fractionation technique was used to identify the potential active principle in the plant, which will be used as the marker for quality control purpose. A flavonoid was isolated from the most active ethyl acetate fraction from the ethanolic extract of the plant and the compound was identified as quercetin by compare to the authentic quercetin.

A quantitative HPLC method for determining quercetin in *Cissus quadrangularis* Linn. has been developed and validated. The optimum HPLC system was comprised of C-18 reverse phase (Aquasil column 5 um, 250 × 4.6 mm i.d.), isocratic elution with 0.05 % ortho-phosphoric acid and acetonitrile (65 : 35) as the mobile phase and UV detection at 260 nm. The retention time of quercetin is at 10.68 min while ethyl paraben which was used as internal standard is at 13.68 min. Validation of the method was assessed from the specificity, linearity, precision, accuracy and system suitability test. The linearity with $R^2 = 0.9996$ was found in the range of 0.3 – 1.5 μg / ml for quercetin. The precision of quercetin calculated from the relative standard deviation was less than 2. The mean recovery of quercetin was found to be 97.53 % (% RSD = 1.31) in plant samples.

The validated HPLC method has been used to determine the quercetin contents in plant raw materials which were collected in different period of time through out a year (February, April, June, August, October and December) from Phitsanuloke and the contents of quercetin was found as following 10.15, 1.54, 2.54, 3.25, 7.88 and 13.59 mg %. Determination of quercetin in the plant collected from Phitsanuloke, Prachinburi and Prachuapkhirikhan province were found 1.54, 1.88 and 0.83 mg %. In addition, determination of quercetin in 2 lots of GPO finished product was also found 13.45 and 9.95 mg %, respectively.

Department Pharmaceutical Chemistry Student's signature ……

Field of study Pharmaceutical Chemistry Advisor's signature ……

Academic year 2003 Co-advisor's signature……...

ACKNOWLEDGEMENTS

No work is completed without any contribution. For this opportunity, I would greatly appreciate all the helps which have been given to me. By far my heaviest debt of gratitude is to Assistant Professor Dr. Chamnan Patarapanich, who not only advises me on the thesis completion but also sets me up as a good researcher. In particular, his guidance, constrictive criticism and encouragement throughout my study is invaluable.

I would like to express my sincere thank to Associate Professor Dr. Ekarin Saifah for his valuable advice during this study.

My special thanks are also expressed to the thesis committee for their constructive suggestions and critical review of the thesis.

I am very much indebted to the Government Pharmaceutical Organization for the Master degree scholarship. My thanks are also extended to friends and colleagues at Research and Development Institute, Government Pharmaceutical Organization for their help and friendship.

I would like to thank the Faculty of Pharmaceutical Sciences, Chulalongkorn University for financial support on technical investigation.

Finally, I wish to express my deepest thank to my family for their love and moral support, without them I would not have been able to complete this work.

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LIST OF ABBREVIATIONS

ABTS	=	2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)
$ABTS^{+\bullet}$	=	ABTS cation radical
AR grade	=	Analytical grade
ACN	=	Acetonitrile
BuOH	=	Butanol
C	=	Concentration
^{o}C	=	Degree Celcius
CH_2Cl_2	=	Dichloromethane
cm	=	Centimetre
DPPH	=	2,2-diphenyl-1-picryl-hydrazyl
$DPPH^{\bullet}$	=	DPPH radical
DMSO	=	Dimethyl sulfoxide
EtOAc	=	Ethyl acetate
EtOH	=	Ethanol
et al.	=	Et alia
g	=	gram
HPLC	=	High Performance Liquid Chromatography
H_2O_2	=	Hydrogenperoxide
h	=	hour
H_2O	=	water
IC_{50}	=	Median inhibitory concentration
i.d.	=	Internal diameter
Kg	=	Kilogram
L	=	Liter
min.	=	minute
mg	=	milligram
mm	=	millimeter

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mM	=	millimolar
ml	=	milliliter
MeOH	=	methanol
mp	=	melting point
M	=	Molar
nm	=	nanometer
no.	=	number
% RSD	=	Percentage of relative standard deviation
TLC	=	Thin layer chromatography
µl	=	microliter
µM	=	micromolar
µg	=	microgram
UV	=	ultraviolet
v/v	=	volume by volume
wt.	=	weight
w/w	=	weight by weight
λmax	=	wavelength at maximal absorption

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CHAPTER I

INTRODUCTION

Medicinal plants which are natural resources, are at present, being used and promoted as therapeutic agents in pharmaceutical industry because plant drugs are frequently considered to be less toxic and more free from side effects than synthetic chemicals. In order to utilized the medicinal plants as therapeutic purposes, scientific evidences in various fields such as pharmacology, immunology and toxicology are essential. In addition, the qualitative and quantitative control of active constituent to assure the consistency of the products is also necessary. The purpose of this research work to determine both quality and quantity of antioxidant constituents in a widely used medicinal plants, *Cissus quadrangularis* Linn.

Cissus quadrangularis Linn.; a well known climbing herb belonging to the Vitaceae family, is known in Thai name as, Phet Sang Khat (เพชรสังฆาต), San Chakuat (สันชะควด), San Chakhat (สันชะฆาต) (Bangkok); Samroito (สามร้อยต่อ), Samroikho (สามร้อยข้อ) (Prachaubchirikhan); Khankho (ขันข้อ), Wai-fai (ไวไฟ), Tamleung Tong (ตำลึงทอง) (Ratchaburi).[1] The plant grows widely throughout the plains of India, Africa and Arabic countries, where are assumed to be its native land.[2] In India, the preparation of the powdered dry shoot of *Cissus quadrangularis* Linn. has been used against indigestion and whooping cough;[3] the leaves of this plant have been used in the treatment of internal ulceration and for external wounds;[4] the roots were used for myaglia[5] the stems were used for irregular mentruation[6] and the plant juice were used for earache[3] and amenorrhea.[7] In Central Africa, decoction of this plant has been taken for blenorrhagia and to calm palpitation.[8] In South Africa, dried root of this plant has been used for inflammation.[9] In Arab countries, the hot water extract of dried entire plant has been used as a “cure-all” medicine.[10]

Figure 1 : *Cissus quadrangularis* Linn. Thai name : Phet Sang Khat

This plant has also been known to have beneficial effects on bone healing for many centuries. The study of the effect of this plant in experimental animals was performed by Udupa et al.[11] in 1961. It was found that this plant promote healing of the fracture and the effect of the drug was not due to the vitamin content in plant alone. This result was supported by the work of Singh and Udupa[12] and by Udupa and Prasad.[13]

In Thailand, the plant has been used as a carminative, anti-haemorrhoid and to promote fracture bone healing.[14] Further investigation indicated that dried plant materials of its whole part could lower the development of haemorrhoids.

Haemorrhoid result from the distal displacement of the anal cushions and are a very common and widespread condition.[15, 16] They are classified as either external or internal expending on their origin (i.e. below or above the dentate line) and both types may thrombose.[17] Symptoms are chronic with recurrent self-resolving acute episodes.[18] Various causes of haemorrhoidal disease have been proposed (e.g. increased maximum resting anal pressure, intrinsic weakness in the blood vessel wall, excessive arterial flow, secondary obstruction of outflow and increased intra-abdominal pressure). However its pathophysiology is not completely understood.[16, 17, 19, 20] The pathogenesis of haemorrhoids may involve the stagnation and stasis of the blood in the vascular plexuses of anal cushions.[21] Venous stasis may lead to inflammation resulting in the increase permeability, fragility and necrosis of the vessel wall in the anal cushion and result in bleeding. Free radicals and other cytotoxic substances released by the inflammatory response may in turn lead to tropic changes that may ultimately cultimate in the development of haemorrhoids.[22] Some antioxidants inhibit the activation of free radical, resulting in a decrease inflammation of haemorhoids.[23] In a nation-wide questionair in the US,[24] the prevalence of haemorrhoids was 4.4 %; the prevalence was greatest between the ages of 45 and 65 years and there was an equal distribution of haemorrhoidal disease between men and women.

The pre-clinical study and clinical study on C*issus quadrangularis* Linn. as a drug for acute haemorrhoids treatment were conducted.[25, 26] In a clinical study, the plant *Cissus quadrangularis* Linn. was formulated as enteric film coated tablet and the physical appearance of the tablet (size, color, shape) was identicle to standard drug of choice (Daflon®). Treatment with this plant's drug to the haemorrhoid patients, decrease the inflammation to the some extent of that treat by Daflon. However, the main active principle of the Daflon® were diosmin and hesperidin which possess the antioxidant property.[27, 28, 29]

The Board of Primary Health Care, Ministry of Public Health has established a policy on promotion of the usage of this plant in the treatment of haemorrhoids in several community hospital of Thailand. Nowadays this medicinal plant are widely used in Thailand.

For a safe utilization of any medicinal plant as a medicine, it's standardization is necessary to assure the plant drug authenticity and its consistency of active principles according of the parameters utilized as quality criteria. However, no chromatographic method for the standardization of antioxidant constituent from *Cissus quadrangularis* Linn. has been reported.

In this study, the antioxidant in the plant was identified by using the standard DPPH and ABTS method together with the conventional isolation, extraction and chromatographic process. Such bioactivity guided fractionation can ultimately indicate that active components which can be used as the marker for the quality control purpose.

A simple, rapid and accurate HPLC method was developed for the determination of the antioxidant constituent, using internal standard techniques.

The quantitative analysis by HPLC based on comparison between the retention and peak areas of samples with authentic standard which can be measured, either manually or electronically. The same methods were applied for the assay of

amount of antioxidant constituent in dried plant raw materials collected monthly through out a year.

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CHAPTER II

HISTORICAL

1. Chemical compounds in *Cissus quadrangularis* Linn.

Cissus quadrangularis Linn. has been widely investigated, especially in the field of chemical constituents studies. Many type of chemical compounds, isolated from this plant, have been reported such as triterpene, steroid, stilbene, flavonoid and miscellaneous compounds. The occurrence of chemical compounds in *Cissus quadrangularis* Linn. are shown in Table 1.

Table 1. The occurrence of chemical compounds in *Cissus quadrangularis* Linn.

Type of compound	Compounds	Part of the plant	% Content found	Reference
Triterpene	δ-amyrin	Whole plant	0.14000 %	30, 31
	δ-amyrone	Whole plant	0.10000 %	31
	Iso-aborenol	Whole plant	0.00030 %	32
	Friedelan-3-one	Aerial parts	-	33
	Epi-friedelinol	Whole plant	0.00024 %	32
	Lupenone	Whole plant	0.00022 %	32
	Onocer-7-ene-3-alpha-21-beta-diol	Whole plant	0.00600 %	31
	Onocer-7-ene-3-beta-21-alpha-diol	Whole plant	0.00300 %	31

Type of compound	Compound	Part of the plant	% Content	Reference
Triterpene	Onocer-8-ene-3-beta-21-alpha-diol	Aerial parts	0.00225 %	34
	Oxo-onoler-7-ene-3-alpha-21-beta-diol	Whole plant	-	30
	Onoler-7-ene-3-beta-alpha-diol	Whole plant	-	30
	Taraxerol	Aerial parts	-	33
	Taraxeryl acetate	Aerial parts	-	33
Steroid	Keto-steroid	Whole plant	-	35
	Oxo-steroid	Whole plant	-	36
	Steroid (MP134-136)	Whole plant	-	37
	β-sitosterol	Whole plant	-	31, 32, 33
Stilbene	Pallidol	Stem	0.00055 %	38
	Parthenocissin A	Stem	0.00070 %	38
	Quadrangularin A	Stem	0.00090 %	38
	Quadrangularin B	Stem	0.00040 %	38
	Quadrangularin C	Stem	0.00080 %	38
	Piceatannol	Stem	0.00020 %	38
	Resveratrol	Stem	0.00050 %	38
Flavonol	Kaemferol	Stem	0.00030 %	38
	Quercetin	Stem	0.00210 %	38
	Quercitrin	Stem	-	39
	Iso-quercitrin	Stem	-	39

Type of compound	Compound	Part of the plant	% Content	Reference
Alkane C-5 or More	Heptadecyl octadecanoate	Aerial parts	-	33
	Icosanyl icosanoate	Aerial parts	-	33
	Tritriacontanoic acid, 31-methyl	Aerial parts	-	33
Alkene C-5 or More	Octadec-9-ene, 9-methyl	Aerial parts	-	33
Alkanol C-5 or More	Docosan-1-ol-cyclohexane, 7-hydroxy-20-oxo.	Aerial parts	-	33
	Tritriacontan-1-ol, 31-methyl	Aerial parts	-	33
Alkenone C-5 or More	Tricos-2-en-22-one, 4-hydroxy-2-methyl	Aerial parts	-	33

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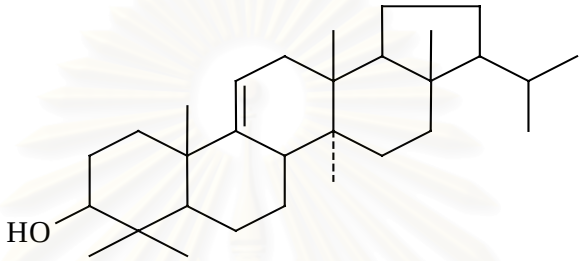
2. Chemistry of the constituents

2.1 Triterpene compounds

2.1.1 Iso-aborenol

The isolation of isoaborenol from *Cissus quadrangularis* Linn. was reported in 1986 by Pluemjai and Saifah.[32] Isoaborenol, a pentacyclic triterpene which has molecular formula $C_{30}H_{49}O$, appeared as white crystals, mp 275–278°C.

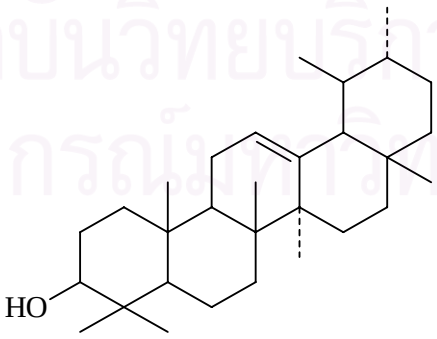


$C_{30}H_{49}O$

Isoaborenol

2.1.2 δ - amyrin [olean –13(18)-en-3-ol]

The isolation of δ-amyrin from the whole plant of *Cissus quadrangularis* Linn. was reported by Bhutani *et al.*[31] in 1984. δ-amyrin is colorless crystals, mp 156-158°C, with molecular formula $C_{30}H_{50}O$.

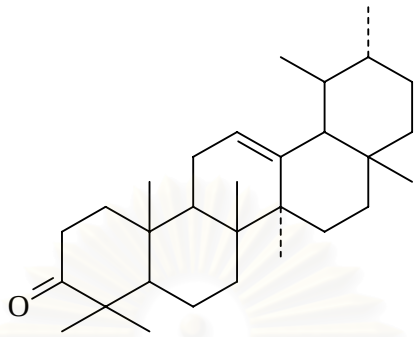


$C_{30}H_{50}O$

δ - amyrin

2.1.3 δ-amyrone [olean 13(18) -en-3-one]

Bhutani *et al*.[31] isolated δ-amyrone from *Cissus quadrangularis* Linn. It is colorless flakes, mp 176-178°C, with molecular formula $C_{30}H_{48}O$.



$C_{30}H_{48}O$

δ-amyrone

2.1.4 Friedelan-3-one

The isolation of firedelan-3-one from *Cissus quadrangularis* Linn. was reported in 1991 by Gupta and Verma.[33] The compound has mp 256–258°C.

2.1.5 Epifriedlinol

In 1986 Pluemjai and Saifah[32] isolated epifriedlinol from *Cissus quadrangularis* Linn. Epifriedelinol, a pentacyclic triterpene which has molecular formula $C_{30}H_{49}O$, appeared as white crystals, mp 272–275°C.

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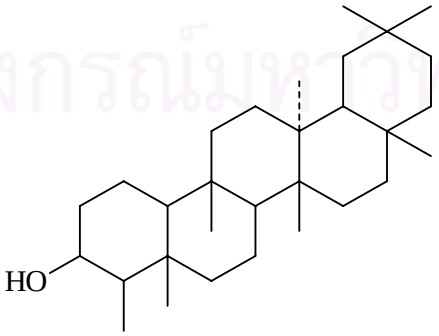
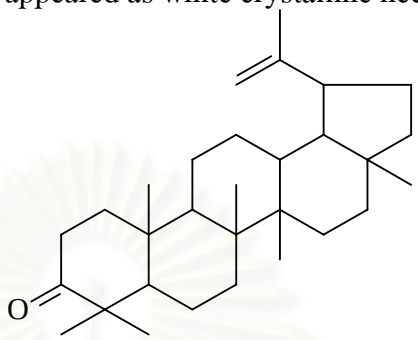


$C_{30}H_{49}O$

Epifriedelinol

2.1.6 Lupenone

The isolation of lupenone from *Cissus quadrangularis* Linn. was reported in 1986 by Pluemjai and Saifah.[32] Lupenone, a pentacyclic triterpene which has molecular $C_{30}H_{48}O$, appeared as white crystalline needles, mp 165-166°C.

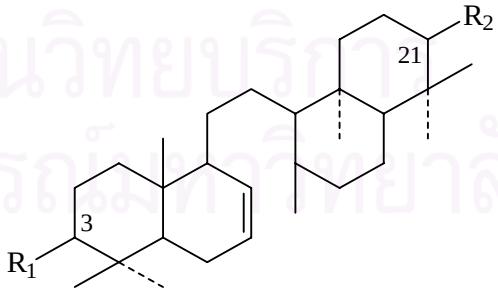


$C_{30}H_{48}O$

Lupenone

2.1.7 Onocer-7-ene-3-alpha-21-beta-diol

The isolation of onocer-7-ene-3-alpha-21-beta-diol from *Cissus quadrangularis* Linn. was reported in 1984 by Bhutani *et al*.[31] Onocer-7-ene-3-alpha-21-beta-diol, an unsymmetric tetracyclic triterpene which has molecular formular $C_{30}H_{52}O$, appeared as colorless crystals, mp 200-202°C, with IR absorbtion bands at 3350 (hydroxy group), 1615 (exocyclic methylene group), 1380, 1055 $cm.^{-1}$



R1= α -OH, R2 = β-OH

$C_{30}H_{52}O$

Onocer-7-ene-3-alpha-21-beta-diol

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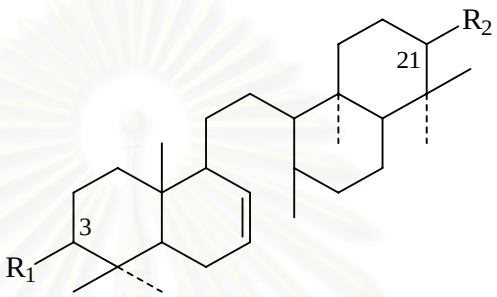
2.1.8 Onocer-7-ene-3-beta-21-alpha-diol

The isolation of onocer -7-ene-3-bata-21-alpha-diol from *Cissus quadrangularis* Linn. was reported in 1984 by Bhutani et al.[31] Onocer-7-ene-3-beta-21-alpha-diol, an unsymmetric tetracyclic triterpene which has molecular formula $C_{30}H_{32}O_2$, appeared as colorless crystals, mp 233-234°C.



R1= β-OH , R2= α-OH

$C_{30}H_{32}O_2$

Onocer-7-ene-3-alpha-21-beta-diol

2.1.9 Taraxerol

The isolation of taraxerol from the aerial part of *Cissus quadrangularis* Linn. was reported in 1991 by Kupta and Verma.[33] Taraxerol has mp 280-283°C.

2.1.10 Taraxeryl acetate

The isolation of taraxeryl acetate from the aerial part of *Cissus quadrangularis* Linn. was reported in 1991 by Kupta and Verma.[33] Taraxeryl acetate has mp 297-299°C.

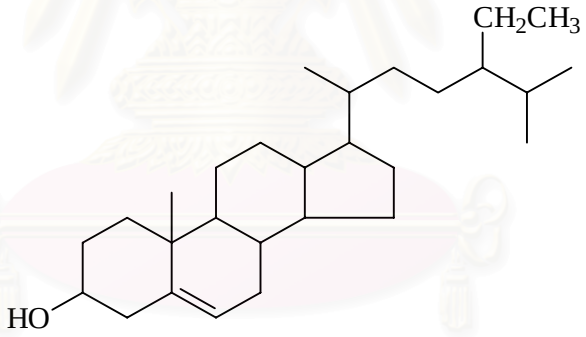
2.2 Steroid compound

2.2.1 Oxo-steroid

In 1964 Sen *et al.*,[36] isolated oxo-steroid from *Cissus guadrangularis* Linn., mp 134-136°C. The UV and IR spectra suggested an α, β-unsaturated ketone.

2.2.2 β-sitosterol

In 1986 Pluemjai and Saifah[32] isolated β-sitosterol from *Cissus quadrangularis* Linn. It is white crystalline needles, mp 132-133°C with molecular formula $C_{29}H_{50}O$.



$C_{29}H_{50}O$

β-sitosterol

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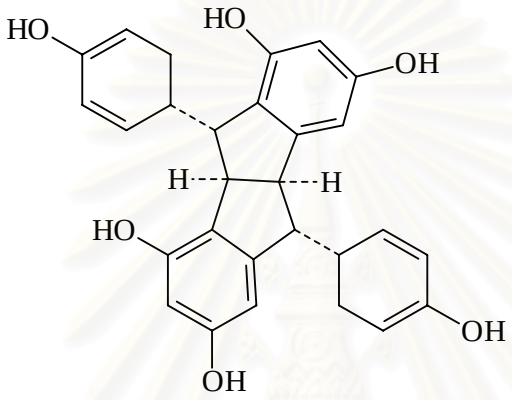
2.3 Stilbene compounds

2.3.1 Pallidol

In 1999 Saburi *et al*.,[38] isolated pallidol from the stem of *Cissus quadrangularis* Linn. Pallidol has molecular formula $C_{28}H_{22}O_6$.



$C_{28}H_{22}O_6$

Pallidol

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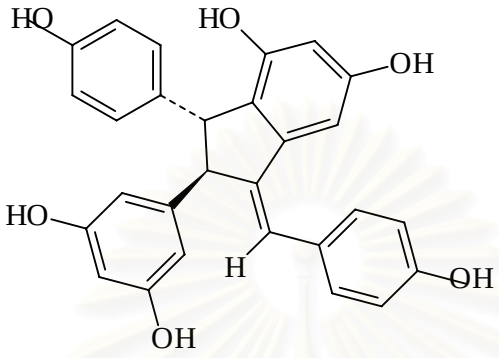
2.3.2 Parthenocissin A

In 1999 Saburi *et al*.,[38] isolated parthenocissin A from the stem *Cissus quadrangularis* Linn. Parthenocissin A has molecular formula $C_{28}H_{22}O_6$.

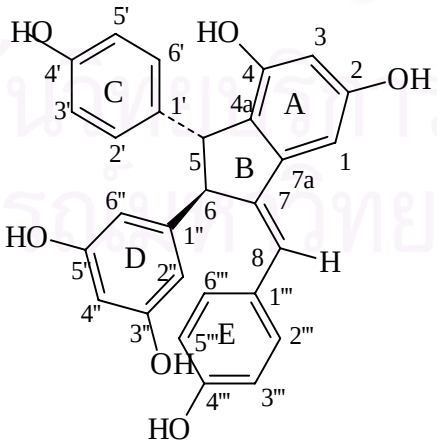


$C_{28}H_{22}O_6$

Parthenocissin A

2.3.3 Quadrangularin A

In 1999 Saburi *et al*.,[38] isolated quadrangularin A from the stem of *Cissus quadrangularin* Linn. It is amorphous gum, $[\alpha]_D$-2° (MeOH), with molecular formula $C_{28}H_{22}O_6$.

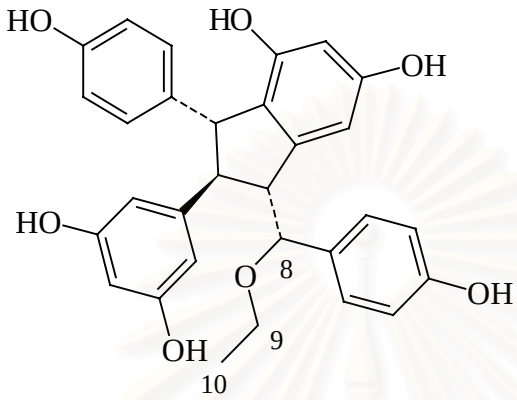


$C_{28}H_{22}O_6$

Quadrangularin A

2.3.4 Quadrangularin B

In 1999 Saburi *et al*. (38), isolated quadrangularin B from the stem of *Cissus quadrangularis* Linn., it is amorphous gum, $[\alpha]_D 0^o$ (MeOH), with EIMS m/z 454, $[M\text{-}C_2H_5OH]^+$.

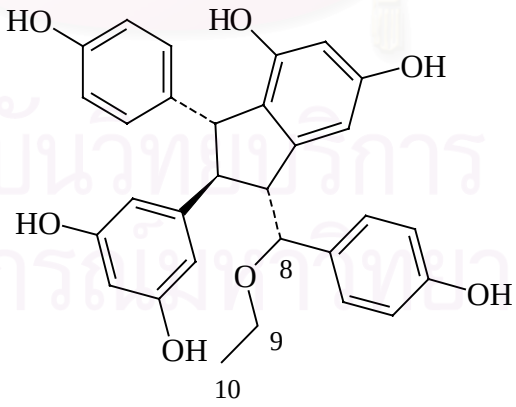


Quadrangularin B

(8S)

2.3.5 Quadrangularin C

Quadrangularin C was a sterioisomer of quadrangularin B which has been isolated from the stem of *Cissus quadrangularis* Linn. by Saburi *et al*.[38] in 1999. It is amorphous gum, $[\alpha]_D$ -1 (MeOH), with EIMS m/z 454, $[M - C_2H_5OH]^+$

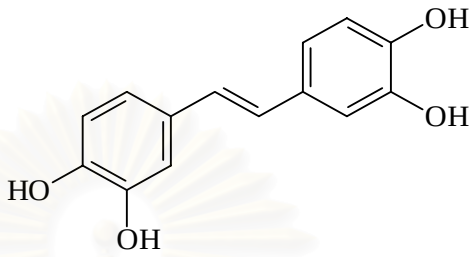


Quadrangularin C

(8R)

2.3.6 Piceatannol

In 1999 Saburi *et al*.,[38] isolated piceatannol from the stem of *Cissus quadrangularis* Linn., it is white crystals, with molecular formula $C_{14}H_{12}O_4$.

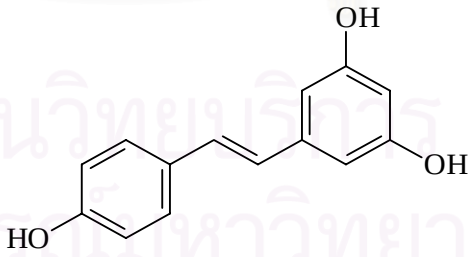


$C_{14}H_{12}O_4$

Piceatannol

2.3.7 Resveratrol

In 1999 Saburi *et al*.,[38] isolated resveratrol from the stem of *Cissus quadrangularis* Linn., it is white crystals, with molecular formula $C_{14}H_{12}O_3$.



$C_{14}H_{12}O_3$

Resveratrol

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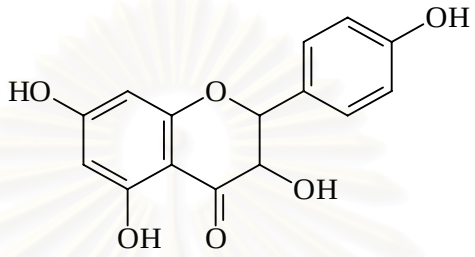
2.4 Flavonoid compounds

2.4.1 Kaemferol

In 1999 Saburi *et al*.,[38] isolated kaemferol from the stem of *Cissus quadrangularis* Linn., it is yellow amorphous powder, with molecular formula $C_{15}H_{10}O_6$.

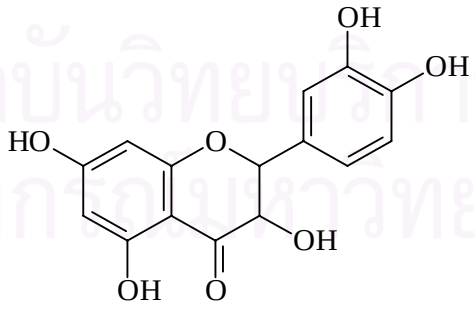


$C_{15}H_{10}O_6$

Kaemferol

2.4.2 Quercetin

In 1999 Saburi *et al*,[38] isolated quercetin from the stem of *Cissus quadrangularis* Linn., it is yellow amorphous powder, mp 315°C, with molecular formula $C_{15}H_{10}O_7$.

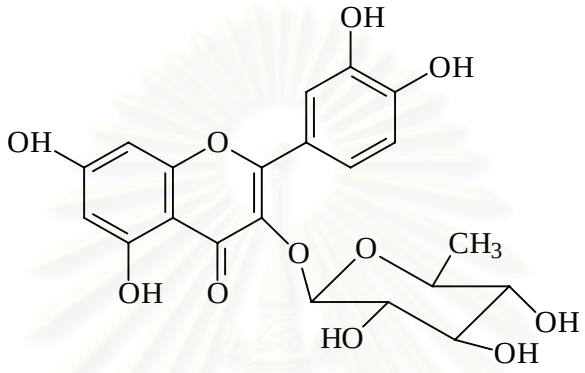


$C_{15}H_{10}O_7$

Quercetin

2.4.3 Quercitrin

Jakikasem *et al.*,[39] isolated quercitrin from the stem of *Cissus quadrangularis* Linn., the compound has molecular formula $C_{21}H_{20}O_{11}$, appeared as a yellow amorphous powder which give a blueish green color with $FeCl_3$ spray reagent.

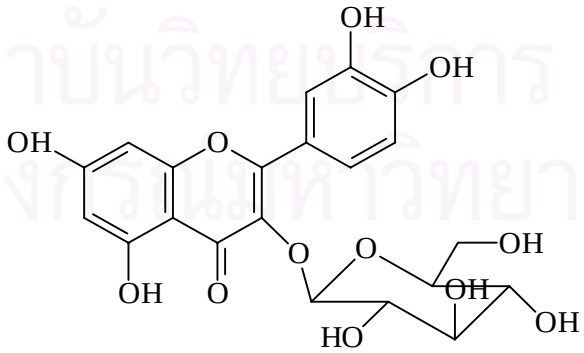


$C_{21}H_{20}O_{11}$

Quercitrin

2.4.4 Iso-quercitrin

Jakikasem *et al.*,[39] isolated iso-quercitrin from the stem of *Cissus quadrangularis* Linn., the compound has molecular formula $C_{21}H_{20}O_{12}$, appeared as a yellow amorphous powder.



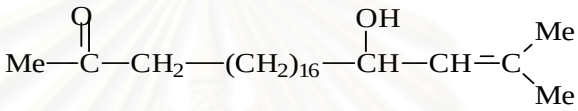
$C_{21}H_{20}O_{12}$

Iso-quercitrin

2.5 Miscellaneous compounds.

2.5.1 4-hydroxy-2-methyl-tricos –2- en-22-one

In 1991 the isolation of 4-hydroxy-2-methyl-tricos–2- en-22-one from the aerial part of *Cissus quadrangularis* Linn. was reported by Gupta and Verma.[33] The compound has molecular formula $C_{24}H_{46}O_2$, IR absorbtion band at 3400, 1720, 1640, 1070, 720 $cm.^{-1}$ and gave a positive 2,4 dinitrophenyl hydrazine test for a long chain unsaturated hydroxy ketone.



$C_{24}H_{46}O_2$

4-hydroxy-2-methyl-tricos–2- en-22-one

2.5.2 9-methyl-octadec–9-ene

Gupta and verma[33] isolated 9-methyl– octadec-9-ene from the aerial part of Linn. the compound has molecular formula $C_{19}H_{38}$, with IR Absorbtion bands at 1640, 730, 720 cm^{-1} for an unsaturated straight chain hydrocarbon.

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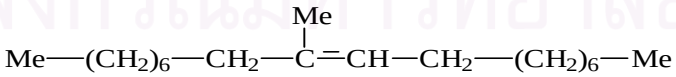
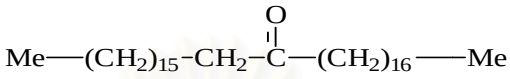


$C_{19}H_{38}$

9-methyl-octadec–9-ene

2.5.3 Hepadecyl octadecanoate

In 1991, Gupta and Verma[33] isolated heptadecyl octadecanoate from the aerial part of *Cissus quadrangularis* Linn. The compound has molecular formula $C_{35}H_{70}O_2$, mp 68–70°C, had IR absorbtion bands at 1740 (ester CO) and 730 cm^{-1} (long chain).

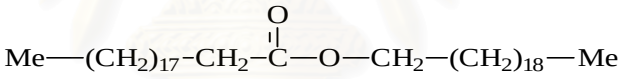


$C_{35}H_{70}O_2$

Hepadecyl octadecanoate

2.5.4 Icosanyl icosanoate

Gupta and Verma[33] isolated Icosanyl icosanoate from the aerial part of *Cissus quadrangularis*. Linn. the compound has molecular formula $C_{40}H_{80}O_2$, mp 67-69°C.

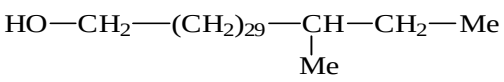


$C_{40}H_{80}O_2$

Icosanyl icosanoate

2.5.5 31-methyl tritriacontan-1-ol

The isolation 3-methyl tritriacontan-1-ol from the aerial part of *Cissus quadrangularis* Linn. was reported by Gupta and Verma[33] in 1991. The compound with molecular $C_{34}H_{70}O$, mp 72-73°C, had IR absorbtion bands at 3380, 1050, 730 and 720 cm^{-1} for its long chain alcohol

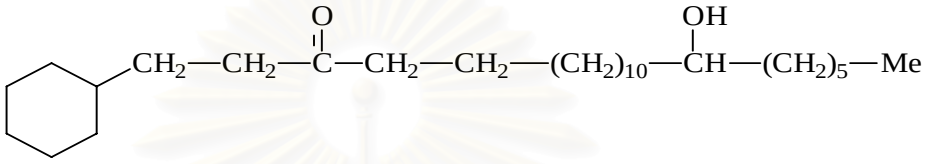


$C_{34}H_{70}O$

31-methyl tritriacontan-1-ol

2.5.6 7- hydroxy-20-oxo-docosanyl cyclohexane

In 1991, Gupta and Verma[33] isolated 7- hydroxy-20-oxo-docosanyl cyclohexane from the aerial part of *Cissus quadrangularis* Linn. The compound has molecular formula $C_{28}H_{54}O_2$, had IR absorbtion bands at 3400, 1040 (hydroxyl group), 1720 (CO) and 720 cm^{-1} (long chain).

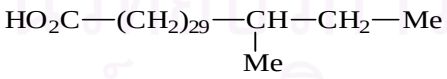


$C_{28}H_{54}O_2$

7-hydroxy-20-oxo-docosanyl cyclohexane

2.5.7 31- methyl tritriacontanoic acid

Gupta and Verma[33] isolated 31- methyl tritriacontanoic acid from the aerial part of *Cissus quadrangularis* Linn. The compound has molecular formula $C_{34}H_{68}O_2$, mp 80-81°C.



$C_{34}H_{68}O_2$

31- methyl tritriacontamoic acid

3. Pharmacological Activity

3.1 Crude Extract of *Cissus quadrangularis* Linn.

3.1.1 Antimicrobial Activity

Lin *et al*.,[9] reported that the aqueous extraction and methanolic extract of the dried root of *Cissus quadrangularis* Linn. exhibited antibacterial acitivity with equivocal potency against *Bacillus cereus*, *Bacillus circulans*, *Bacillus pumilus*, *Bacillus stearothermophilus*, *Bacillus subtilis*, *Proteus mirabilis*, *Pseudomonas sola*cearum, *Salmonella typhimurium*, S*higella boydii*, *Shigella flexneri*, *Staphylococcus aureus* and *Staphylococcus epidermidis in vitro*.

3.1.2 Hypotensive activity

Bhukani *et al*.,[40] reported that the extraction by EtOH-H_2O (1:1) of the dried aerial part of *Cissus quadrangularis* Linn. showed hypotensive activity in dog's experiment.

3.1.3 Anti-implantation activity

Dhawan *et al*.,[41] reported that oral administration of a single dose of the ethanolic extract of the dried aerial part of *Cissus quadrangularis* Linn. (340 mg/kg) to adult female rat showed anti- implantation activity.

3.1.4 Antiproliferation activity

Opolu *et al*.,[42] reported the aqueous extraction and methanol extract from the dried root of *Cissus quadrangularis* Linn. showed antiproliferation activity in cell culture.

3.1.5 Mutagenic activity

Sivasvamy *et al*.,[46] reported that the fresh fruit of *Cissus quadrangularis* Linn. posses a very low mutagenic activity against *Salmonella typhimurium* (TA 1537) and *Samonella typhimurium* (TA 98), but not against *Salmonella typhimurium* (TA1535) and *Salmonella typhimurium* (TA1538).

3.1.6 Molluscicidal activity

Abdel-Azizi *et al*.,[47] reported that the ethaolic extract from the dried leaves of *Cissus quodrangularis* Linn. was no molluscicidal activity against *Biomphalaria pfeifferi* and *Bolinus truncatus*.

3.1.7 Chromosome aberrations induced and clastogenic activity

Balachandran *et al*., reported that intra gastric administration of *Cissus quadrangularis* Linn. extract (0.1 g/animal) to mouse posses a very low chromosome aberrations induced and clastogenic activity.

3.1.8 Anti-inflammatory activity

The preliminary anti-inflammatory activity was reported by Thisayakorn *et al*.[49] The suspension of *Cissus quadrangularis* Linn. in soy bean oil was given orally in concentration of 500, 1,000, 2,000 and 3,000 mg/kg to rates. It showed that *Cissus quadrangularis* Linn. at 500 and 1,000 mg/kg gave negative results, where as 1500, 2000 and 3000 mg/kg could slightly inhibit inflammation at 1 and 2 h. It appeared that *Cissus quadranularis* Linn. might not inhibit acute inflammation in rats.

3.1.9 Miscellaneous.

Chopra *et al*., [14,43] reported the aqueous extraction of the dried aerial

part of *Cissus quadrangularis* Linn. (1.0 mg/kg) was given to the dog by intramuscular administration, increased fracture bone healing. The tensile strength of healing of fracture bones was measured by the tensiometer and the rate of calcium deposit in the callus was measured by used of ^{45}Ca. The extract had a definite beneficial effect the increasing tensile strength in treated animals correlated with an increase in the components of the bones, such as mucopolysaccharides, collagen, calcium and phosphorus.

Lin *et al*.,[9] reported that the methanolic extract of the dried root of *Cissus quadrangularis* Linn. possesses very low cox-1 hydroperoxidase inhibition. Insecticidal activity of the ethanolic extract of the dried aerial part of *Cissus quadrangularis* Linn. at concentration of 1.0 % was reported by Atal *et al*.[44] No insecticidal activity against Mosca domestica and Tribolium castaneum.

Masscle *et al*.,[45] reported that the methanolic extract of the dried aerial part of *Cissus quadrangularis* Linn. concentration more than 1.0 mg/ml had no anticrustacean activity against *Artemiasalina*.

4. Toxicity

4.1 Crude Extract of *Cissus quadrangularis* Linn.

4.1.1 An acute toxicity

Lhieochaiphunt and Sangdee,[50] studied an acute toxicity of the aqueous extract of whole part of *Cissus quadrangularis* Linn. in mice, rats and rabbits by intraperitoneal injection and orally through gastric tubing. The result showed that the degree of toxicity of this medicinal plant ranged from slightly toxic to practically non toxic.

In 1999, Intarapuak,[51] studied acute oral toxicity of *Cissus quadrangularis* Linn. in rats which was designed to meet the requirement of

method defined in the Limit test of OECD (1993). The Wistar rats of both sexs were feed with the drug at the dose of 2,000 mg/kg. It found that no toxic signs and no mortalities occurred within the observation period of 14 days.

In addition, Limpanussorn[52] studied acute oral toxicity of *Cissus quadrangularis* Linn. in mice. Ten mice (Swiss albino), 5 males and 5 females, 8 weeks age, were used in each group. The suspension of *Cissus quadrangularis* Linn. were gave at the dose of 2,000 mg/kg body weight. The result showed that no toxic signs and no mortalities occurred during 14 days of observation period.

4.1.2 Subchronic toxicity

Limpanussorn *et al.*[53] studied a subchronic toxicity of dried powder of *Cissus quadrangularis* Linn. which was conducted according to OECD guidelines (1993). Wistar rats, 5 weeks of age, 10 of each sex in each group, were given with suspension of *Cissus quadrangularis* Linn. at the dose 50, 350 and 2,450 mg/kg body weight for 30 consecutive days and observed thereafter for 14 days in order to study reversibility of adverse effects. No toxic sign and no changes of growth rate, food consumption and relative internal organ weights were found. Clinical blood chemistry and haematological parameters of rat were not affected after treated with the low and medium doses of *Cissus quadrangolaris* Linn. whereas the highest dose caused significant changes in clinical chemistry and haematological parameters, including decrease in serum creatinine in female rats ($p=0.044$), decrease in serum albumin in both sexs of rats ($p=0.046$ and $p= 0.044$, respectively) and also decrease in total white blood cell count of male rats ($p=0.044$). The level of serum creatinine, serum albumin and total white blood cell count of rats were returned to normal levels after drug withdrawal for 14 days.

In 2002, Attawish *et al.*[54] studied a subchronic toxicity of the dried powder of *Cissus quadrangolaris* Linn. in five groups of 12 wistar rats of each sex, were given with the dried stem powder at the dose of 0.03, 0.3 and 3.0 g/kg body weight/day,

which were equivalent to 1, 10, 100 folds of the therapeutic dose in human for 3 months. The result showed that no difference of initial or final body weights between *Cissus quadrangolaris* Linn. treated and control groups was detected. It was found that *Cissus quadrangolaris* Linn. did not produce any significant dose related changes of hematological parameters or serum clinical chemistry, and no histopathological lesion of any internal organ that could be due to the toxic effect of *Cissus quadrangularis* Linn. was observed.

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CHAPTER III

Experimental

1. Source of Plant Materials

The whole plant of *Cissus quadrangularis* Linn. (Vitaceae) were collected from different wild locations in the central (Prachinburi), northern (Phitsanulok) and eastern (Prachuabchirikhan) geographic zones of Thailand. The plant sample were purchased from suppliers who supplied this plant to the hospital in that province for production of haemorrhoid product and prescribed to haemorrhoids patients. After collecting the aerial parts, the whole plant were cleaned with tap water, dried in hot air oven at 60°C for 72 hours and then ground into powder by an electric mill and passed through sieve No. 80.

2. General Techniques

2.1 Thin Layer Chromatography (TLC). The experimental details are summarized as follows :-

Technique	:	One dimension, ascending
Adsorbent	:	Silica gel 60 F_{254} (E. Merck) precoated plate
Plate size	:	20 cm ×5 cm
Layer thickness	:	0.2 mm
Temperature	:	Laboratory temperature (25-30°C)
Detection	:	1. Ultraviolet light at wavelengths of 254 and 365 nm
		2. 10 % Sulfuric acid in ethanol and heated at 105°C for 1-2 min
		3. 0.2 % DPPH in methanol solution
Solvent system	:	Toluene – EtOAc- Formic acid (5 : 4 : 1)

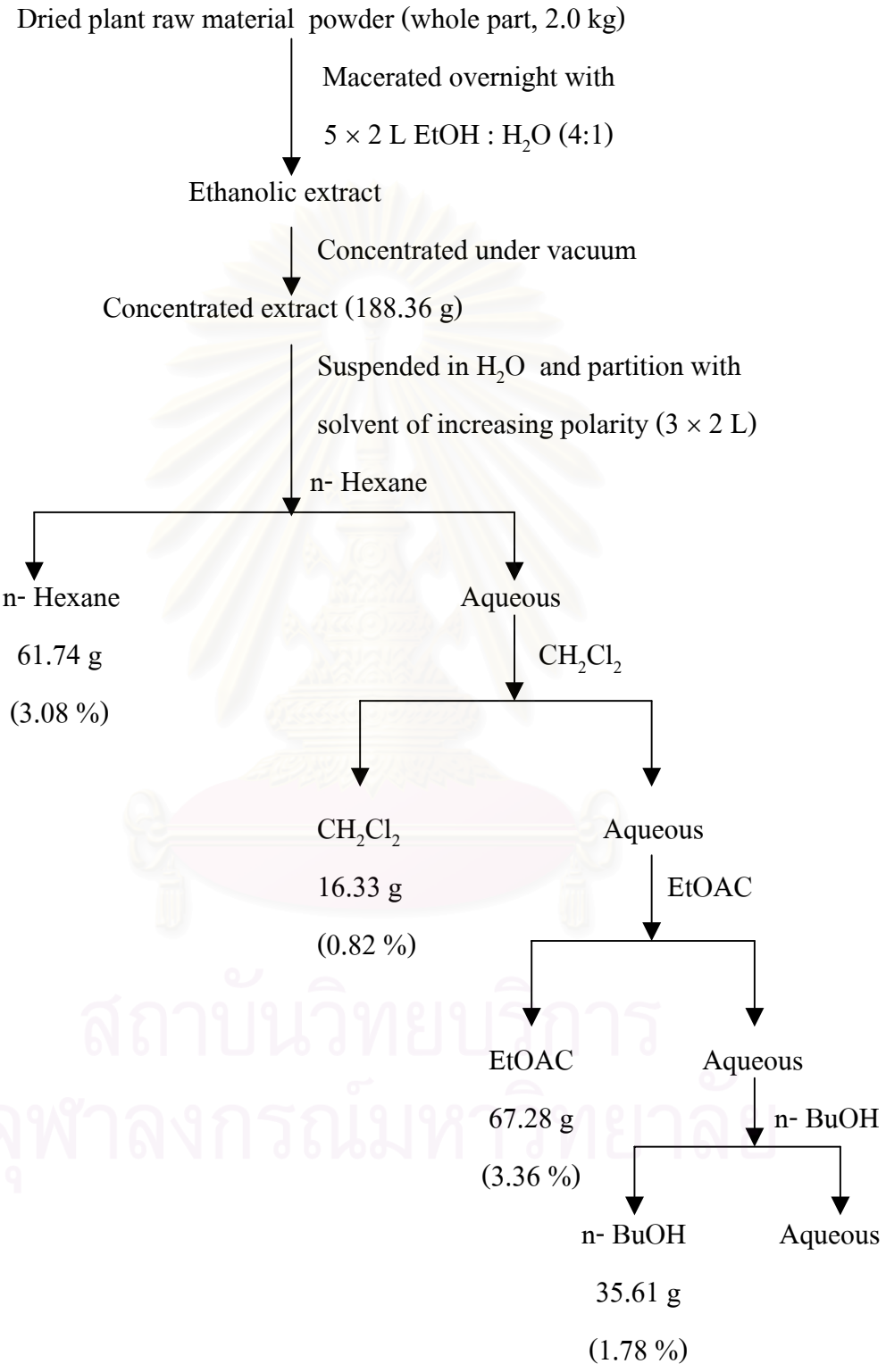
2.2 Solvents

All organic solvents for extraction were of commercial grade which were redistilled prior to use. For the analysis, all organic solvent were AR grade and HPLC grade.

3. Extraction

Two kg of the dried plant raw materials of *Cissus quadrangularis* Linn. were extracted by maceration with 2 litres of EtOH : H_2O (4 :1) for five times.[38,59] The filtrates were pooled and evaporated under reduced pressure at temperature not exceeding 40°C to yield ethanolic extract (syrupy mass 188.36 g, 9.418 % based on dried weight of the plant materials). Then the mass was suspended in H_2O and partitioned with 2 litres of n–hexane for 3 times. Combined hexane extract after evaporated of the solvent, to yield hexane extract as syrupy mass 61.74 g, 3.08 % based on dried weight of the plant materials. Then the aqueous fraction was partitioned with 2 litres of dichloromethane for 3 times. The combined dichloromethane extract after evaporated of the solvent, yielded dichloromethane extract as syrupy mass 16.33 g, 0.82 % based on dried weight of plant materials. Than the aqueous fraction was partitioned with 2 litres of ethylacetate for 3 times. The combined ethylacetate extract after evaporated of the solvent, yielded ethylacetate extract as syrupy mass 67.28 g, 3.36 % based on dried weight of the plant materials. Then the aqueous fraction was partitioned with 2 litres of n-butanol for 3 times. The combined butanol extract after evaporated of the solvent, yielded butanol extract as syrupy mass 35.61 g, 1.78 % based on dried weight of the plant materials, respectively. The diagram of extraction is shown in scheme l. Dichlormethane extraction (3 × 2 L) yielded a dichloromethane extract (syrupy mass 16.33g, 0.8165 % based on dried weight of plant materials), ethyl acetate (3 × 2 L) to yield an ethyl acetate extract 67.28 g, 3.364 % based on dried weight of the plant materials) and n-

butanol extraction (3 × 2 L) yielded butanol extract (syrupy mass 35.61 g, 1.7805 % based on dried weight of the plant materials), respectively.



Scheme 1. Extraction of *Cissus quadrangularis* Linn.

4. Determination of DPPH Free Radical Scavenging Activity

1,1-diphenyl –2-picryl hydrazyl (DPPH) radical scavenging activity has been determined by generating the DPPH radical which used as the substrate.[60] The violet color of DPPH radical can be detected by a UV-Visible spectrophotometer (Lambda 35) with 514 nm and quantitation any potential of DPPH radical scavenger would decrease the DPPH radical generated which in turn would decrease the DPPH radical absorption. This method was modified from the method of Brand-Williams *et al*.[61] and Chen *et al.* [62]

4.1 Instrument

UV-Vis spectrophotometer

4.2 Chemical

4.2.1 1,1 – diphenyl- 2- picryl – hydrazyl (DPPH), (Sigma)

4.2.2 Methanol AR grade (Lab-scan)

4.2.3 Sodium acetate

4.2.4 0.1 % phosphoric acid

4.3 Preparation of the reaction mixture.

4.3.1 Preparation of 5 mM DPPH

DPPH$^{\bullet}$ (9.85 mg) was dissolved with 5 ml of 50 mM acetate buffer (pH 6.5)

4.3.2 Preparation of 50 mM acetate buffer (pH 6.5)

Sodium acetate (0.41 g) was dissolved with 100 ml of distilled water, adjusted with 0.1 % phosphoric acid until pH 6.5 was reached.

4.3.3 Preparation of the test sample

Fifty mg of the test sample was dissolved in 25 ml of methanol (or suiTable solvent) and diluted with methanol until a suiTable range of concentration was obtained. The unit of each concentration was mg/ml. For each well 0.1 ml of 5 mM DPPH solution was added to the 4.9 ml test solution. to furnish the total volume of 5 ml. The final concentration was calculated by the formula below.

$$N_1V_1 = N_2V_2$$

N_1 = Beginning concentration (mg/ml)

V_1 = Beginning volume (ml)

N_2 = Final concentration (mg/ml)

V_2 = Final volume (ml)

4.4 Measurement of activity.

The reaction mixture (5 ml) was measured into four wells (A, B,C and D), each well contains the following reagents.

Solution A	(Control)	4.9 ml of methanol
		0.1 ml of 5 mM DPPH solution
Solution B	(Blank of A)	4.9 ml of methanol
		0.1 ml of 50 mM acetate buffer (pH 6.5)
Solution C	(Test sample)	4.9 ml of test sample in methanol
		0.1 ml of 5 mM DPPH solution
Solution D	(Blank of C)	4.9 ml of test sample in methanol
		0.1 ml of 50 mM acetate buffer (pH 6.5)

After each well was mixed and incubated at 37°C for 30 min, the absorbance of each well was measured at 514 nm with UV-Vis spectrophotometer.

In addition to the four extracts to be evaluated from the activities, ascorbic acid and authentic quercetin were also evaluated as the positive control.

4.5 Calculation of the percent inhibition of DPPH radical.

The percent inhibition of DPPH radical reaction was calculated as follows.

$$\%\text{DPPH}^{\bullet}\ \text{inhibition} = \left[\frac{(A\text{-}B) - (C\text{-}D)}{(A\text{-}B)} \right] \times 100$$

A = The optical density after incubation at 514 nm without test sample

B = The optical density after incubation at 514 nm without test sample and DPPH radical solution

C = The optical density after incubation at 514 nm with test sample

D = The optical density after incubation at 514 nm with test sample, but without DPPH radical solution.

4.6 Calculation of IC_{50}

After the % DPPH radical inhibition of the test sample in each concentration was calculated, as shown in Table 2 the curve of each versus % DPPH radical inhibition was plotted. The IC_{50} of each test sample was then obtained from the graph as shown in Figure 3.

Table 2 % DPPH cation radical inhibition of the extract from *Cissus quadrangularis* Linn.

Test Sample	Concentration (ug/ml)	Optical Density 514 nm				% DPPH inhibition ± SD
		X1	X2	X3	Mean	
Hexane extract	0.00	0.5938	0.5924	0.5956	0.5929	0.00 ± 0.001
	119.40	0.4811	0.4825	0.4802	0.4812	18.85 ±0.001
	238.80	0.3893	0.3896	0.3899	0.3896	34.28 ±0.001
	358.20	0.3059	0.3069	0.3061	0.3063	48.34 ± 0.001
	477.60	0.2312	0.2301	0.2294	0.2300	61.20 ±0.002
	597.00	0.1699	0.1687	0.1677	0.1689	71.50 ±0.002
Dichloromethane Extract	0.00	0.6233	0.6246	0.6254	0.6244	0.00 ±0.001
	49.47	0.461	0.4621	0.4602	0.4609	26.17 ±0.001
	98.94	0.3405	0.3391	0.3397	0.3397	45.58 ±0.001
	148.84	0.2711	0.2702	0.2723	0.2711	56.60 ± 0.001
	197.80	0.1937	0.1925	0.1914	0.1924	69.18 ± 0.001
	247.30	0.1343	0.1356	0.1332	0.1343	78.48 ± 0.002
Ethyl acetate Extract	0.00	0.6802	0.6823	0.6812	0.6811	0.00 ± 0.001
	33.60	0.5114	0.5121	0.5133	0.5122	24.81 ± 0.002
	67.20	0.397	0.398	0.396	0.397	41.71 ± 0.001
	100.80	0.2951	0.2962	0.2943	0.2951	56.68 ± 0.001
	134.40	0.2231	0.2235	0.2227	0.223	67.25 ± 0.001
	168.00	0.1491	0.1495	0.1495	0.1495	78.03 ± 0.001
Butanol extract	0.00	0.5741	0.5749	0.57	0.5743	0.00 ± 0.001
	57.94	0.4749	0.4741	0.4757	0.4748	17.33 ± 0.001
	115.80	0.3671	0.3681	0.3692	0.3681	35.91 ± 0.001
	173.80	0.2973	0.2998	0.2983	0.2985	48.05 ± 0.001
	231.70	0.2281	0.2273	0.2293	0.2282	60.27 ± 0.001
Quercetin	0.00	0.8002	0.8121	0.8111	0.8078	0.00 ± 0.006
	0.50	0.6704	0.6711	0.6709	0.6708	16.95 ± 0.007

Test Sample	Concentration (ug/ml)	Optical Density 514 nm				% DPPH inhibition ± SD
		X1	X2	X3	Mean	
	1.00	0.5654	0.5659	0.5651	0.5654	30.12 ± 0.004
	1.50	0.442	0.4416	0.4481	0.4416	45.87 ± 0.004
	2.00	0.3231	0.3239	0.3233	0.3234	59.18 ± 0.003
	2.50	0.2584	0.2589	0.2576	0.2583	67.83 ± 0.006
Ascorbic acid	0.00	0.8168	0.8145	0.8156	0.8157	0.00 ± 0.0011
	1.00	0.7013	0.6984	0.6993	0.6996	14.12 ± 0.0014
	2.00	0.5824	0.5804	0.5812	0.5814	28.72 ± 0.0010
	3.00	0.4829	0.4806	0.4815	0.4817	40.94 ± 0.0012
	4.00	0.3636	0.3638	0.3612	0.3628	55.41 ± 0.01014
	5.00	0.2835	0.2837	0.2836	0.2836	62.23 ± 0.0010

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5. Determination of antioxidant capacity by reaction of ABTS / Hydrogen peroxide / Methmyoglobin

A Total antioxidant capacity has been determined by the spectroscopic method using the reaction of 2,2' –azinobis- (3 ethylbenzothiazoline- 6- sulphonic acid) (ABTS) / H_2O_2 / Methmyoglobin to yield ABTS cation radical as a substrate.[63] The green color of ABTS cation radical can be detected by visible light. In this experiment a microplate reader (Biotex, model Elx 808) with 750 nm interference filter was used for detection.

The potential antioxidant capacity would show decreasing ABTS cation radical absorption. This method was modified from the method of Kim *et al*.[64] and Miller *et al*.[63]

5.1 Instrument

Microplate Reader (Biotex, modle Elx 808)

5.2 Chemicals

5.2.1 Myoglobin type III (sigma)

5.2.2 Iron in ferric state (sigma)

5.2.3 L- ascorbic acid (sigma)

5.2.4 Quercetin (sigma)

5.2.5 Heparin (sodium salt), (sigma)

5.2.6 Sephadex G –15 – 120 (sigma)

5.2.7 2,2' –azinobis –(3- ethyl benzothiazoline – 6- sulphonic acid) (ABTS), (Aldrich)

5.2.8 Hydrogen peroxide (BDH)

5.2.9 Dihydrogenphosphate

5.2.10 Potassium ferricyanide (BDH)

5.3 Preparation of the reaction mixture

5.3.1 Preparation of 20 mM Phosphate buffer, pH 7.4

KH_2PO_4 (3.72 g) was dissolved with 100 ml of distilled water, adjusted with 1 N NaOH until pH 7.4 was reached.

5.3.2 Preparation of 0.45 mM H_2O_2

H_2O_2 3 % (0.5 ml) was dissolved with 100 ml of distilled water.

5.3.3 Preparation of 740 μM potassium ferricyanide

Potassium ferricyanide (2.5 mg) was dissolved with 10 ml of phosphate buffer saline (pH 7.4).

5.3.4 Preparation of 70 μM methmyoglobin.

Myoglobin (400 μM) was dissolved in phosphate buffer solution, pH 7.4, was added to an equal volume of freshly prepared 740 μM potassium ferricyanide. To purify methmyoglobin, the mixture was loaded into a prepared sephacyl S-100 HR column equilibrated in the buffer. The brown solution of methmyoglobin fraction was collected. The final concentration of purified methmyoglobin was calculated by applying the Whitburn equations :

$$[\text{Met mb}] = 146A_{490} - 108A_{560} + 2.1A_{580}$$

$$[\text{FerrylMb}] = -62A_{490} + 242A_{560} - 123A_{580}$$

$$[MbO_2] = 2.8A_{490} - 127A_{560} + 153A_{580}$$

Where Mb is myoglobin. The purity of the methmyoglobin was estimated by applying all three equations.

5.3.5 Preparation of 500 μM ABTS

ABTS (2.5 mg) was dissolved with 9 ml of 20 mM phosphate buffer saline (pH 7.4).

5.3.6 Preparation of the test sample

Fifty mg of the test sample was dissolved in 25 ml of methanol (or suitable solvent) and diluted with methanol until a suitable range of concentration was obtained. The unit of each concentration was mg/ml. For each well, 10 μl of test solution was added to the reaction mixture to finish the total volume of 1000 μl. The final concentration was calculated by the formula below.

$$N_1V_1 = N_2V_2$$

N_1 = Beginning concentration (mg/ml)

V_1 = Beginning volume (ml)

N_2 = Final concentration (mg/ml)

V_2 = Final volume (ml)

The final concentration of other dilutions were calculated by the same method.

5.4 Measurement of activity

The reaction mixture (1000 μl) was measured in four wells (A, B, C and D)

Solution A (Control)	36 μl of 70 uM methmyoglobin
	167 μl of 0.45 mM H_2O_2
	487 μl of 20 mM phosphate buffer, pH 7.4
	10 μl of methanol
Solution B (Blank of A)	690 μl of 20 mM phosphate buffer , pH 7.4

	10 µl of methanol
Solution C (Test sample)	36 µl of 70 uM methmyoglobin
	167 µl of 0.45 mM H_2O_2
	487 µl of 20 mM phosphate buffer, pH 7.4
	µl of test sample in methanol
Solution D (Blank of C)	690 µl of 20 mM phosphate buffer, pH 7.4
	10 µl of test sample in methanol

After each well was mixed and pre-incubated at 25°C for 10 min, 300 µl of 500 µM of $ABTS^{+\bullet}$ was added and incubated at 25°C for 60 minutes. The absorbance of each well was measured at 750 nm with the microplate reader.

5.5 Calculation of the percent inhibition of ABTS cation radical.

$$\%ABTS^{+\bullet} \text{ inhibition} = \left[\frac{(A-B)-(C-D)}{(A-B)} \right] \times 100$$

A = The optical density after incubation at 750 nm without test sample

B = The optical density after incubation at 750 nm without test sample and reactant

C = The optical density after incubation at 750 nm with test sample

D = The optical density after incubation at 750 nm with test sample, but without reactant.

5.6 Calculation of IC_{50}

After the % $ABTS^{+\bullet}$ inhibition of the test sample in each concentration was calculated as shown in Table 3, the curve of each concentration and its % $ABTS^{+\bullet}$ inhibition was plotted. The IC_{50} of each test sample was then obtained from the graph as shown in Figure 4.

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Table 3 % ABTS cation radical inhibition of the extract from *Cissus quadrangularis* Linn.

Test sample	Concentration (ug/ml)	Optical Density 750 nm				% ABTS inhibition ± SD
		X1	X2	X3	Mean	
Hexane extract	0.00	0.716	0.722	0.703	0.714	0.00 ± 0.009
	100.00	0.506	0.512	0.509	0.509	28.72 ± 0.003
	200.00	0.425	0.441	0.432	0.433	39.35 ± 0.008
	300.00	0.302	0.312	0.308	0.307	56.95 ± 0.005
Dichloromethane extract	0.00	0.788	0.791	0.782	0.787	0.00 ± 0.0046
	25.00	0.551	0.542	0.565	0.552	29.77 ± 0.0110
	50.00	0.346	0.355	0.361	0.354	55.01 ± 0.0075
	100.00	0.275	0.266	0.271	0.270	65.61 ± 0.0045
Ethyl acetate extract	0.00	0.760	0.748	0.711	0.740	0.00 ± 0.0255
	25.00	0.417	0.435	0.455	0.436	41.13 ± 0.0190
	50.00	0.259	0.251	0.268	0.259	64.95 ± 0.0085
	100.00	0.076	0.079	0.088	0.081	89.05 ± 0.0063
Butanol extract	0.00	0.773	0.787	0.791	0.784	0.00 ± 0.0090
	50.00	0.477	0.459	0.451	0.462	41.03 ± 0.0133
	100.00	0.324	0.311	0.329	0.321	59.02 ± 0.0093
	200.00	0.092	0.090	0.096	0.093	88.18 ± 0.0031
Quercetin	0.00	0.687	0.693	0.672	0.684	0.00 ± 0.0108
	0.50	0.594	0.596	0.585	0.592	13.50 ± 0.0051
	1.50	0.428	0.424	0.433	0.428	37.43 ± 0.0045
	2.50	0.249	0.258	0.266	0.257	62.42 ± 0.0085
	3.75	0.112	0.118	0.115	0.115	83.18 ± 0.003

Test sample	**Concentration** (ug/ml)	Optical Density 750 nm				% ABTS inhibition ± SD
		X1	X2	X3	Mean	
Ascorbic acid	0.00	0.814	0.794	0.605	0.738	0.00 ± 0.1153
	2.50	0.618	0.653	0.677	0.656	11.07 ± 0.0279
	5.00	0.480	0.474	0.496	0.483	34.51 ± 0.0114
	7.50	0.223	0.239	0.277	0.293	60.30 ± 0.0277
	10.00	0.000	0.001	0.000	0.000	100.00 ± 0.0005

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CHAPTER IV

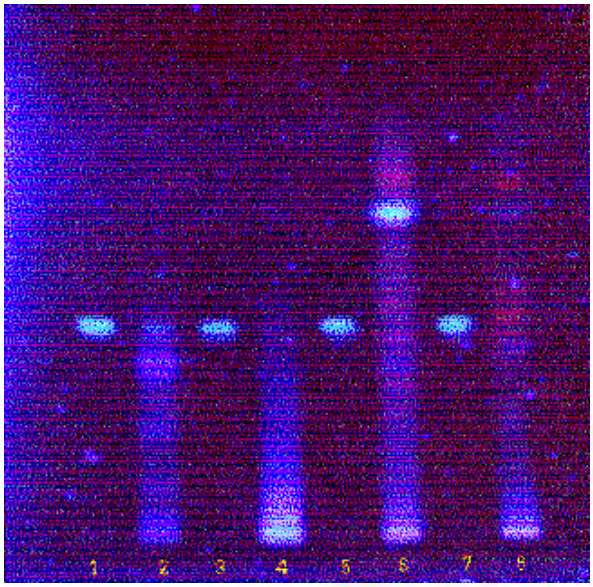
RESULTS AND DICUSSION

1. Extraction of plant sample

The dried plant materials (whole part, 2 kg) was successively extracted by maceration with EtOH : H_2O (4 : 1), the solvent was evaporated, and then the crude extract was partitioning partition with four solvents of increasing polarity using (n-hexane, dichloromethane, ethyl acetate and n-butanol, respectively.) The extracts were evaporated under reduced pressure, and the weight and characteristic of the mass obtained are shown in Table 4. The four mass obtained were then studied by TLC developing with mixtures of toluene : ethyl acetate : formic acid (5 : 4 : 1)[56]. Visualization of those TLC plates were done by either spray with 10 % H_2SO_4 and 0.2 % DPPH solution. The pictures of the TLC (A, B) were demonstrated in Figure 2.

Table 4 : The extract of *Cissus quadrangularis* Linn. from dried plant materials.

Plant part	weight of powdered drug (g) used	weight and characteristic of crude extraction obtained (g) (% yield)				
		ethanol : H_2O (4 :1)	n-hexane	dichloro-methane	ethyl-acetate	n-butanol
Whole part	2,000	188.36 (9.418 %) dark brown syrup	61.74 (3.087 %) dark green syrup	16.33 (0.8165 %) dark green syrup	67.28 (3.364 %) dark brown syrup	35.61 (0.781 %) brown syrup



A. 10 % H_2SO_4

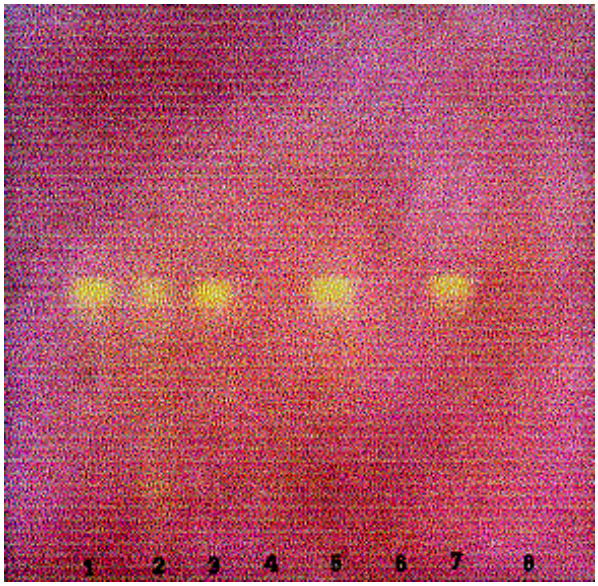
1, 3, 5, 7 = reference quercetin

2 = ethyl acetate extract

4 = butanol extract

6 = hexane extract

8 = dichloromethane extract



B. 0.2 % DPPH

1, 3, 5, 7 = reference quercetin

2 = ethyl acetate extract

4 = butanol extract

6 = hexane extract

8 = dichloromethane extract

Adsorbent : Siliga gel GF_{254}

Solvent system : Toluene - EtOAc - Formic acid (5 : 4 : 1)

Detection A : Spray with 10 % aq. H_2SO_4 and heat in the oven at 110°C for 1-2 min.

Detection B : Spray with 0.2 % DPPH in methanol solution

Fig 2. Thin-layer chromatograms of the solvent extracts sample of dried plant material.

From the ethyl acetate fraction, the TLC show a major spot which give the positive test to the DPPH spraying reagent and such spot move in the TLC with the Rf of 0.52 as shown in Table 5 which is identical to the spot of authentic quercetin in terms of response to spraying reagent or its Rf value. Quercetin was previously reported to contain in this plant.[38] However, in hexane , butanol fraction, no such spot were detected, the dichloromethane fraction was also shown the spot but to a very small extent.

Table 5. Rf. value of compound A from ethyl acetate extract compare with standard quercetin.

Sample	Rf. value	
	Spray with H_2SO_4	Spray with DPPH's reagent
Std. Quercetin	0.52	0.52
Compound A	0.52	0.52

Bioassay guided fractionation of *Cissus quadrangularis* Linn. revealed that ethyl acetate extract possess antioxidation properties against DPPH's reagent, it was then further investigated to identify active constituent in this fraction. From the reported literature, the compound bearing antioxidant property are those in the group of flavonoid, stilbene and polyphenol . Quercetin was previously reported as major flavonoid in *Cissus quadrangularis* Linn. Therefore, in this research works quercetin was selected and proposed as the chemical marker for quantitative analysis in this plant.

2. Determination of DPPH radical scavenging activity

The stable free radical α,α-diphenyl-β-picrylhydrazyl, which is generally available in laboratories in which electron spin resonance experiment are conducted, because of the paramagnetism conferred by its odd electron. It can be seen from its structures that while this compound can accept an electron of hydrogen radical to become a stable, diamagnetic molecule, it can be oxidized only with difficulty, and then, irreversibly. Because of its odd electron, α,α-diphenyl-β-picrylhydrazyl shows a strong absorption band at 514 nm (in ethanol), its solutions appearing a deep violet color. As this electron becomes paired off, the absorption vanishes and the resulting decolorization is stoichiometric with respect to the number of electron taken up. The strong absorption is fortunate because the solubility of α,α-diphenyl-β-picrylhydrazyl is not great, however, alcoholic solution coving concentrations of approximately 5×10^{-4} M are nevertheless densely colored and tow concentration, the Lem bert-Beer law is obeyed over the useful range of absorption.[60]

DPPH is a stable free radical compound and has been widely used to test the radical scavenging activity of various chemicals including the natural product.[55, 57, 58] In this study, determination of the presence of DPPH scavenging activity of the extract fraction from hexane, dichlorometrane, ethyl acetated and butanol of *Cissus quadrangularis* Linn. was conducted and using quercetin and ascorbic acid, which were well known antioxidant as the references. The result is shown in Table 6 . The IC_{50} of the extract sample is determined from the curve are shown in Fig. 3.

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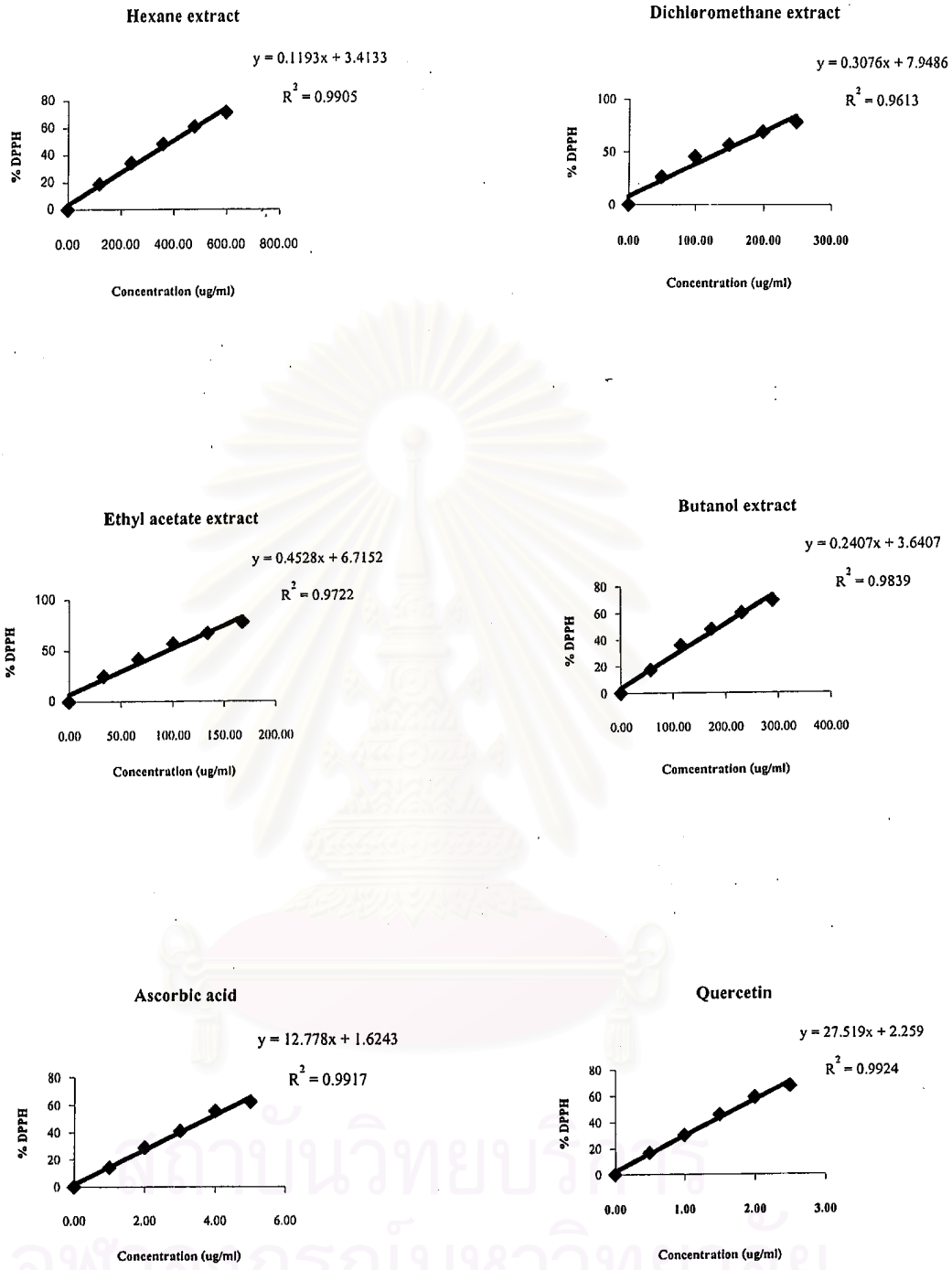

Fig. 3 Activity of the several extract from *Cissus quadrangularis* Linn. in experiment on DPPH test, IC_{50} is determined from the curve.

Table 6. DPPH radical scavenging activity of the extract from *Cissus quadrangularis* Linn.

Sample	DPPH, IC_{50} (μg/ml)
Hexane extract	375.81 ± 0.82
Dicholromethane extract	130.23 ± 0.21
Ethyl acetate extract	94.54 ± 0.62
Butanol extract	187.50 ± 0.37
Ascorbic acid	3.65 ± 0.06
Quercetin	1.73 ± 0.09

Each value is the mean of three independent experiments

From table 6, ethyl acetate fraction exhibited the highest DPPH radical scavenging activity with the IC_{50} = 94.5 μg/ml while the hexane extract exhibited the lowest activity with IC_{50} = 375.8 μg/ml.

When the ethyl acetate extract fraction was separated by TLC and visualized the compound by spraying with DPPH's reagent. It was evident that spot which gave the positive test to DPPH's reagent identical to the spot of authentic quercetin also.

3. Determination of antioxidant capacity by reaction of ABTS/H_2O_2/ Metmyoglobin.

The method for measuring the antioxidant status derives from the observation that when 2-2′-azinobis-(3-ethybenzothiazoline-6-sulphonic acid (ABTS) is incubated with a peroxidase (myoglobin) and hydrogen peroxide, the relatively long-lived radical cation $ABTS^{+\bullet}$ is formed. A large number of free radicals, such as hydroxyl, peroxyl, alkoxyl and inorganic radicals also reach rapidly with ABTS to form this species. When the peroxidase is metmyoglobin, the formation of the

$ABTS^{+\bullet}$ radical cation an interaction with ferryl myoglobin has absorption maxima at 650, 734, at 820 nm, beyond the region of absorption of the haem proteins. In the presence of antioxidant reductant and hydrogen donors, the absorbance of this radical cation is quenched to an extract related to the antioxidant capacity of the added fluid.

The total antioxidant activity assay originally developed by NJ Miller *et al*. was also used to measure the antioxidant activity of the extract in *Cissus quadrangularis* Linn. The modified method can be extended to study other food and plant extracts provided the extracts could be dissolved in the assay mixture. This assay employs the ABTS radical cation; a green chromophore absorbed at 750 nm that is easily monitored by a spectophotometer. Test samples if containing antioxidants, scavenge radical cation of ABTS, resulting in the decrease of the absorbance at 750 nm. This method is simple and requires only a conventional spectrophotometer.

Determination of antioxidant activity of the extract from *Cissus quadrangularis* Linn. was carried out when compare with quercetin and ascorbic acid, which were well known antioxidants and the results were summarised in Table 7. Antioxidant activity of several extract from *Cissus quadrangularis* Linn. by reaction of ABTS / H_2O_2/ Metmyoglobin, IC_{50} is determined from the curve are shown in Fig. 4.

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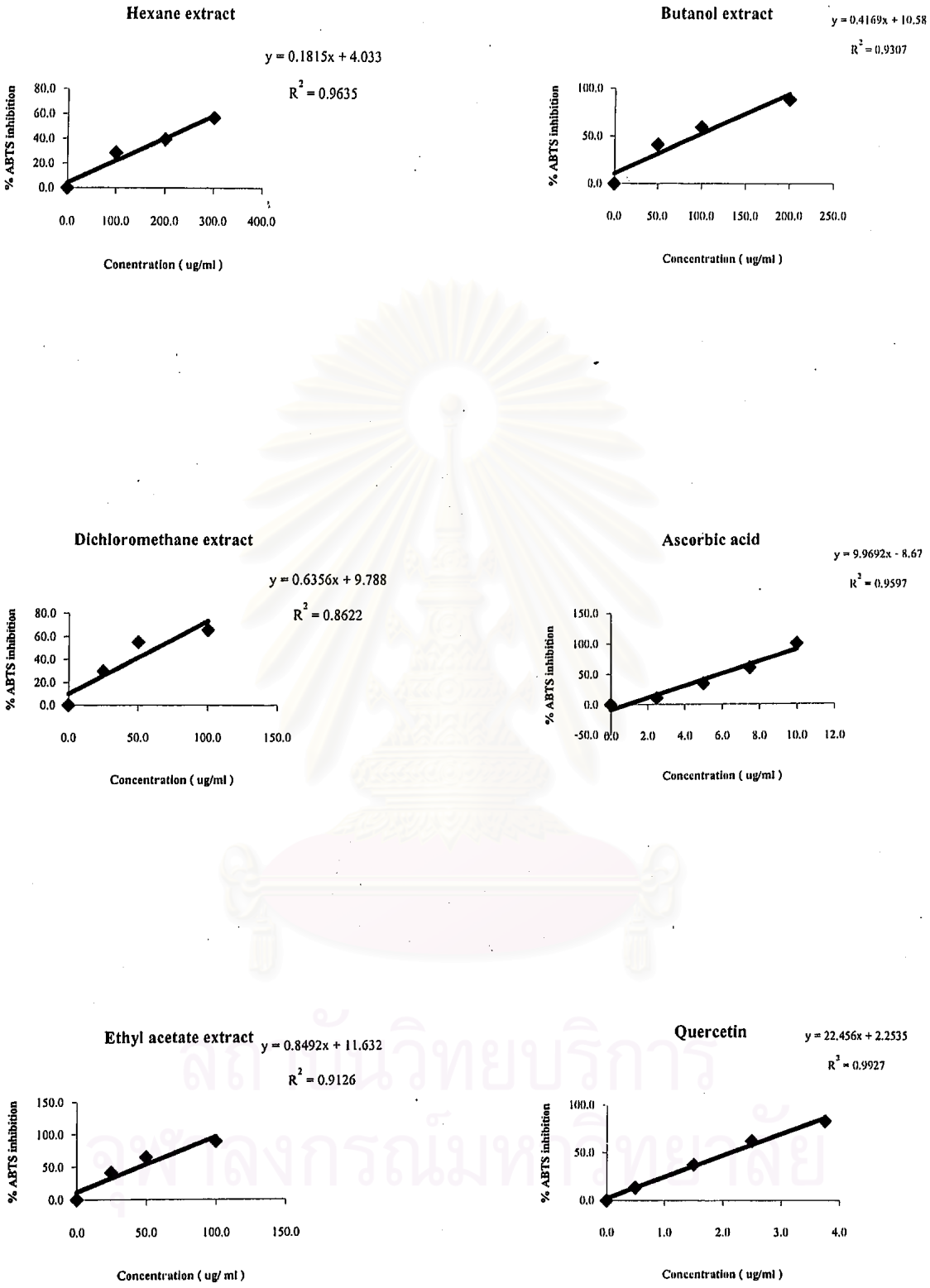

Fig. 4 : Antioxidant capacity of several extract from *Cissus quadrangularis* Linn. by reaction of ABTS/ H_2O_2/ Metmgoglobin, IC_{50} is determined from the curve.

Table 7. Antioxidant activity of the extract from *Cissus quadrangularis* Linn. by reaction of ABTS/ H_2O_2/ Metmgoglobin.

Sample	$ABTS^{+\bullet}$ IC_{50} (μg/ml)
Hexane extract	263.2 ± 0.018
Dichlomethane extract	58.5 ± 0.032
Ethyl acetate extract	34.7 ± 0.013
Butanol extract	76.2 ± 0.013
Ascorbic acid	4.5 ± 0.036
Quercetin	2.03 ± 0.084

Each value is the mean of at independent experiments ± SD

The ABTS/H_2O_2/Metmyoglobin test method also confirm that ethyl acetate fraction constituted the antioxidant fraction since the result exhibit the lowest also. The result also confirm that quercetin possess the stronger antioxidant properlees than ascorbic acid.

From the two in vitro anti oxidation testing, we concluded that the antioxidant constituent remain in the ethyl acetate fraction. While the TLC of this fraction when detected by spraying with DPPH, the chromatogram exhibit only one single spot that response to DPPH, the other chromatogram from other solvent extract do not exhibit other spots that response to DPPH. Such responsive spot was identified as quercetin by compare the Rf. value with the authentic sample and latter conferm by HPLC as shown in the chromatogram (Fig. 5) Therefore, quercetin was selected as the maker for the quantitative analytical method.

4. HPLC method for determination of quercetin

4.1 Development of HPLC method

The HPLC condition which give best chromatogram were

Column : Aquasil® C18, 5 μm, 4.6 × 250 mm i.d.

Mobile phase : 0.05 % ortho-phosphoric acid : Acetonitrile (65 : 35) v/v

Flow rate : 1.0 ml/min

Detector Wavelength : 260 nm

Injection Volume : 20 μl

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4.2 Validation of HPLC quantitative method

The developed method was validated by the following tests

4.2.1 Specificity

The specificity of the method was determined by injecting the standard solution of quercetin, ethyl paraben and sample onto the chromatographic system previously described.

The retention times of each compound are given below :

Specificity	Absolute retention time (min)	
	standard	sample
Quercetin	10.68	10.77
Ethyl paraben	13.68	13.63

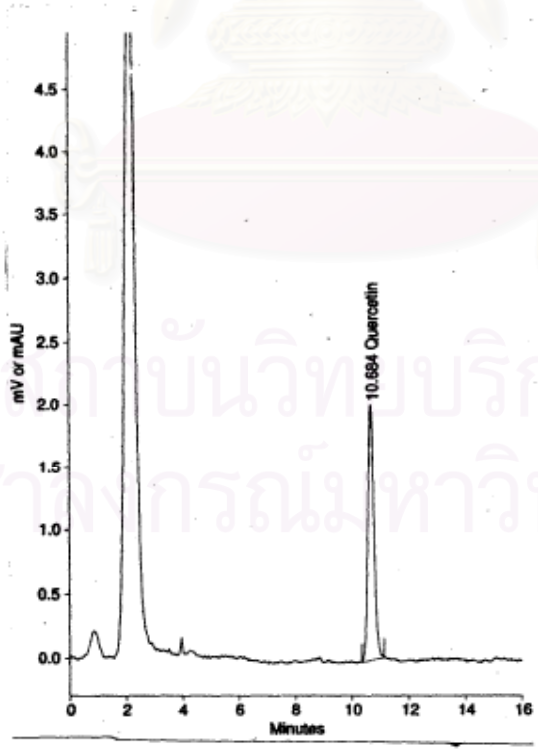

Figure 5 : Chromatogram of quercetin

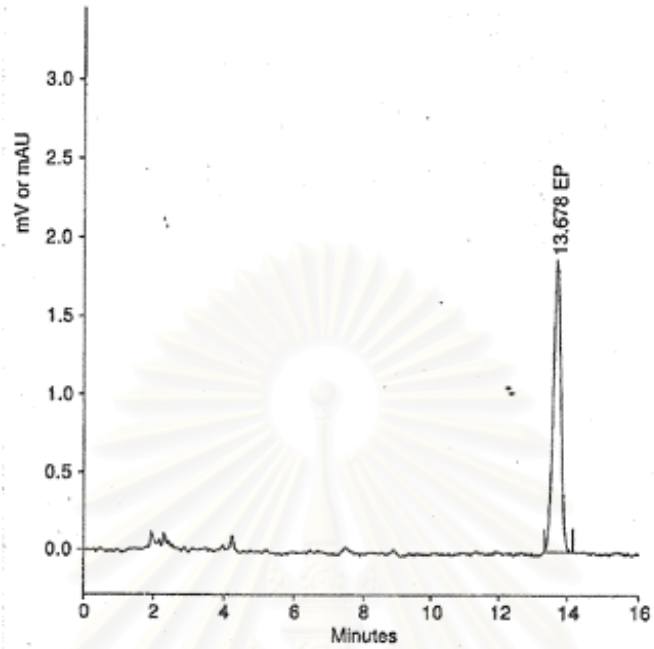

Figure 6 : Chromatogram of ethyl paraben

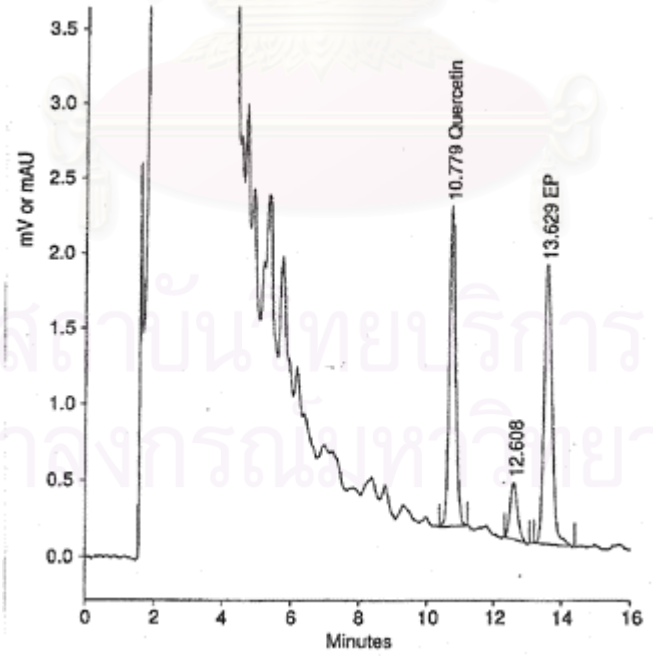

Figure 7 : Chromatogram of sample

4.2.2 Linearity

The concentration of standard solution for determination of linearity were prepared in the range 0.3 to 1.5 μg/ml. The calibration curve of quercetin was shown in Fig. 8. The result of the regression coefficient (r^2) was 0.9996. The linear regression equation was y = 1068.9 X + 0.0537. The standard curve was established between concentration and the the peak area ratio and used for the quantitation.

Concentration	Peak Area Ratio		Mean PAR
(μg/ ml)	A	B	
0.3	0.3780	0.3833	0.3806
0.6	0.6779	0.6808	0.6793
0.9	1.0244	1.0243	1.0243
1.2	1.3438	1.3393	1.3415
1.5	1.6520	1.6538	1.6529

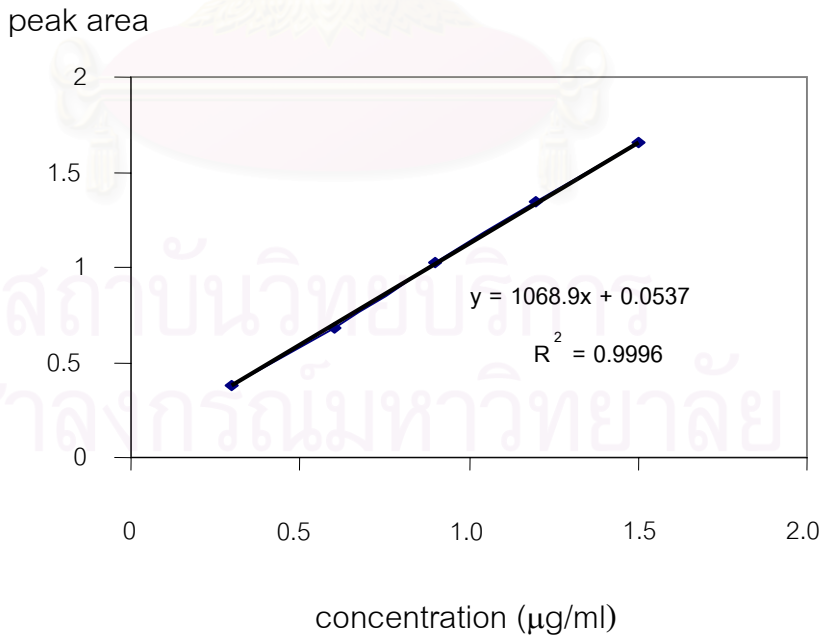

Fig. 8 : Calibration curve of quercetin

4.2.3 Precision

The precision of the method was determined in term of repeatability and intermediate precision. Repeatability test was performed by five consecutive injection of six different concentrations of the authentic photochemical substance on the same day. Intermediate precision test was similarly performed but for different days, analysts and equipment.

Six sample solutions of the homogeneous sample were used for intra-day and intermediate precision. Intra-day precision was performed with two injections of each sample in a day while intermediate precision was performed with two injections of each sample for different days, different analysts and different equipment.

The results present in Table 8, 9 and 10 show that the percentage of coefficient of variation of the intra-day, inter-day and intermediate precision were 0.4252, 0.2084 and 0.8278, respectively.

Table 8 : Intra-day precision of analysis of quercetin in the dried plant materials

Sample	Wt. of sample (mg)	Conc. of sample found (mg/ml)	Calculated mg % of quercetin
1	5081.0	0.919	9.043
2	5132.9	0.934	9.098
3	5233.5	0.944	9.016
4	5089.3	0.923	9.070
5	5191.3	0.947	9.123
6	5345.9	0.968	9.055
Mean		0.939	9.068
% RSD		1.911	0.425

Table 9 : Inter-day precision of analysis of quercetin in the dried plant material

Day	Mean of concentraion (μg/ml)	mg % of quercetin
1	1.047	9.103
2	1.024	9.073
3	0.939	9.068
Mean	1.003	9.081
% RSD	5.670	0.208

Table 10 : Intermediate precision of analysis of quercetin in the dried plant material

Different	Mean of concentration (μg/ml)	mg % of quercetin
Day	1.003	9.081
Analyst	1.042	8.955
Equipment	1.036	9.088
mean	1.027	9.041
% RSD	2.448	0.828

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Table 11 : Inter-analyst precision of analysis of quercetin in the dried plant material

Trial No.	Analyst 1	Analyst 2
1	0.9026	0.8916
2	0.8949	0.8956
3	0.8844	0.9039
4	0.8800	0.9142
5	0.8804	0.9077
6	0.8838	0.9060
X	0.8878	0.9032
%RSD	1.0232	0.9156

Table 12: Inter-instrument precision of analysis of quercetin

Trial No.	Instrument 1	Instrument 2
1	0.9076	0.9056
2	0.9171	0.9012
3	0.9123	0.9111
4	0.9098	0.9076
5	0.9087	0.9132
6	0.9061	0.9051
X	0.9103	0.9073
% RSD	0.4338	0.4786

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4.2.4 Accuracy

The accuracy of the method was determined using spiking method (standard addition). Three concentraions i.e. 0.6, 0.9 and 1.2 ug/ml of quercetin were injected for each sample, and the percentage recoveries were determined. The result was shown in Table 13 The recommended % R range from 80 to 120 %.

Table 13 : Percentage recovery of quercetin in the dried plant material

Quercetin (μg/ml)	Amount added (μg/ml)	Amount found (μg/ml)	% Recovery (% R)
0.60	0.68	0.67	98.92
0.90	1.02	0.98	96.41
1.20	1.36	1.32	97.25
		Mean	97.53
		SD	1.27
		% RSD	1.31

4.2.5 System suitability test

The system suitability test was performed to verify the suitability of the chromatographic system. The standard solution of quercetin was injected, the seperation was isocratic system using 0.05 % phosphoric acid : acetonitrile (65:35) as mobile phase. Retention time, peak area, tailing factor, resolution and plate count were measured. The result of system suitability test were shown in Table 14 and 15.

Table 14 : System suitability test of quercetin.

Standard injection no.	Retention Time (min)	Peak area	Tailing factor (TF, ≤ 2)	Theoritical-plates (N, ≥ 2000)	Resolution (Rs, ≥ 2)
1	11.330	65044	1.1280	14422	7.16
2	11.321	64596	1.1279	14410	7.16
3	11.304	66699	1.1268	14567	7.15
4	11.283	65067	1.1278	14531	7.17
5	11.282	65700	1.1282	14429	7.15
6	11.285	65255	1.1275	14433	7.16
Mean	11.3008	65393	1.1277	14465	7.16
% RSD	0.18	1.12	0.04	0.46	0.11

Table 15 : System suitability test of ethyl paraben

Standard Injection no.	Retention time (min)	Peak area	Tailing factor (TF, ≤ 2)	Theoretical Plates (N, ≥ 2000)	Resolution (Rs, ≥ 2)
1	14.252	59740	1.0172	16788	7.21
2	14.240	60454	1.0461	16865	7.19
3	14.225	61269	1.0421	16910	7.20
4	14.207	60056	1.0425	16543	7.19
5	14.204	61020	1.0433	16777	7.18
6	14.203	59388	1.0471	16912	7.19
Mean	14.2218	60321	1.0447	16765	7.19
% RSD	0.1456	1.2144	0.2246	0.9901	0.1435

5. Application of the developed HPLC method

5.1 Method of sample preparation

Two methods were used to extract the quercetin from the plant raw material powder, firstly, direct reflux of the plant sample with aqueous alcoholic solution. The amount of quercetin extract were determined and the result is shown in table 12. With this method, the maximun quercetin can be deteced at the refluxing time of 1.5 hr.

Secondly, the plant was extract by using the soxhlet apparatus in aqueous alcoholic solution, the result is shown in table 13. Maximum quercetin content can be extracted in this method after 6 hr of extraction. Therefore, method of refluxing the sample for 1.5 hr was chosen in order to minimize the analysis time.

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Table 16 : Direct Reflux : time 0, 0.5, 1.0, 1.5, 2.0, 2.5 hrs. and After reflux 1 hr

Time	Quercetin (mg %)
0.0	1.167
0.5	7.000
1.0	7.800
1.5	8.300
2.0	8.200
2.5	7.800
After reflux 1 hr.	0.000

Table 17 : Soxhlet Phet Sang Khaat raw materials at time 0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0 and After reflux 1 hr

Time (hr)	Quercetin (mg %)
0.0	0.420
0.5	0.840
1.0	1.884
2.0	3.933
3.0	7.474
4.0	7.886
5.0	7.792
6.0	8.713
7.0	8.151
After reflux 1 hr	0.000

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5.2 Determination of quercetin in dried plant materials

The quantiative analysis quercetin content in *Cissus quadrangularis* Linn. extracts was dertermined using the validated HPLC method. Each determination was thrice carried out. The qurecetin content in dried plant materials collected monthly, provincely and finished product were shown in Table 14, 15 and 16.

Table 18 : The percent yield (mg %) of quercetin content in the dried plant materials* from *Cissus quadrangularis* Linn.

Sample solution (injection)	Quercetin conteny (mg %)		
	Phitsanuloke*	Prachinburi**	Prachuapchirikhon**
1(A)	1.515	1.900	0.8245
1(B)	1.571	1.871	0.8306
2(A)	1.550	1.889	0.8105
2(B)	1.561	1.902	0.8469
3(A)	1.527	1.895	0.8419
3(B)	1.544	1.885	0.8469
Mean	1.544	1.885	0.834
%RSD	1.352	1.003	1.809

* Plant material collected in April 2001

** Plant material collected in April 2003

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Table 19 : The percent yield (w/w) of quercetin content in the dried plant material* of *Cissus quadrangularis* Linn.

Sample solution (injection)	Quercetin content (mg %)					
	Feb.	Apr.	Jun.	Aug.	Oct.	Dec.
1(A)	9.984	1.515	2.553	3.255	7.916	13.56
1(B)	10.14	1.571	2.548	3.287	7.839	13.64
2(A)	10.29	1.550	2.581	3.211	7.887	13.61
2(B)	10.16	1.561	2.527	3.244	7.852	13.53
3(A)	10.21	1.527	2.533	3.216	7.834	13.59
3(B)	10.15	1.544	2.517	3.307	7.921	13.62
Mean	10.15	1.544	2.543	3.253	7.875	13.59
% RSD	0.9922	1.3522	0.8968	1.1736	0.4907	0.2995

* Plant material collected from Phitsanuloke (June 2000 – April 2001)

Table 20 :The percent yield (mg %) of quercetin content in finished product* from GPO

Sample solution (injection)	Quercetin content (mg %)	
	Lot. Dec001	Lot. Feb001
1(A)	13.46	10.02
1(B)	13.25	9.998
2(A)	13.36	9.929
2(B)	13.53	9.928
3(A)	13.50	9.927
3(B)	13.59	9.896
Mean	13.45	9.950
% RSD	0.9209	0.4835

*** GPO *Cissus quadrangularis* Linn. tablet, 500 mg**

The quercetin content throughout a year period was found to be in the range of 0.8 – 13.6 mg % therefore, a 500 mg *Cissus quadrangularis* Linn. tablet may possibly contain only 4 – 68 μg /tablet which is too much less than the commercial product Daflon®, which contain diosmin and hesperidin (450mg, 50 mg). It seems possible that the therapeutic effect of *Cissus quadrangularis* Linn. not only resulted from quercetin but may also from other compounds present in the plant, possibly from the triterpene compounds

In addition, the poor absorbtion property of quercetin is well documented[66]. Therefore, the concentration of quercetin that survives metabolism (0.003 – 0.012 μ mol/kg body weight) seem to not correlate with the reported IC_{50} value affecting the system. What needs to be explored is how common path ways inhibited by flavonoids in laboratory schedule result in the known beneficial effect of dietary flavonoids, and we also need to know more about the pharmacokinetic, absorbtion, distribution and metabolism of flavonoids.

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CHAPTER V

CONCLUSION

Maceration of the plant raw material with 80 % ethanol yielded 9.418 % dark brown syrup. Partitioning of the syrup in water mixed with solvents in increasing polarity (hexane, dichloromethane, ethyl acetate and butanol) yielded 3.087 %, 0.8165 %, 3.364 % and 0.781 %, successively.

The antioxidant properties of those four extract fractions were determined by the DPPH method and ABTS / H_2O_2 / Metmyoglobin method and found that ethyl acetate fraction exhibited the strongest antioxidant properties. TLC pattern of the ethyl acetate fraction indicated that the spot of quercetin (as compared to the authentic quercetin) is the major antioxidant constituent .

The HPLC method for quercetin analysis was developed. The optimum conditions were comprised of C-18 reverse phase (Aquasil® column 5 um, 250 × 4.6 mm i.d.), isocratic elution with 0.05 % ortho-phosphoric acid and acetonitrile (65 : 35) as the mobile phase, and UV detection at 260 nm. The retention time of quercetin was at 10.68 min while that of ethyl paraben used as internal standard was at 13.68 min.

Validation of the developed method on specificity, linearity, precision, accuracy and system suitability test (according to the ICH guideline) were performed. All parameters were found in the acceptable limit ranges.

Application of the validation method for plant samples was performed. The quercetin content from plant samples collected from different locations e.g. Phitsanuloke, Prachinburi, Prachuabkhirikhan were found as 1.54, 1.88 and 0.83 mg %, respectively. The quercetin contents from plant samples collected from different periods of time (Feb., Apr., Jun., Aug., Oct., Dec.) were found to be 10.15, 1.54, 2.54, 3.25, 7.88 and 13.59 mg %, respectively. The quercetin content from 2 lots of *Cissus quadrangularis* Linn. tablets were found to be 13.45 and 9.95 mg %.

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