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EXTRACTION OF PHENOLIC COMPOUND FROM FRUITS OF BITTER MELON (*MOMORDICA CHARANTIA*) WITH SUBCRTTICAL WATER AND ANTIOXIDANT ACTIVITIES

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A Thesis Submitted in Partial Fulfillment of the Requirements
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อ. ที่ปรึกษา: ผศ. ดร. อาทิวรรณ โชติพฤกษ์, 95 หน้า.

มะระขี้นกเป็นที่รู้กันดีว่ามีคุณสมบัติทางการยามากมาย เช่น ต้านโรคเบาหวาน, ต้านมะเร็ง, ลดการอักเสบ, ต้านเชื้อไวรัส, และลดไขมันในเส้นเลือด เป็นต้น ในมะระขี้นกจะประกอบไปด้วยสารประกอบฟีนอลิกต่างๆ ซึ่งมันมีคุณสมบัติในการต่อต้านอนุมูลอิสระ และต่อต้านการกลายพันธุของ DNA ถึงแม้ว่าเราจะตระหนักถึงคุณประโยชน์ต่างๆของมะระขี้นก แต่ข้อมูลที่เกี่ยวข้องกับสารประกอบฟีนอลิกจากมะระขี้นกและคุณสมบัติในการต่อต้านอนุมูลอิสระ และต่อต้านการกลายพันธุ์ของ DNA ของสารสกัดที่ได้จากมะระขี้นกก็ยังคงมีจำกัด ดังนั้นในงานวิจัยนี้จึงศึกษาถึงปริมาณสารประกอบฟีนอลิกที่อยู่ในมะระขี้นกโดยวิธีการสกัดแบบน้ำกึ่งวิกฤติ และการต่อต้านอนุมูลอิสระของสารสกัด โดยพิจารณาถึงผลกระทบของอุณหภูมิและอัตราการไหลของน้ำที่ใช้ในการสกัด และเปรียบเทียบกับสารสกัดที่ได้จากวิธีการใช้ตัวทำละลายและวิธีซอคเลท การสกัดปริมาณสารประกอบฟีนอลิกที่อยู่ในมะระขี้นกโดยวิธีการสกัดแบบน้ำกึ่งวิกฤติจะสกัดที่อุณหภูมิ 130 - 200 องศาเซลเซียส และอัตราการไหลของน้ำที่ 2-5 มิลลิลิตรต่อนาที โดยใช้ความดันคงที่ 10 เมกกะปาสคาล โดยสภาวะที่ดีที่สุดในการสกัดคือที่ อุณหภูมิ 150-200 องศาเซลเซียส และอัตราการไหลของน้ำ 2 มิลลิลิตรต่อนาที ปริมาณสารประกอบฟีนอลิกในมะระขี้นกที่สกัดโดยวิธีน้ำกึ่งวิกฤติ วิธีการใช้ตัวทำละลายและวิธีซอคเลท คือ 52.63 6.00 และ 6.68 มิลลิกรัมแกลลิกต่อกรัมน้ำหนักแห้ง ตามลำดับ โดยรวมแล้ววิธีน้ำกึ่งวิกฤติจะให้ปริมาณสารประกอบฟีนอลิกที่สูงกว่าวิธีใช้ตัวทำละลายและวิธีซอคเลท สารประกอบฟีนอนิกที่มีมากที่สุดในมะระขี้นก คือ คาเทชิน ซึ่งในวิธีน้ำกึงวิกฤติ วิธีการใช้ตัวทำละลายและวิธีซอคเลท มีปริมาณคาเทชินอยู่ 46.16 1.61 และ 1.77 มิลลิกรัมต่อกรัมน้ำหนักแห้ง ตามลำดับ ในการสกัดโดยวิธีน้ำกึ่งวิกฤติที่อุณหภูมิต่ำจะให้คุณสมบัติการต่อต้านอนุมูลอิสระที่สูงกว่าอุณหภูมิสูง และสูงกว่าวิธีการใช้ตัวทำละลายและวิธีซอคเลท ถึงแม้ว่าที่อุณหภูมิและวิธีการสกัดที่ต่างกันปริมาณสารประกอบฟีนอลิกและคุณสมบัติการต่อต้านอนุมูลอิสระจะมีความแตกต่างไม่เป็นไปในทิศทางเดียวกัน ซึ่งผลแสดงให้เห็นว่าการต่อต้านอนุมูลอิสระไม่ขึ้นกับปริมาณสารประกอบฟีนอลิกเพียงอย่างเดียว ดังนั้นสรุปได้ว่ามะระขี้นกเป็นแหล่งที่ดีของสารประกอบฟีนอลิกและมีคุณสมบัติสูงในการต่อต้านอนุมูลอิสระ

ภาควิชา.....วิศวกรรมเคมี.....ลายมือชื่อนิสิต.....ปาริฉัตร บุตรราช.....

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PARICHAT BUDRAT: EXTRACTION OF PHENOLIC COMPOUND FROM FRUITS OF BITTER MELON (*MOMORDICA CHARANTIA*) WITH SUBCRITICAL WATER AND ANTIOXIDANT ACTIVITIES. THESIS ADVISOR: ASST. PROF. ARTIWAN SHOTIPRUK, Ph.D., 95 pp.

Bitter melon (*Momordica charantia*) is traditionally known for its medicinal properties such as antidiabetic, anticancer, anti-inflammation, antivirus, and cholesterol lowering effects. It contains many phenolic compounds that may have the potential as antioxidant and antimutagen. Although the value of bitter melon is realized, scientific information on phenolic composition of bitter melon and antioxidant and antimutagenic activities of its extracts from food grade solvents are limited. This study were investigated the total phenolic contents of bitter melon obtained by subcritical water extraction (SCWE) and antioxidant activities of these extracts. The effects of extraction temperature and water flow rate were considered and the results were compared with the extracts obtained by solvent extraction and soxhlet extraction. The subcritical water extraction of the total phenolic contents in bitter melon was carried out at the temperature between 130 °C to 200 °C and the effect of water flow rate was investigated in the range of 2 to 5 ml/min, while the constant pressure of 10 MPa. The most suitable extraction condition was found to be at the temperature of 150-200 °C and flow rate of 2 ml/min. The total phenolic contents of bitter melon obtained by the SCWE, the solvent extraction, and soxhlet extraction were 52.63, 6.00, and 6.68 mg gallic acid equivalents (GAE)/g dry weight (DW), respectively. Overall, the extract obtained by SCWE was significantly higher than solvent extraction and soxhlet extraction. The most phenolic acids contained in bitter melon were catechin. Catechin was calculated from HPLC analysis of the extracts from bitter melon obtained by the SCWE, the solvent extraction, and soxhlet extraction were 46.16, 1.61, and 1.77 mg/g DW, respectively. The subcritical water extraction at lower temperature gave higher antioxidant activity than the extracts obtained at higher temperature. The extract obtained by subcritical water extraction gave the higher antioxidant activity than solvent extraction, and soxhlet extraction. Even though the total phenolic contents between extraction method and extraction temperature were significantly different, their different antioxidant activities indicated that antioxidant activity was not depended only by their total phenolic contents. Thus, bitter melon is a good source of phenolic compounds and has high potent antioxidant activity.

Department.....Chemical Engineering..... .Student's signature....ปาริฉัตร บุตรวาช

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CHAPTER I

INTRODUCTION

1. Rationale

Phenolic compounds are categorized as secondary metabolites essential for growth and reproduction of plants. They are known as hydrophilic antioxidants, and are produced as a response for defending injured plants against pathogens. They potentially show antioxidant, antimutagen, antitumor, anti-inflammatory, and anticarcinogenic properties (Lee et al., 2004). In general, deep–colored vegetables and fruits including bitter melon are good sources of phenolic compounds.

Bitter melon (*Momordica charantia*), or commonly as *Ma-ra-khee-nok*, is an herbal plant grown in Thailand and other tropical regions. It is traditionally known for its medicinal properties such as antidiabetic, antitumor, anticancer, anti-inflamatory, antiviral, and cholesterol lowering effects (Ahmed et al., 2001; Grover et al., 2004; Taylor, 2002). The main constituents of bitter melon which are responsible for these effects are such as triterpene, proteid, steroid, alkaloid, inorganic, lipid, and phenolic compounds (Grover et al., 2004). The protein in bitter melon including MAP-30, alpha–momorcharin, and beta–momorcharin were shown to have the ability for fighting against HIV (Luetrakul et al., 1998). A steroid, charantin, contained mainly in the aerial parts, has been proven for its antidiabetic activity (Chanchai et al., 2002). The phenolic compounds from bitter melon extracted by solvent extraction were reported to exhibit antioxidant activity (Horax et al., 2005).

Recently, subcritical and pressurized fluids have become an interesting alternative in the extraction of herbal plants and the most benign and available solvent for pressurized solvent extraction is water. The subcritical fluid extraction is a technique for extraction of plants based on the use of solvent whose temperature lies between boiling and critical temperatures, and at high pressure enough to maintain the liquid state (Ayala et al., 2001). The important advantages of this method are its simplicity, reduced extraction time, higher quality of the extract, lower cost of the extracting agent, and an environmentally friendly technique (Herrero et al., 2006). Extraction with subcritical fluid using water as a solvent has been shown to be

effective for several compounds, such as essential oils from majoram (Carmona et al., 1999), oregano (Ayala et al., 2001), and coriander seeds (Eikani et al., 2006) etc. Moreover, Jesada et al. (2006) showed that charantin which is a fat soluble steroid could be successfully extracted benignly from fruit of bitter melon using polar solvents at subcritical condition such as acetone and ethanol. Although the authors showed that subcritical water was not suitable for extraction of charantin from fruit of bitter melon, water is an adequately good solvent for extraction of phenolic compounds from bitter melon due to the high solubility of phenolic compounds in water.

In this study, we investigated the total phenolic contents of bitter melon obtained by subcritical water extraction. The effect of extraction temperature was considered and the results were compared with the extracts obtained by solvent extraction. Moreover, the antioxidant activities of these extracts were determined.

1.2 Objectives

1.2.1 To investigate the suitable conditions for extraction of phenolic compounds from bitter melon with subcritical water extraction.

1.2.2 To compare the efficiency of subcritical water extraction with soxhlet extraction method.

1.3 Working scopes

1.3.1 Evaluation of the suitable conditions for subcritical water extraction of phenolic compounds from bitter melon by determining the effects of two factors: temperatures (100-200 °C), and flow rate (2-5 ml/min) at a fixed pressure of 10 MPa on the amount of the compounds extracted and the extraction rate. The extraction time is approximately 2 hr.

1.3.2 Determination of antioxidant activities of the extracts using ABTS assay.

1.4 Expected benefits

1.4.1 Provide a new benign alternative for extraction of high quality phenolic compounds

1.4.2 Provide fundamental information useful for industrial scale-up of the extraction process.

CHAPTER II

BACKGROUNDS AND LITERATURE REVIEWS

Backgrounds

2.1 *Momordica charantia*

Momordica charantia (Figure 2.1.1-4), also commonly know as bitter melon, papailla, balsam pear, mergose, la-kwa, leprosy gourd, and karela, is an herbal plant, belonging to the cucurbitaceae family. It is commonly grown in tropical and subtropical areas, including part of the Amazone east, Afica, Caribbean, and Asia. It grows vigorously in warm climates and is resistant to rain. The variety commonly grown in Thailand is called Ma-ra-khee-nok, whose fruits are smaller and taste more bitter than the Chinese variety. Ma-ra-khee-nok is used as food, health berverages, and medicine, and all parts are used such as leaves, vines, roots, fruits, and seeds. However, the fruits are the most widely used medicinally, as they are the safest and most prevalent part. So this study, we focus about fruits of bitter melon.

2.2 Momordica charantia fruits: constituents and biological activity

The fruits of bitter melon are similar to cucumbers but bitter melon fruits have warty skin and taste very bitter. The young fruit is emerald green and has white seed cost as shown in Figure 2.1.3. The color of the old fruit changes to orange yellow and the seed coat turns red when it is ripe. The red seed coat is reportedly toxic in children causing vomiting, diarrhea, and death (Figure 2.1.4).

Figure 2.1.1 *Momordica charantia* or Bitter melon.

Figure 2.1.2 The flower of *Momordica charantia*.

Figure 2.1.3 The young fruit of *Momordica charantia*.

Figure 2.1.4 The old fruit of *Momordica charantia*.

The fruit of bitter melon is known to contain many constituents such as triterpene, proteid, steroid, alkaloid, inorganic, lipid, phenolic compounds. The main constituients are charantin, momordin, momordicine, stearic acid, gentisic acid, glycoside, protein MAP-30, vicine, alpha–momorcharin, and beta–momorcharin, which are found mostly in the pulp and seed of bitter melon and are summarized in Table 2.1 and Table 2.2. The fruits of bitter melon have many medicinal properties such as hypoglycemic, hypocholesterolemic, antibacterial, carminative, antimalarial, anti-inflamatory, antiviral, antiprotozoal, anti-fertility, digestive stimulant, antiseptic, febrifuge, antivenin, hypotensive, and antioxidant (Ahmed et al., 2001). The fruits of bitter melon are used in several countries for various remedies, and these are summarized in Table 2.3. Proteins found in bitter melon such as alpha-momorcharin, beta-momorcharin, and protein MAP-30, have clinically been demonstrated to be anticancerous, effective for treating tumors, and known to inhibit HIV virus in vitro studies (Luetrakul et al., 1998). Charantin is used for diabetes to reduce blood sugar (Chanchai et al., 2002). In addition, phenolic compounds bitter melon were shown to exhibit antioxidant, antimutagen, antitumor, anti-inflammatory, and anticarcinogenic properties (Cheng et al., 2003; Lee et al., 2004). In this study, phenolic compounds are of particular interest.

Table 2.1 Compounds in the flesh of *Momordica charantia* (Taylor, 2002).

Chemical type	Compound	Quantity
Proteid	Alanine	Not stated
	Alanine,beta	Not stated
	Alanine,phenyl	Not stated
	Butyric acid,gamma-amino	Not stated
	Citrulline	41.2 ppm
	Glutamic acid	Not stated
	Momorcharin,delta	Not stated
	Momordica charintia cytostatic factor	Not stated
	Momordica charintia cytostatic factor 11000 daltons	Not stated
	Momordica charintia cytostatic factor 40000 daltons	Not stated
	Momordica protein (mw-34000)	Not stated
	Momordica protein map-30	Not stated
	P-insulin	Not stated
	Peptide mc-6	Not stated
	Peptide m6.1	Not stated
	Peptide m6.2	Not stated
	Peptide m6.3	Not stated
	Proline	Not stated
	V-insuline	Not stated
	Protein	9000-181000 ppm
Inorganic	Ascorbic acid	570-36444 ppm
	Calcium	130-4333 ppm
	Copper	30 ppm
	Iodine	0.41 ppm

Table 2.1 Compounds in the flesh of *Momordica charantia* (cont.).

Chemical type	Compound	Quantity
Inorganic	Iron	2-560 ppm
	Magnesium	195-3800 ppm
	Manganese	10 ppm
	Niacin	3-50 ppm
	Nitrogen	33800 ppm
	Potassium	2700-45000 ppm
	Phosphorus	320-8333 ppm
	Riboflavin	0.4-9 ppm
	Sodium	20-333 ppm
	Thiamin	0.2-12 ppm
Steroid	Charantin	Not stated
	Momordica charantia steroid glycoside	Not stated
	Momordenol	0.00003 %
	Sitosterol,beta	Not stated
	Sitosterol,beta : D-glucoside	Not stated
	Spinasterol,alpha	Not stated
	Stigmasta-5-ene-3-beta-25-diol	Not stated
	Stigmasta-5-25(27)-dien-3-beta-ol,3-o-(6'-o-palmitoyl-beta-d-glucosyl)	Not stated
	Stigmasta-5-25-dien-3-beta-ol	Not stated
	Stigmasta-7-22-25-trien-3-beta-ol	Not stated
	Stigmasta-5-25-diene-3-beta-d-glucoside	Not stated
	Stigmasta-7-22-dien-3-beta-ol	Not stated
	Stigmasterol	Not stated
Unknow	Ascorbigen	Not stated
	Flavochrome	Not stated

Table 2.1 Compounds in the flesh of *Momordica charantia* (cont.).

Chemical type	Compound	Quantity
Unknown	Kakara l-B	Not stated
	Kakara lll-A	Not stated
	Kakara lll-B	Not stated
	Oxalic-acid	5 ppm
	Oxalate	185-1444 ppm
	Peroxidase	Not stated
	Pipecolic acid	Not stated
	Polypeptide-p	Not stated
Alkaloid	Charine	Not stated
	Tryptamine,5-hydroxy	Not stated
Lipid	Cholesterol	Not stated
	Elaeostearic acid, alpha	Not stated
	Lauric acid	Not stated
	Linoleic acid	Not stated
	Linolenic acid	Not stated
	Myristic acid	Not stated
	Palmitic acid	Not stated
	Oleic acid	Not stated
	Palmitoleic acid	Not stated
	Stearic acid	Not stated
	Soya cerebroside l	Not stated
Triterpene	Goyaglycoside A	0.00007%
	Goyaglycoside B	0.00005%
	Goyaglycoside C	0.00006%
	Goyaglycoside D	0.00008%

Table 2.1 Compounds in the flesh of *Momordica charantia* (cont.).

Chemical type	Compound	Quantity
Triterpene	Goyaglycoside E	0.0001%
	Goyaglycoside F	0.00008%
	Goyaglycoside G	0.00006%
	Goyaglycoside H	0.00007%
	Goyasaponin l	0.0001%
	Goyasaponin l	0.00027%
	Goyasaponin l	0.00008%
	Momordicilin	0.000015%
	Momordicin	0.000018%
	Momordicine l	0.05%
	Momordicinin	0.00002%
	Momordicoside A	0.00128%
	Momordicoside C	0.00016%
	Momordicoside E′	0.00104%
	Momordicoside E-1	0.07560%
	Momordicoside Ex	0.00126%
	Momordicoside F	0.00720%
	Momordicoside F-1	0.04340%
	Momordicoside F-2	0.00400%
	Momordicoside G	0.01236%
	Momordicoside H	0.00740%
	Momordicoside I	0.00818%
	Momordicoside J	0.00080%

Table 2.1 Compounds in the flesh of *Momordica charantia* (cont.).

Chemical type	Compound	Quantity
Triterpene	Momordicoside K	0.00002%
	Momordicoside L	0.00360%
	Momordol	0.00004%
Sepogenin	Diosgenin	1.69 mg/gm
Alkene to c3	Ethylene	Not stated
Non-alkaloid nitrogen heterocy	Uracil	Not stated
Carbohydrate	Galacturonic acid, D:	0.06%
	Inulin	Not stated
	Pectin	Not stated
Sterol	Lanosterol	Not stated
Carotenoid	Lycopene	0.02310%

Table 2.2 Compounds in the seed of *Momordica charantia* (Taylor, 2002).

Chemical type	Compound	Quantity
proteid	Alanine	0.01577%
	Arginine	0.0323%
	Asparagine	Traces
	Aspartic acid	0.009%
	BGIT	Not stated
	Bitter gourd inhibitor bg-I-a	Not stated
	Butyric acid, gamma-amino	Traces
	Glutamic acid	0.0212%
	Glycine	0.00382%
	Gourd protein mrk-29	Not stated
	Histidine	0.00436%
	Lectin inhibitor	Not stated
	Lectin	Not stated
	Leucine, iso:	0.00384%
	Leucine	0.00416%
	Map-30	Not stated
	Momorcharin l	Not stated
	Momorcharin ll	Not stated
	Momorcharin , alpha	Not stated
	Momorcharin , beta:	Not stated
	Momorcharin , delta:	Not stated
	Momorcharin , agamma:	Not stated
	Momodica agglutinin	Not stated
	Momodica anti-HIV protein map-30	Not stated
	Momodica charanthia inhibitor protein	Not stated

Table 2.2 Compounds in the seed of *Momordica charantia* (cont.).

Chemical type	Compound	Quantity
	Momodica charanthia lectin	0.016%
Proteid	Momodica elastase inhibitor mcei-l	Not stated
	Momodica lectin	Not stated
	Momodica protein map-30	Not stated
	Momodica protein ms-1	Not stated
	Momodica protein ms-2	Not stated
	Momodica protein ms-3	Not stated
	Momodica protein ms-4	Not stated
	Momodica trypsin inhibitor mcti-l	Not stated
	Momodica trypsin inhibitor mcti-ll	Not stated
	Momordin	Not stated
	Momordin 2#	Not stated
	Momordin A	Not stated
	Momordin B	Not stated
	Momordin ll#	Not stated
	Ornithine	0.00632%
	P-insulin	Not stated
	Ribosome-inactivating protein 1	Not stated
	Ribosome-inactivating protein 2	Not stated
	Ribosome-inactivating protein 3	Not stated
	Ribosome-inactivating protein 4	Not stated
	Serine	0.00399%
	Threonine	0.00178%
	Trypsin inhibitor mci-3	Not stated
	Tyrosine	0.0517%

Table 2.2 Compounds in the seed of *Momordica charantia* (cont.).

Chemical type	Compound	Quantity
Triterpene	Amyrin,beta :	Not stated
	Cucurbita-5-24-dien-3-beta-ol,1o-alpha	Not stated
	Cucurbitacin K	Not stated
	Cycloart-cis-23-ene-3-beta-25-diol	Not stated
	Cucurbitacin B	Not stated
	Cycloartenol	Not stated
	Cycloartenol, 24-methylene	Not stated
	Erythrodiol	Not stated
	Karounidiol	Not stated
	Karounidiol, dihydro :7-oxo	Not stated
	Karounidiol, iso :	Not stated
	Karounidiol, iso: 3-para-methoxy-benzoate	Not stated
	Karounidiol-3-benzoate	Not stated
	Momorcharaside A	Not stated
	Momorcharaside B	Not stated
	Momordicoside A	0.12874%
	Momordicoside B	0.00898%
	Momordicoside C	0.01141%
	Momordicoside D	0.00228%
	Momordicoside E	0.00365%
	Multiflorenol	Not stated
	Squalene	Not stated

Table 2.2 Compounds in the seed of *Momordica charantia* (cont.).

Chemical type	Compound	Quantity
Triterpene	Taraxerol	Not stated
	Charantin	Not stated
Steroid	Cholesta-trans-7-trans-22-25(27)-trien-3-beta-ol,5-alpha:3-o-beta-d-glucosyl-24-beta-ethyl	Not stated
Unknow	Ribonuclease MC1	Not stated
	Ureas	Not stated
Alkaloid	Zeatin	Not stated
	Zeatin riboside	Not stated
Lipid	Capric acid	Not stated
	Lauric acid	Not stated
	Linoleic acid	Not stated
	Linolenic acid	Not stated
	Myristic acid	Not stated
	Palmitic acid	Not stated
	Petroselinic	Not stated
	Stearic acid	Not stated
Monoterpene	Cymene,para:	Not stated
	Menthol,(-) :	Not stated
Carbohydrate	Glucose, alpha :	Not stated
	Glucose, beta :	Not stated
	Mycose	Not stated
	Trehalose	0.39600%
	Trehalose, alpha-alpha:	Not stated
Alkanol c5 or more	Hexadecan-1-ol	Not stated
	Pentacecan-1-ol	Not stated

Table 2.2 Compounds in the seed of *Momordica charantia* (cont.).

Chemical type	Compound	Quantity
Sesquiterpene	Nerolidol	Not stated
Carotenoid	Lycopene	Not stated
Non-alkaloid nitrogen heterocy	Vicine	0.40000%

Table 2.3 Various properties of the fruits of *Momordica charantia* used in various countries (Taylor, 2002).

Country	Properties for Use	Type Extract
India	- Used foe diabetes	Hot H_2O Ext
	- Used as s remedy for diabetes mellitus.	
	- Used foe diabetes	Decoction
	- Used for hydrophobia. Notonia grandiflora juice is mixed with better gourd powder and taken internally.	Power
	- Used as an abortifacient in large doses.	Fruit
	- Used as an anthelmintic.	
	- Used as a common vegetable	
	- Used as an antivenin	Oil Ext
	- Used as an antileprotic.	Not stated
	- Used for jaundice, piles, leprosy, rheumatism and gout. Used as a tonic and laxative, dysmenorrheal and as a emmenagogue.	
	- Used for malarial fevers.	Juice
Australia	- Used as an anthelmintic and emetic.	Plant
Bimini	- Used as a food.	Fruit
Brazil	- Used for tumors.	-
	- Used to treat wounds.	Hot H_2O Ext
	- Used as an anthelmintic and to lower blood sugar.	Fruit
China	- Used as a male aphrodisiac.	Decoction
	- Used as a food.	Fruit
	- Used for diabetes mellitus both mild-moderate chronic cases.	-
	- Used to reduce glucose in blood and urine.	-
Columbia	- Used for snakebite.	Infusion
England	- Used for diabetes.	Fruit , Hot H_2O Ext
	- Used as an ingredient in curries eaten by immigrants.	Fruit
Fiji	- Used for stomach worms, fever, phlegm and diabetes.	Fruit
Guadeloupe	- Reported to have hypoglycemic activity.	
Traq	- Used for leprosy.	-
	- Used as an anthelmintic.	
Jamaica	- Used for diabetes.	Hot H_2O Ext
Nigeria	- Eaten as a pot herb.	Fruit
Pakistan	- Used for diabetes.	Fruit
	- Eaten as a food.	
Peru	- Used as a purgative.	Hot H_2O Ext
	- Used for contusions , respiratory.	

Table 2.3 Various properties of the fruits of *Momordica charantia* used in various country (cont.).

Country	Documented Ethnomedical Use	Type Extract
Peru	- Used to treat hepatitis.	Infusion
	- Used as a suppurative.	-
	- Used as a vermifuge , an emetic and a febrifuge.	
	- Used as an emmenagogue.	
	- Used for diarrhea and colic	Juice
Islands	- Used for a bad heart and diabetes.	Fruit
Saudi Arabia	- Used for diabetes , rheumatism, gout, liver disorders, pyrexia, colic, flatulence.	Hot H_2O Ext
	- Used for menstrual suppression.	
Sri Lanka	- Used as a hypoglycemic agent.	
	- Used as an anthelmintic.	-
	- Used to treat diabetes mellitus.	Juice
	- Used for diabetes.	Hot H_2O Ext
Thailand	- Used as an anti-inflammatory.	Decoction
	- Used for diabetes.	Hot H_2O Ext
	- Used as a food.	Fruit
Turkey	- Used as a treatment for peptic ulcers.	
	- Used as an antiallergic, antihepatic, antipruritic.	Plant
	- Used as an anti-inflammatory.	
USA	- Used for remedy for hemorrhoids.	Hot H_2O Ext
	- Used in large doses as an abortifacient.	Plant
	- Used to treat snakebite, leprosy, itching skin, burns and wounds.	
	- Used for bacillary dysentery and to relieve chronic colitis.	
	- Used for thrush.	
	- Used as a substitute for quinine in intermittent fever, liver and spleen ailments, gout, menstrual difficulties and rheumatism.	Hot H_2O Ext
	- Used as a vermifuge and purgative.	
West Africa	- Used as an antidiabetic remedy.	-
	- Used as an abortifacient.	Fruit

2.3 Phenolic compounds

Phenolic compounds constitute a group of compound containing one or more aromatic benzene rings which it attached to more hydroxyl functional groups (-OH). Phenolic compounds are diverse in structure. If they are attached to sugar molecules, they are called glucosides or glycosides, depending on the type of sugar. The basic structure of phenolic compound consists of one aromatic benzene ring attached with one hydroxyl functional group as shown in Figure 2.3.1. Phenolic compounds are categorized as secondary metabolites and are essential for growth and reproduction of the plant. They are known as hydrophilic antioxidants, and are produced as a response for defending injured plants against pathogens.

Deep–colored vegetables and fruits including bitter melon are good sources of phenolic compounds. The main phenolic compounds in bitter melon are gallic acid, gentisic acid, chlorogenic acid, and catechin (Horax et al., 2005). The structures of these compounds are shown in Figure 2.3.2. They are believed to exhibit antioxidant, antimutagen, antitumor, anti-inflammatory, and anticarcinogenic properties (Cheng et al., 2003; Lee et al., 2004).

Gallic acid ($C_7H_6O_5$) occurs as a free molecule or as part of tannin molecule. Gallic acid has hydroxyl groups and carboxylic acid groups in the molecule. It has anti-fungal and anti-virus properties and acts as an antioxidant that helps protect our cells against oxidative damage. Moreover it has been found to show cytotoxicity against cancer cells, without harming healthy cells. In addition, the compound has been used to treat albuminuria and diabetes.

Chlorogenic acid ($C_{16}H_{18}O_9$) is a family of esters formed between certain *trans* cinnamic acids and (-)-quinic acid. This acid is an important factor in plant metabolism. It is also an antioxidant. Chlorogenic acid is claimed to have antiviral, antibacterial, and antifungal effect with relatively low toxicity. Moreover, it is used to prevent diabetes mellitus and cardiovascular disease.

Catechin is polyphenolic that belongs to the family of flavan-3-ol. It is an antioxidant whose health benefits have been studied extensively in human model, particularly in its ability to reduce the risk of four of major health problems: stroke, heart failure, cancer, and diabetes.

Gentisic acid ($C_7H_6O_4$) contains a carboxylic group and two -OH at quinol position, and is a crystalline powder that forms monoclinic prism in water solution. Gentisic acid had anti-bacterial properties and used as an anticarcinogenic agent.

The total phenolic contents of some Thai plants obtained by ethanol extraction are summarized in Table 2.4 (Pasuk et al., 2008)

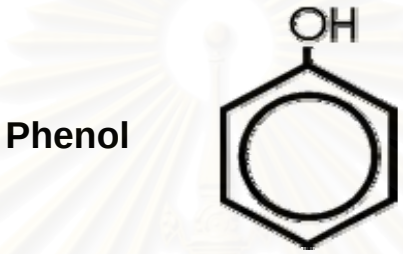
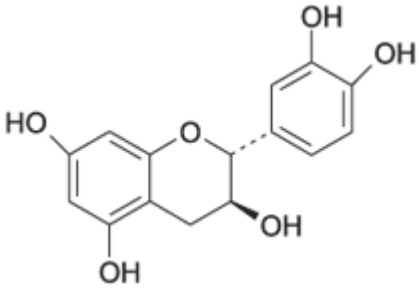
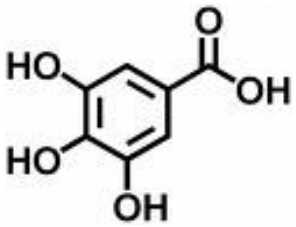


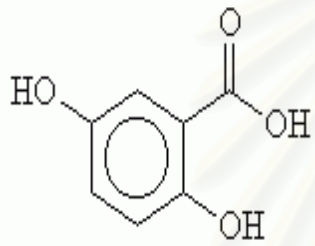
Figure 2.3.1 Chemical structure of phenol



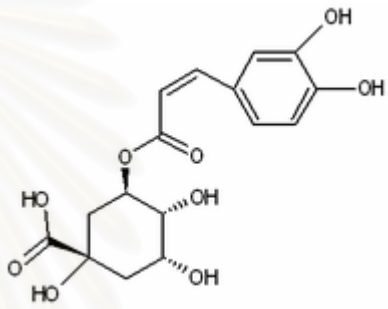
Catechin



Gallic acid



Gentisic acid



Chlorogenic acid

Figure 2.3.2 Chemical structures of catechin, gallic acid, gentisic acid and chlorogenic acid

Table 2.4 Total phenolic content of some Thai plants[1]

Scientific name	Plant part	Total phenolics (mg GAE/g db[a])
Herb and vegetable leaves		
Basella alba Linn.	Leaf	15.5
Careya sphaerica Roxb.	Young leaf and leaf	54.5
Centella asiatica Linn.	Leaf	12.4
Cratoxylum formosum Dyer.	Young leaf and leaf	63.4
Erythrina crista Galli.	Leaf	67.5

Lasia spinosa (Linn.) Thw.	Leaf	6.4
Leucaena glauca Benth	Young leaf and leaf	51.2
Limnocharis flava Buch.	Leaf	5.4
Ocimum basilicum Linn.	Young leaf and leaf	50.5
Ocimum sanctum Linn.	Young leaf and leaf	41.9
Sauropus androgynus Linn.	Young leaf and leaf	11.5
Spondias pinnata Kurz.	Young leaf and leaf	42.6
Syzygium gratum (Wight) S.N.Mitra var. gratum	Young leaf and leaf	57.3
Herbs and vegetable flowers		
Allium ascalonicum Linn.	Flower	55.7
Azadirachta indeca A. Juss Var. siamensis valeton	Flower	40.3
Cassia siamea Britt.	Flower	51.5
Musa spiantum Linn.	Flower	45.3
Sesbania grandiflora Desv.	Flower	58.6
Berries and fruits		
Capsicum frutescus Linn.	Fruit	40.3
Eugenia siamensis Craib.	Fruit	82.4
Euginia malaccenses Linn.	Fruit	69.2
Momordica charantia Linn.	Fruit	50.9
Phyllanthus emblica	Fruit	69.1
Spondias pinnata Kurz.	Fruit	47.2
Berry and fruit seeds		
Nephelium lappaceum Linn.	Seed	43.5
Parkia speciosa Hassk.	Seed	51.9
Piper nigrum Linn.	Seed	53.1
Tamarindus indica Linn	Seed	40.7

[a] Dry weight basis of the original sample of plant parts.
[1] source: Maisuthisakul et al. (2008)

2.4 Subcritical water extraction technology

Subcritical water extraction or superheated water extraction (SCWE), also called pressurized hot water extraction (PHWE) or pressurized low polarity water extraction (PLPWE), was a technique that has been used for extraction of plants. Subcritical water was water that has the temperature between boiling point temperature (100 °C) and the critical point temperature (374 °C) and at a pressure high enough to maintain the liquid state (Ayala et al., 2001) as was shown in Figure 2.4. At such conditions, organic solutes were much more soluble in water than at room temperature. The important factor that affects these results was the variability of the dielectric constant with temperature. Normally, water at room temperature was very polar, with a dielectric constant (ε) of approximately 80. However, the ε value was significantly decreased to less than 30 when water temperature rises to 250ºC, (see figure 2.5) which was a value similar to that of ethanol at room temperature (King et al., 2004).

This technique was a powerful alternative for benign extraction of solid sample. The important advantage of subcritical water extraction were its simplicity, non-toxic, non-flammable, economical, reduced extraction time, higher quality of the extract, lower cost of the extracting agent, and an environmentally friendly teachnique (Herrero et al., 2006). For extraction of medicinally active compounds from natural materials, water at milder subcritical conditions rather than corrosive supercritical condition is more effective. At subcritical conditions, water has been shown to be capable of extracting a wide range of organic solutes from different matrixes.

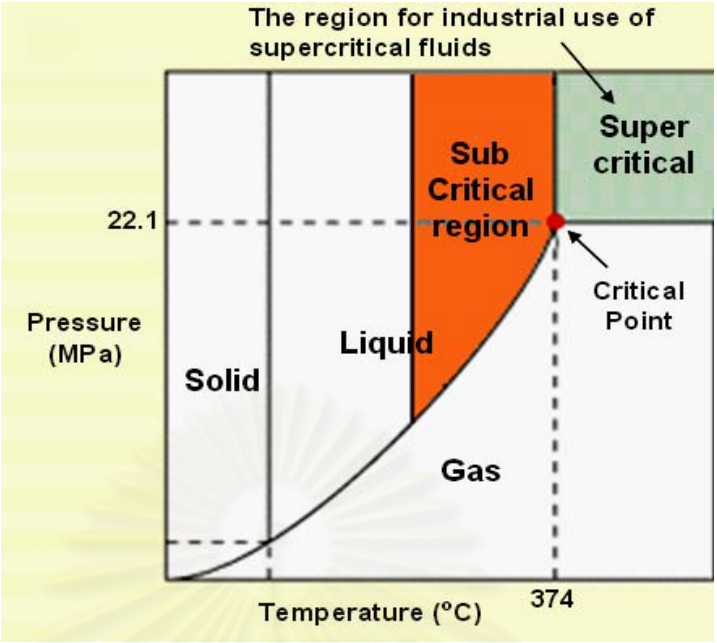


Figure 2.4 Theoretical Pressure – Temperature phase diagram for pure compound.

Source: http://www.kobelco.co.jp/eng/p14/sfe01.htm

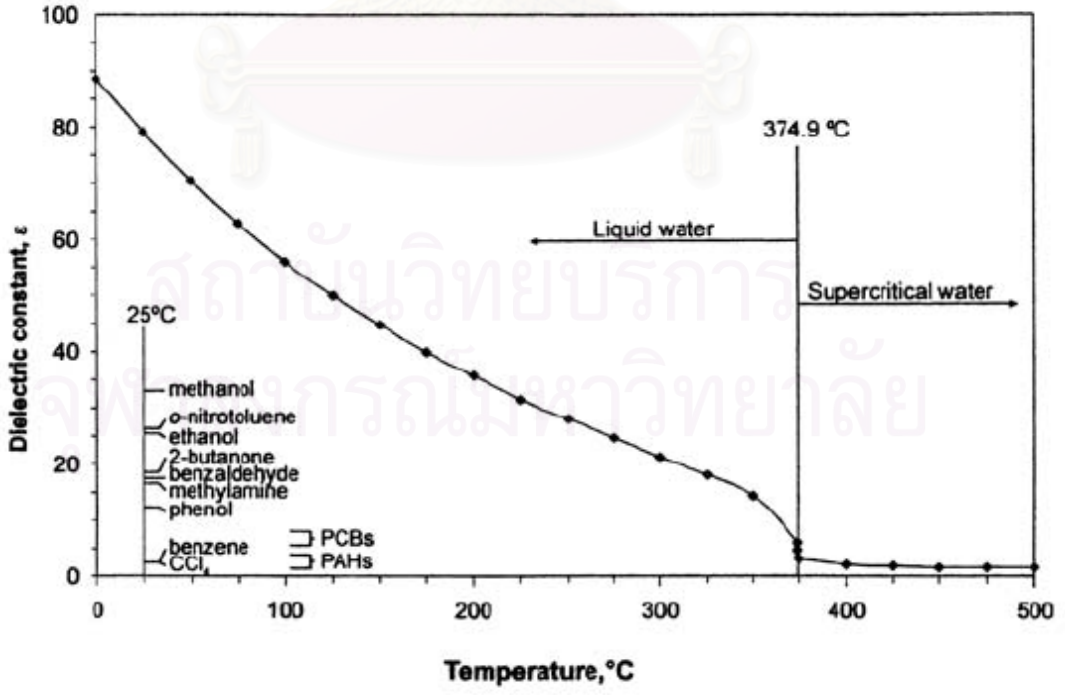


Figure 2.5 Dielectric constant of water versus temperature (King, 2004)

2.5 Antioxidant activitiy

Antioxidants are specific organic compounds that prevent the oxidation of other chemicals in the body by destroying single oxygen molecules, also called free radicals, thereby protecting against oxidative damage to cells. Oxidation is a redox chemical reaction that transfers unpaired electrons in a substance to an oxidizing agent. This reaction involves dangerous chain reactions of the production of free radicals, which would then stabilize themselves by oxidizing other molecules, including proteins, carbohydrates, lipids, and DNA. Examples of reactive oxygen species (ROS), or free radicals are such as, singlet oxygen (O_2), superoxide anion ($\bullet O_2^-$), hydroxyl radical (•OH), hydrogen peroxide (H_2O_2), peroxyl radical ($\bullet O_2$), hypochlorite ion (OCl^-), and peroxynitrite (ONOO-). They are therefore the major cause of various chronic and degenerative diseases, including ageing, coronary heart disease, stroke, atherosclerosis, inflammation, diabetes mellitus, dementia, and cancer. The oxidative reaction can however be terminated by means of an antioxidant, which can remove radical intermediates and inhibit the oxidation reaction by being oxidized themselves.

Many vegetables, fruits, grain cereals, herbs, and medicinal plants are good natural sources of antioxidants that protect the human body free radicals. The main antioxidants have three major groups, including vitamins, phenolic compounds, and carotenoids as shown in Table 2.5. These antioxidants are divided into two types, which include hydrophilic antioxidant and lipophilic antioxidant. Some vitamin and phenolic compounds are known as hydrophilic antioxidant, while carotenoids are known as lipophilic antioxidant (Halliwell, 1996). The antioxidant properties of bitter melon extracts have been attributed to their polyphenol contents.

The methods for which antioxidant activities measurement of natural products are evaluated include the 2,2-azinobis (3-ethyl-bezothiazoline-6-sulfonic acid) (ABTS) assay (Leong and shui, 2002), 2,2-diphenyl-l-picrylhydrazyl (DPPH) assay (Williams et al., 1995), the oxygen radical absorption capacity (OARC) assay (Prior et al., 2003), ferric reducing antioxidant power (FRAP) assay (Benzie and Strain, 1999), xanthine oxidase inhibition test (Chang et al., 1994), and anti lipid peroxidation assay (Yoshiyuki et al., 1981) etc.

Antioxidant activity of some medicinal plants and some tropical fruits were measured using DPPH assay and summarized in table 2.6 and 2.7, respectively.

Table 2.5 Antioxidant compounds found in various foods

Antioxidant compounds	Foods containing high levels of these antioxidants
Vitamin C (ascorbic acid)	Fruits and vegetables
Vitamin E (tocopherols, tocotrienols)	Vegetable oils
Polyphenolic antioxidants (resveratrol, flavonoids)	Tea, coffee, soy, fruit, chocolate and red wine
Carotenoids (lycopene, carotenes)	Fruit and vegetables

Source : http://en.wikipedia.org/wiki/Antioxidant

Table 2.6 The antioxidant activities of extracts from tropical fruits, measure by DPPH assay [1]

Fruits	IC_{50} (mg/ml) [a]
Guava (seeded)	1.71 ± 0.61
Guava (seedless)	2.11 ± 0.63
Banana (mas)	13.4 ± 2.5
Gragon fruit	27.5 ± 3.9
Star friut	3.8 ± 2.1
Sugar apple (brown)	3.9 ± 0.4
Sugar apple (green)	4.6 ± 0.8
Water apple	12.0 ± 3.8
Orange	5.4 ± 1.3

[a]The IC_{50} values (the concentration required to inhibit radical formation by 50%)

[1]Source: Lim et al. (2007)

Table 2.7 The antioxidant activities of extracts from medicinal plants, measure by DPPH assay[1]

Plant	Part used	IC_{50} (μg/ml)
Copernicia cerifera	Leaves	23.5 ± 0.1
	Mesocarp of fruits	15.3 ± 0.4
	Epicarp of fruits	41.9 ± 0.8
Mauritia vinifera	Mesocarp of fruits	538.3 ± 2.0
	Epicarp of fruits	71.0 ± 1.0
Syagrus oleracea	Epicarp of fruits	425.5 ± 1.9
	Epicarp/mesocarp of fruits	27.0 ± 0.2
Orbigynia speciosa	Endocarp of fruits	4104.3 ± 6.7
	Flowers	427.4 ± 1.8
	Leaves	895.9 ± 2.3
Bauhinia variegata	Aerial parts	37.0 ± 0.4
Bauhinia purpurea	Aerial parts	137.9 ± 1.1
Bauhinia candida	Aerial parts	45.4 ± 0.9
Bauhinia monandra	Aerial parts	199.8 ± 1.6
Bauhinia angulosa	Aerial parts	106.4 ± 1.1
	Aerial parts	68.4 1.0
	Aerial parts	35.1 0.3
Polygala paniculata	Aerial parts	135.4 ± 1.1
	Roots	325.6 ± 1.6
Hyptis fasciculata	Aerial parts	57.9 ± 0.9
	Aerial parts	35.0 ± 0.3
Hyptis heterodon	Aerial parts	233.4 ± 1.3
Ginkgo biloba	Leaves	41.5 ± 0.1

[1]Source: Silva et al. (2005)

Literature reviews

Thailand is in the tropical region whose climate is suitable for cultivation of several fruits and vegetables. Bitter melon also can grow vigorously in this climate and are consumed as food and medicine. All parts of bitter melon are used as traditional medicine such as leaves, vines, roots, fruits, and seeds. Bitter melon contain a wide range of compounds such as charantin, momordin, momordicine, stearic acid, gentisic acid, glycoside, protein MAP-30, vicine, alpha–momorcharin, and beta–momorcharin. Charantin was demonstrated for the ability to reduce blood sugar and was found in the roots (0.0139 %), vine (0.0417 %) s, and fruits (0.0301%) (Chanchai et al., 2002). The compound could be extracted in Soxhlet apparatus with organic solvents such as 95%ethanol and chloroform. Alternatively, Jesada et al. (2004) proposed a method of extraction of is study charantin from fruits of bitter melon with subcritical fluids such as acetone, dichloromethane, ethyl acetate, ethanol, and water and found that acetone and ethanol gave the highest extraction efficiency. This method allowed the compound to be extracted with more benign solvents than conventional solvent extraction. Other compound in bitter melon were also examined including vicine from seeds, leaves, and fruits with ultrasonic water extraction (Zhang et al., 2003) and phenolic acid constituents studied in four varieties from flesh, inner tissue, and seeds with methanol extraction (Horax et al., 2005). The phenolic content of the flesh were significantly higher than those of inner tissue and seed and the main phenolic acids in the bitter melon flesh and inner tissues were gallic acid, gentisic acid, catechin, and epicatechin, while the main phenolic acids in the bitter melon seeds were gallic acid, catechin, and epicatechin (Horax et al., 2005). In addition, the antioxidant activity of the water and ethanol extract of bitter melon was also investigated (Wu et al., 2007). The results showed that both the water extract of bitter melon (IC50 = 129.94 mg/ml) and the ethanol extract of bitter melon (IC50 = 156.78 mg/ml) possess highly potent DPPH radical scavenging activity that was higher than vitamin E (IC50 = 172.21 mg/ml). Table 2.8 summarizes the review on the investigation of extraction of various compounds in *Momordica charantia*.

Extraction methods for extraction of natural products from plants include solvent extraction, soxhlet extraction, distillation, supercritical fluid extraction, and subcritical fluid extraction. This study aims to investigate subcritical water extraction

of bitter melon. The main advantage of subcritical water extraction are its simplicity, reduced extraction time, higher quality of the extract which can be directly used for pharmacological and toxicological testing, lower cost of the extracting agent, and an environmentally friendly technique. Previous reports demonstrated that SCWE was an effective method for lactones extraction from kava root (Kubátová et al., 2001) and for anthraquinones extraction from roots of *Morinda citrifolia* (Pongnaravane et al., 2005). Moreover this technique is widely used for essential oils extraction from marjoram (Carmona et al., 1999), oregano (Ayala et al., 2001), coriander seeds (Eikani et al., 2006), and some terpenes from basil and oregano leaves (Yang et al., 2006). Reviews of subcritical water extraction of natural plants are summarized in Table 2.9.

Table 2.8: Reviews on investigation of extraction of compounds in *Momordica charantia*.

Author	***Variety***	***Parts used***	***Solvent***	***Extraction methods***	***Product***	***Analysis***	***Contents***
1. Chanchai et al., 2002	Dark green Thai	Root, vine, and fruit	- Petroleum ether - Chloroform - 95 % ethanol	Soxhlet extraction	Charantin	HPLC, thin-layer choma-tograms	Charantin in vine : 0.0417 % Charantin in fruit : 0.0301 % Charantin in root : 0.0139 %
2. Zhang et al., 2003	Large green chaina	Seeds, leaves, and fruits	Water	Ultrasonic extraction	Vicine	HPLC	Vicine in seeds : 0.524 % Vicine in leaves : 0.0456 % Vicine in fruits : 0.115 %
3.Pitiphanpon et al., 2004	Dark green Thai	Fruits	- Acetone -Dichloromethane - Ethyl acetate - Methanol - Water	Accelerated solvent extraction	Charantin	HPLC	Charantin : 0.126 ± 0.018mg/ g dried fruit

Table 2.8: Reviews on investigation of extraction of compounds in *Momordica charantia* (cont.).

Author	*Variety*	*Parts used*	*Solvent*	*Extraction method*	*Product*	*Analysis*	*Contents*
4. Horax et al., 2005	- India green - India white - China green - China white	Flesh, inner tissue, and seed	Methanol	Solvent extraction	Phenolic acid	HPLC	The main phenolic acids in flesh - gallic acid: 8.04-39.76mg/100gDW - gentisic acid: 16.99-32.39mg/100gDW - catechin: 23.06-82.45 mg/100gDW - chlogenic acid:4.55-15.83mg/100gDW - epicatechin: 16.14-44.28 mg/100gDW The main phenolic acids in inner tissues - gallic acid: 2.57-18.05 mg/100gDW - gentisic acid: 5.39-32.61 mg/100gDW - catechin: 13.54-39.74 mg/100gDW - epicatechin: 2.96-40.91 mg/100gDW The main phenolic acids in seeds - gallic acid: 4.61-18.9 mg/100gDW - catechin: 13.2-57.61 mg/100gDW

Table 2.8: Reviews on investigation of extraction of compounds in *Momordica charantia* (cont.).

Author	***Variety***	***Parts used***	***Solvent***	***Extraction method***	***Product***	***Analysis***	***Contents***
5. Wu et al., 2007	Wild bitter melon in Taiwan	Fruits	- Water - 95 %ethanol	Solvent extraction	Total flavonoid and phenolic contents	Spectropho-tometer	The water extract -Total flavonoids : 62.0 mg/g -Total phenol contents: 51.6mg/g The ethanol extract -Total flavonoids : 62.0 mg/g -Total phenol contents: 51.6mg/g

Table 2.9: Reviews on investigation of subcritical water extraction of natural product.

Author	*Plants*	*Parts used*	*Product*	*Condition*	*Analysis*	*Objective*
1. Carmona et al.,1999	Thymus mastichina (marjoram)	Leaves	α-Pinene, β-Pinene, β-Myrcene, Linalool, Geraniol, etc.	- Temperature: 100-175 °C - Pressure: 20-200 bar - Flow rate: 0.5-3 ml/min - Time: 30 min	GC-FID, GC-MS	To develop a rapid, efficient and inexpensive methods (SCWE and hydrodistillation) for the extraction of essential oil from marjoram.
2. Gracia et al., 1999	Foeniculum vulgare (fennel)	Fennel	α-Pinene, β-Pinene, β-Myrcene, Limonene,Camphor , Aneyhol, etc.	- Temperature: 50-200 °C - Pressure: 20 bar - Flow rate: 0.5-3 ml/min - Time: 50 min - Amount of sample: 2-5 g	GC-FID, GC-MS	To develop a method for the SCWE of medical essential oils, and compare the results with those obtained by conventional techniques.

Table 2.9: Reviews on investigation of subcritical water extraction of natural product (cont.).

Author	*Plants*	*Parts used*	*Product*	*Condition*	*Analysis*	*Objective*
3. Ayala et al., 2001	Lippia graveolens	Leaves	Phenol,Thymol, 3- Carene, 1,3-Cyclohexadiene, α-Phellandrene, etc.	- Temperature: 100-175 °C - Pressure: 1-5.1 MPa - Flow rate: 1-4 ml/min - Time: 30min	GC-FID, GC-MS	To develop a method for the SCWE of oregano essential oils, and compare the results with those obtained by hydrodistillation.
4. Kubátová et al., 2001	Piper methysticum (kava)	Roots	Dihydrokawain, kawain, Yangonin, Desmethoxyyangonin, Tetrahydroyangonin, Methysticin, etc.	- Temperature: 50-200 °C - Pressure: 60 bar - Flow rate: 1 ml/min - Time: 120 min	GC-FID, GC-MS	To compare kava lactone extraction efficiencies using traditional water extraction and organic solvent extraction, as well as the new technique of subcritical water extraction.

Table 2.9: Reviews on investigation of subcritical water extraction of natural product (cont.).

Author	*Plants*	*Parts used*	*Product*	*Condition*	*Analysis*	*Objective*
5. Ozel et al., 2002	Thymbra spicata	Leaves	Thymol, α-Thujene, Camphene, Sabinen, Limonene, etc.	- Temperature: 100-175 °C - Pressure: 20, 60, 90 bar - Flow rate: 1-3 ml/min - Time: 30 min	GC-FID, GC-TOF/MS	To determine the optimum condition for CSWE of essential oils of Thymbra spicata and investigate the effect of temperature on composition of extracted essential oils.
6. Pongnara-vane et al., 2005	Morinda citrifolia	Roots	Anthraquinones	- Temperature: 150-200 °C - Pressure: 4 MPa - Flow rate: 2-6 ml/min - Time: 4 hr	Spectropho-tometer	To determine the antioxidant activity of anthraquinones extracted with PHW and compare it with the extracts obtain by other conventional extraction methods.

Table 2.9: Reviews on investigation of subcritical water extraction of natural product (cont.).

Author	*Plants*	*Parts used*	*Product*	*Condition*	*Analysis*	*Objective*
7. Mazza et al., 2006	Voccaria pyramidata	Seeds	Saponins	- Temperature: 125-175 °C - Pressure: 5.17 MPa - Flow rate: 2 ml/min - Time: 3 hr	HPLC	To investigate the potential of PLPW for the extraction of saponins from cow cockle seed and determined the effect of PLPW extraction parameters.
8. Eikani et al., 2006	Cariandrum sativum L.	Seeds	Linalool, β-Pinene, Limonene, Camphor, Citronellal, γ-Terpinene, etc.	- Temperature: 50-200 °C - Pressure: 60 bar - Flow rate: 1 ml/min - Time: 120 min	GC-FID, GC- MS	To investigate the SCWE and identificational of coriander essential oil and compared with those obtained by hydrodistillation and soxhlet extraction.

Table 2.9: Reviews on investigation of subcritical water extraction of natural product (cont.).

Author	*Plants*	*Parts used*	*Product*	*Condition*	*Analysis*	*Objective*
9. Yang et al., 2006	Basil and oregano	Leaves	α-Pinene, Limonene, Camphor, Citronellol, and Carvacrol	- Temperature: 100-250 °C - Pressure: 15 atm - Flow rate: 0.5 ml/min - Time: 30 and 300 min	GC-FID	To investigate the stability of five selected terpenes often found in basil and oregano leaves under SWC condition.
10. Ho et al., 2006	Flaxseed meal	Seeds	Lignans, Proteins and Carbohydrates	- Temperature: 50-200 °C - Pressure: 60 bar - Flow rate: 1 ml/min - Time: 120 min	HPLC	To optimize the extraction of lignans from flaxseed meal using PLPW

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and chemicals

The fruits of bitter melon were obtained from the market in Bangkok, Thailand. Gallic acid, chlorogenic acid, catechin, and gentisic were obtained from Sigma Chemical Co. (St Louis, Mo, USA). Methanol was purchased from Fisher Scientific, UK. Water used in the experiments was distilled and deionized water.

3.2 Sample preparation

The fruits of bitter melon were cleaned and cut into small pieces, and then oven dried at 50 °C about 2 days until it reached a constant weight. The dried sample was pulverized into fine homogeneous powder in a grinder, and the fine homogeneous powder was then stored at 4 °C until used.

3.3 Subcritical water extraction (SCWE)

Subcritical water extraction was carried out in a laboratory-built apparatus shown in Figure 3.1. The extraction system consisted of two HPLC pumps (PU 980, JASCO, Japan) used to deliver the water and solvent through the system at constant flow rates, a degassing instrument (ERC 3215, CE, Japan), an oven (D63450, HARAEUS, Germany), where the extraction vessel (10 ml, Thar Design, USA) was mounted, a pressure gauge, and a back pressure regulator valve (AKICO, Japan). All connections were made with stainless steel capillaries (1/16 inch inside diameter).

Water was passed through a degassing equipment to remove dissolved oxygen. The degassed water was then delivered to preheating coil, made from 3 m length stainless steel tubing, installed in the oven before the extraction vessel, and delivered through to the extraction vessel, which was preloaded with 1.0 g of sample. The back pressure regulator valve placed at the outlet of the extraction system was used to maintain the system pressure to ensure that the water was in liquid state at all temperature tested. Before starting the extraction, all connections were checked for possible leakage. The second pump was then turned on to deliver degassed water at

constant flow rate of 1 ml/min to wash off any residual product in the outlet line behind the extractor to prevented clogged up the line. The extract was cooled in a coil immersed in a water bath to prevent possible product degradation, and the extract was collected in fractions in sample collecting vials every 10 minutes in a first hour and after which it was collected every 20-30 minutes. After extraction, the compound remained in the sample residue was extracted repeatedly in 30 ml methanol until the extract was clear. The extracts were then evaporated under vacuum to remove the water until volume of the extracts were remained about 10 ml and stored at 4 °C until analysis.

The experimental variables to be studied and their ranges were listed in Table 3.1

Table 3.1: Condition for experiment

Variables	**Condition**
Temperature	130-200°C
Flow rate	2-5 ml/min
Pressure	10 MPa

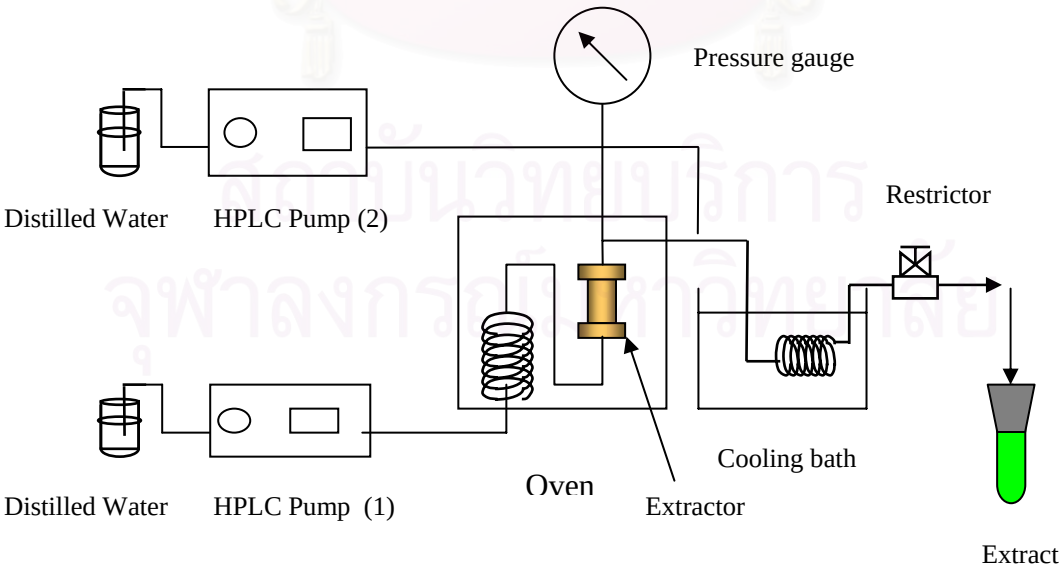


Figure 3.1 Diagram of experimental setup subcritical water extraction.

3.4 Soxhlet extraction

One gram of the fine ground sample was weighed into a thimble and was extracted with 200 ml of methanol or water for up to 6 hours. The sample residue was extracted repeatedly with 30 ml of methanol using ultrasonication. The extract was filtered with filter paper (No.4, Whatman, England) and evaporated under vacuum to remove the volume was about 10 ml. The concentrate extract was then stored at 4 °C until analysis.

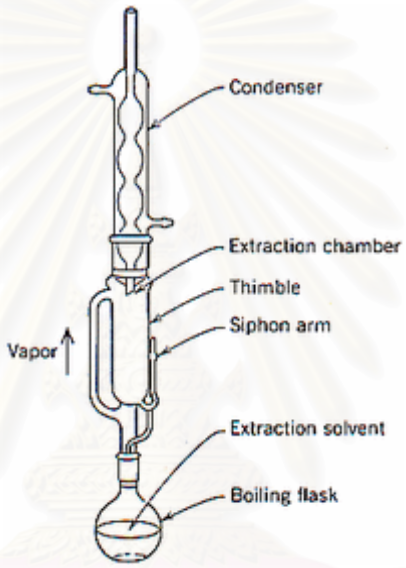


Figure 3.2 Diagram of soxhlet apparatus.

3.5 Solvent extraction

One gram of the fine ground sample was weighed into a beaker and 200 ml methanol was added and the sample was then extracted for 2 h in an ultrasonic bath (275DAG, Crest, Malaysia) at 65 °C. After extraction, the sample was cooled to room temperature and then centrifuged at 1500 rpm for 15 min. The sample residue was extracted repeatedly with 30 ml of methanol. The extract was filtered with a filter paper (No.4, Whatman, England) and evaporated under vacuum to remove the methanol until the remaining volume was about 10 ml. The concentrated extract was then stored at 4 °C until analysis.

3.6 Analysis

3.6.1 Total phenolic analysis

The total phenolic content was analyzed with the Folin-Ciocalteau method modified from Cliffe et al. (1994). 0.1 ml of the extract was mixed with 2.8 ml of distilled water, 0.1 ml of 50% Folin-Ciocalteau reagent, and 2 ml of Na_2CO_3 (2 g/100ml). The mixture was incubated at room temperature for 30 minutes. The mixture absorbance was measured spectrophotometrically at 750 nm. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry weight (DW).

3.6.2 Phenolic acid constituent analysis

The phenolic acid constituents of the extracts were determined using HPLC modified from the method of Cai et al. (2003). HPLC were performed with a C-18 Inertsil ODS-3 column (5 µm particle, 4.6 ×250 mm ID) and equipped with UV detector. The UV absorbance was monitored at 254 nm. The mobile phases consisted of solvent A (0.1% trifluoroacetic acid in acetonitrile), solvent B (0.1% trifluoroacetic acid in HPLC grade water), and solvent C (100% methanol, HPLC grade). The Flow rate was set at 1.0 ml/min, and the column temperature was maintained at 37 °C throughout the test. The initial solvent composition was 0% solvent A and 100% solvent B. A linear gradient was used to increase solvent A from 0% to 10% within 7 minutes. This solvent composition was maintained at an isocratic flow for 3 min. The solvent A was then increased from 10% to 40% using a 20-min linear gradient. This composition was maintained for 2 min and was returned to the initial composition in 3 min. Solvent C was used for washing the column after each run. The sample injection volume was 10 µl. The concentrations of phenolic acids in the sample were calculated from standard curves, from a plot of peak areas versus concentrations for a series of standard solutions.

3.6.3 Antioxidant activity determination

Antioxidatant activity was determined using ABTS (2, 2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay which was carried out following the method of Re et al. (1999) with some modifications. The extract was diluted in series in water and each diluted samples were added with the $ABTS^{\bullet+}$ stock

solution ,which included 7mM ABTS and 2.45 mM potassium persulfate, with the volume ratio of 1:10 (sample solution: $ABTS^{\bullet+}$ stock solution). The $ABTS^{\bullet+}$ stock solution had absorbance of 0.70 ± 0.02 units at 734 nm using the spectrophotometer. The solutions were mixed using a vortex and the mixtures were incubated at room temperature for 10 minutes, and then the absorbance was taken at 734 nm using the spectrophotometer.

For comparing the antioxidant activity of the extracts obtained at various conditions, concentration of sample producing 50% reduction of the radical absorbance (IC_{50}) was used as an index. The IC_{50} values for various extracts were found from the plots of percent inhibition (PI) versus the corresponding concentration of the sample. The values of PI were calculated using the following equation:

$$PI\ (\%) = [1-(A_t / A_r)] \times 100$$

Where A_t and A_r are absorbance of test sample and absorbance of the reference, respectively.

CHAPTER IV

RESULTS AND DISCUSSION

This chapter presents the experimental results of extraction of the total phenolic contents from bitter melon by subcritical water and the antioxidant activities of these extracts. Firstly, the effects of temperature and the water flow rate on the extraction performance were presented and the suitable extraction conditions were determined and discussed. Secondly, the phenolic acid constituents of the subcritical water extracts were investigated, and lastly, the antioxidant activities of the extracts obtained with subcritical water extraction were determined. In addition, the efficiencies, in terms of both the total phenolic contents and antioxidant activities, were compared between the extracts obtained by subcritical water and by other methods such as soxhlet extraction and solvent extraction.

4.1 Subcritical water extraction of total phenolic compounds

4.1.1 Effect of temperature on subcritical water extraction

In this research, subcritical water extraction was carried out to determine the effect of water temperature between 130 °C to 230 °C on the total phenolic contents. A constant water flow rate of 3 ml/min was used and the extraction was carried out at a constant pressure of 10 MPa for up to 2 hours. Figure 4.2 shows the concentration of the extracts collected at different time intervals. The results indicated that the first collected fractions contained the highest amount of total phenolics, and that most of the total phenolic contents in bitter melon were extracted within the first hour. In the second hour, the amount of the total phenolics extracted was small. During the first 10 to 30 min of extraction, the concentration of the extract increased when the temperature increased from 130 °C to 150 C. As the temperature increased further, the amount of phenolic compound extracted decreased due to degradation that occurred at high temperature. The extract obtained after 10 min at the extraction temperature of 150 °C contained the highest amount of total phenolic contents, followed by that obtained at temperature of 200 °C and 180 °C, respectively. The possible reason for

this was because at the temperature higher than 150 °C, the degradation rate was higher than the extraction rate.

The cumulative amount of total phenolic compound obtained during the extraction is shown in figure 4.3 which demonstrated that at 130 °C, the amount of the compound total phenolic extracted was small (7.688 mg/g DW), and increased as the temperature increased. In the first 30 min, the amount of total phenolic compounds extracted at 150 °C, was the highest, followed by the amounts extracted at 200 °C and 180 °C. As the extraction proceeded to 60 min, the amount extracted at 200 °C (54.94 mg/g DW) and 180 °C (51.49 mg/g DW) exceeded that obtained at 150 °C (47.71 mg/g DW). One reason for the increase in the amount of the compound extracted with increasing temperature is attributed to the decrease in water polarity (low dielectric constants) at higher temperature, which causes the increase in the solubilization capability of total phenolic compounds. In addition, the significantly high amount of the phenolic compound extracted with subcritical water, particularly at 150 C and higher temperature was possibly due to the effect of hydrolysis reaction caused by the increase in the ionization constant (Kw) of water at subcritical conditions. The presence of water as the reactant leads to hydrolysis reaction and cleavage of bonds between hetero-atoms and carbon atoms. This hydrolysis is known to be one of the key reactions to promote the degradation of many compounds. Therefore at this condition, larger aromatic molecular compound such as lignin whose molecular structure is shown in figure 4.1, which is a component of plant cell walls, could be decomosed into smaller aromatic molecular compound including phenolic compounds (Goto et al., 2007). Nevertheless, when the temperature increased to 230 °C, the amount of total phenolic compounds recovered was decreased to 32.626 mg/g DW as a result of the compound degradation at such high temperature. From these results, the temperature between 150-200 °C would be suitable for subcritical water extraction of total phenolic compounds from the fruits of bitter melon because the amount of the compound total phenolic extracted was high.

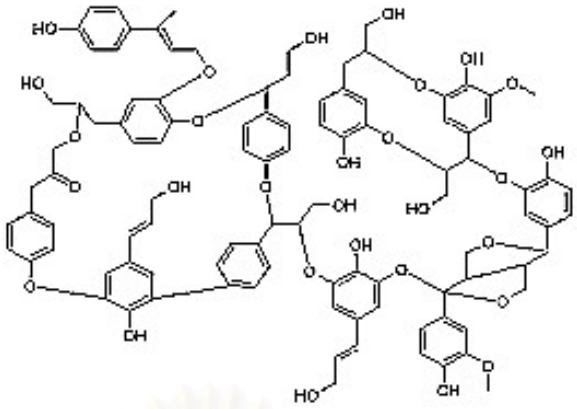


Figure 4.1 The structure of lignin.

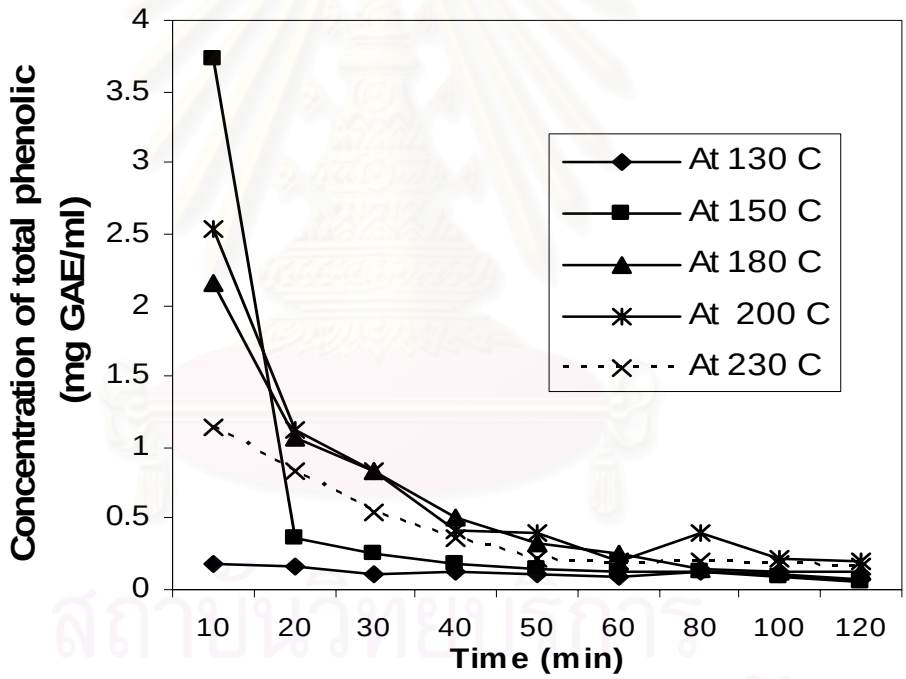


Figure 4.2 Effect of temperature on concentration of total phenolic compounds in different fractions collected at 10 min interval. Operating condition: flow rate = 3 ml/min, pressure = 10 MPa

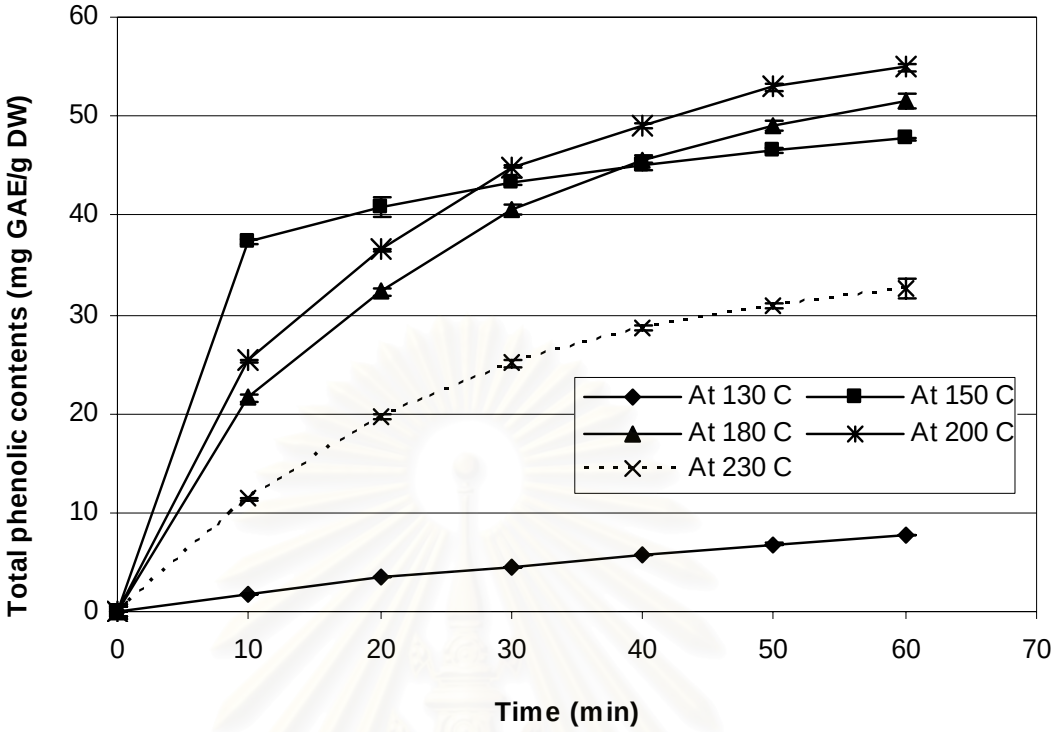


Figure 4.3 Cummulative amount of total phenolic contents obtained at various temperatures. Operating condition: flow rate = 3 ml/min, pressure = 10 MPa.

4.1.2 Effect of water flow rate on subcritical water extraction

The effect of water flow rate was investigated in the range of 2 to 5 ml/min, at a fixed extraction temperature of 200 °C and at 10 MPa. The results were presented by plotting the total phenolic contents versus extraction time and versus volume of the water used, as shown in figure 4.4 and 4.5, respectively.

Figure 4.4 showed that the extraction curves with respect to extraction time were similar for various water flow rate, while the curves with respect to extraction liquid volume differ with water flow rate (Figure 4.5), that is for the same amout of water used, the amount of the total phenolic compounds extracted into subcritical water flowing at a lower flow rate was greater than that extracted at higher flow rate. Then the total amount of total phenolic contents obtained would reached a comparable values at various water flow rates, i.e., after 600 ml of water passed the extractor. The fact that the total phenolic compound was not dependent on water flow rate (Figure 4.4) indicated that the extraction behavior was not dependent on external mass

transfer of the total phenolic component from the surface of solid phase into water phase, and the result in Figure 4.5 suggested that the subcritical extraction was also not limited by the compound solubility in water in the extraction conditions tested in this study. On the other hand, these results would imply that the extraction efficiency would largely be influenced by intraparticle mass transfer. In practice, the best flow rate must be selected considering two important factors, extraction time and extract concentration. It was therefore concluded from this experiment that the lower flow rate was the most suitable as it led to the more efficient use of water, and thus more concentrated extracts, but requiring comparable extraction times compared with extraction with higher flow rates.

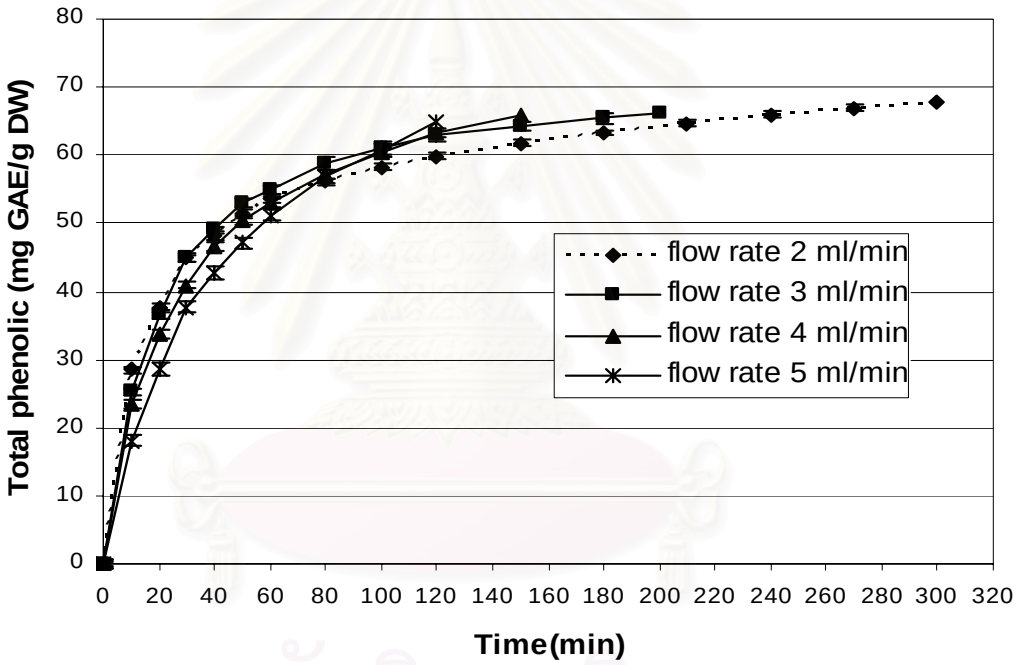


Figure 4.4 Effect of water flow rate on extraction efficiency. Operating condition: Temperature = 200 °C, pressure = 10 MPa. Plotting total phenolic contents versus time

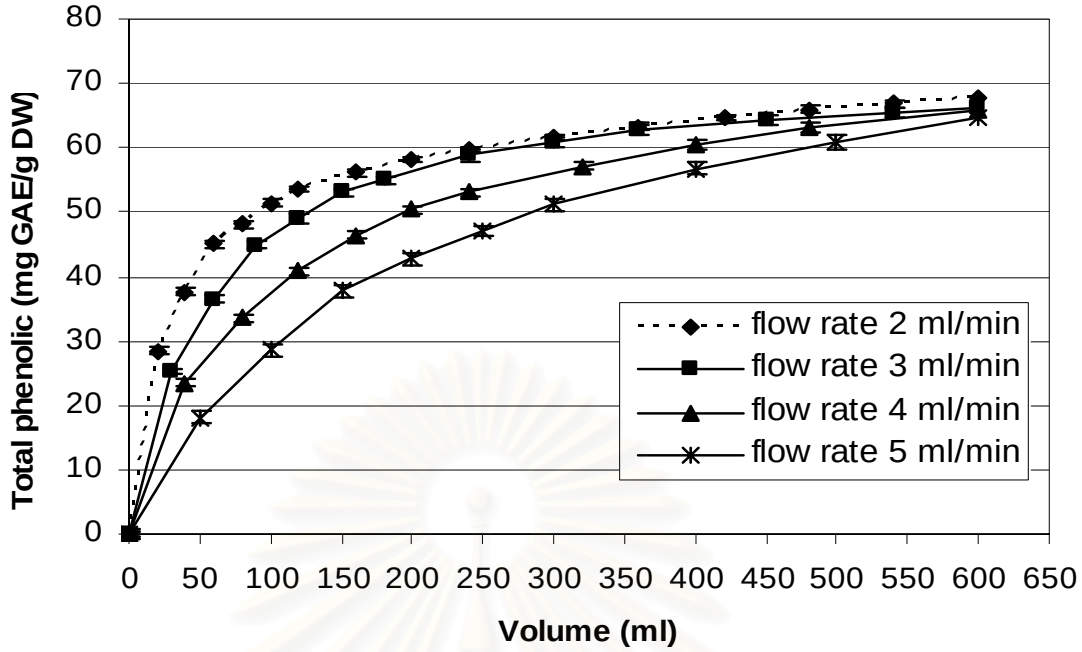
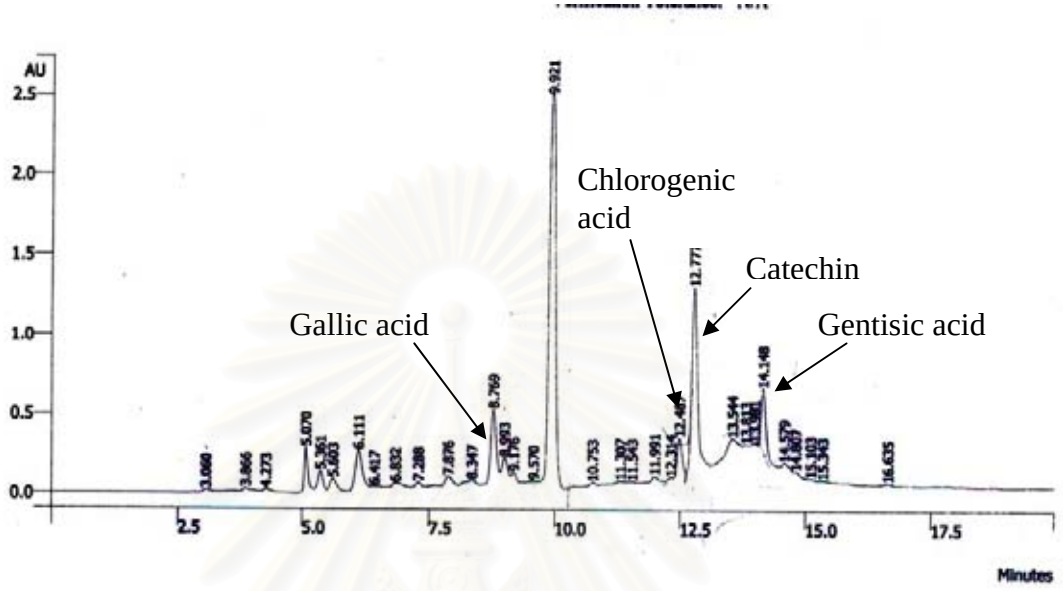


Figure 4.5 Effect of water flow rate on extraction efficiency. Operating condition: Temperature = 200 °C, pressure = 10 MPa. Plotting total phenolic contents versus volume

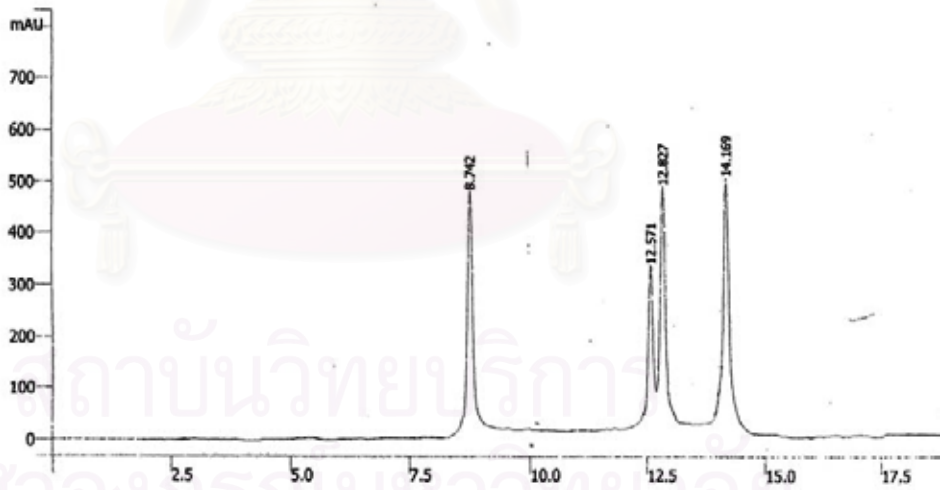
4.2 Phenolic acid constituents in bitter melon extract

Phenolic acid constituents from fruits of bitter melon in subcritical water extract were quantified by HPLC, using 4 standard compounds including catechin, gallic acid, gentisic acid, and chlorogenic acid following the procedure described in previous work (Horax et al., 2005). The chromatograms the sample extract and the four standard compounds were shown in figure 4.6. The amount of these phenolic acid constituents of bitter melon extracted after 1 h with subcritical water at various temperatures at 3 ml/min and 10 MPa are shown in Table 4.1. The extract contained the highest amount of catechin (72-86 % of the total phenolic contents), then followed by gentisic acid (4 -12 %), gallic acid (0.25-0.87 %), and chlorogenic acid (0-0.26 %), respectively. Similar to the results of total phenolic content, the amount of all four phenolic constituents generally increased with increasing temperature except for gallic acid, in which a slight decrease in the compound extracted was observed at temperature of 200 °C. On the other hand water flow rate had little effect on the four

phenolic acid constituents, in a similar manner as for the total phenolic contents (results are shown in Appendix B).



a)



b)

Figure 4.6 Chromatograms of a) phenolic acid extract from fruits of bitter melon with subcritical water extraction b) 4 phenolic acids standards.

Table 4.1 Phenolic acid constituents of subcritical water extraction at various temperature, constant flow rate = 3 ml/min, pressure = 10 MPa and extraction time = 1 hour

Temp. (°C)	Phenolic acid constituents							
	Catechin		Gentisic acid		Gallic acid		Chlorogenic acid	
	mg/g DW	%[a]	mg/g DW	%[a]	mg/g DW	%[a]	mg/g DW	%[a]
130	3.33	77.66	0.90	11.71	0.02	0.27	0	0
150	36.74	77.00	2.09	4.38	0.12	0.25	0.08	0.16
180	38.37	74.52	2.86	5.55	0.45	0.87	0.09	0.18
200	47.34	85.16	5.91	10.76	0.42	0.76	0.12	0.26

[a] % = mg phenolic acid / mg total phenolic contents

4.3 Antioxidant activity

In addition to the amount of total phenolici compounds and the four main phenolic constituents, the antioxidatant activities of the bitter melon extracts were determined using a simple ABTS (2, 2'- azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical scavenging assay (Thaipong et al., 2006). The antioxidant activity was represented by IC_{50} index which is the concentration of sample producing 50% reduction of the radical absorbance.

As shown in figure 4.7, the IC_{50} values of the subcritical water extracts obtained after one hour at 130 °C, 150 °C, 180 °C, and 200 °C were 4.37, 3.90, 5.42, and 5.49 µg/ml, respectively. The IC_{50} values of the subcritical water extraction at all temperatures were rather low, suggesting high antioxidant activity of the extract. At lower temperatures, the IC_{50} values of the extracts obtained were slightly lower than that obtained at higher temperatures, which suggested the extracts with subcritical water extraction at lower temperature gave higher antioxidant activity than the extracts obtained at higher temperature. On the other hand, no significant difference in

the antioxidant activities of the extracts with the extraction flow rate (data not shown). Even though the total phenolic contents differ significantly in the extracts obtained at different extraction temperatures, the effect of different temperature on antioxidant activities was not significant. This result suggested that that the antioxidant activity of the bitter melon extract could be attributed to combination of the contents of phenolic compounds as well as the other compounds that could be coextracted into subcritical water such as vitamin C, which could easily decomposed at high extraction temperature, causing the decrease in the overall antioxidant activity of the extract, despite the increase in the phenolic content.

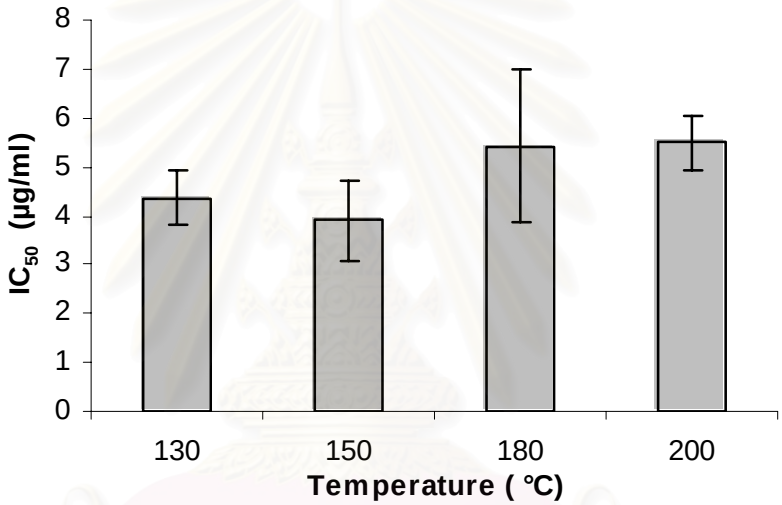


Figure 4.7 Antioxidant activity of subcritical water extracts at various temperatures.

4.4 Comparision of subcritical water extraction and conventional extraction methods

In this section, the performance of subcritical water extraction in terms of the extracted total phenolic contents and phenolic acid constituents, antioxidant activity capacity, as well as the time required for extraction was compared with other conventional methods such as MeOH extraction and soxhlet extraction with water and MeOH.

For soxhlet extraction, two solvents including water and methanol were investigated. The optimal extraction time was found to be 4 hours for both solvents. The results of the total phenolic contents obtained with water and methanol as

extraction solvents at various extraction times are shown in figure 4.8, which indicated that water gave higher amount of the total phenolic compounds than methanol. Similar to water, phenolic compounds are polar compounds containing several OH group, thus causing the compound to be highly soluble in water.

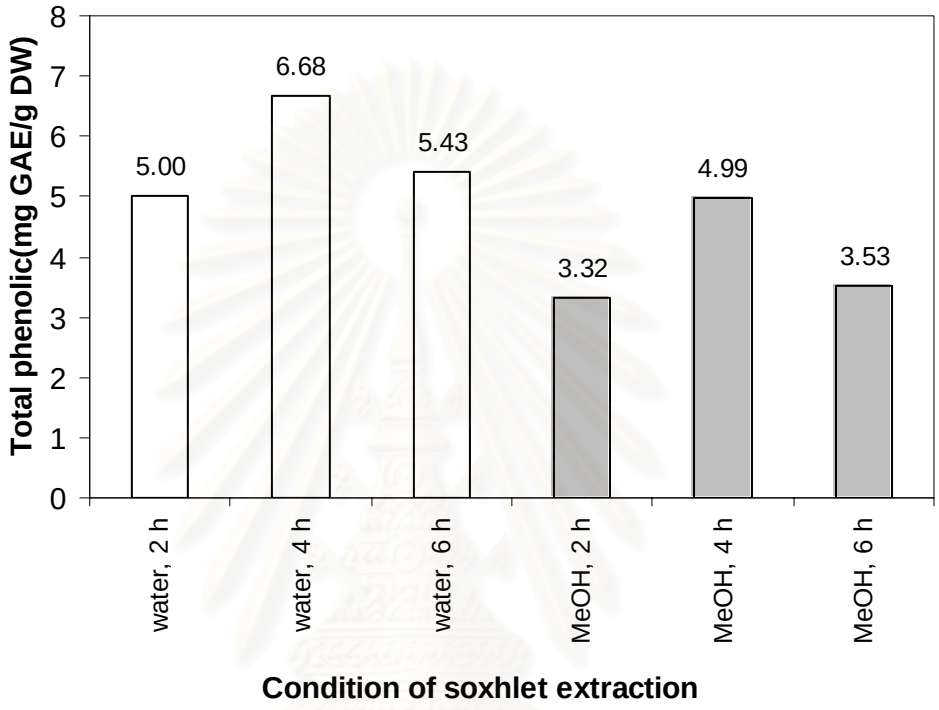


Figure 4.8 The results of total phenolic contents obtained with soxhlet extraction.

Table 4.2 summarizes the extraction results of the amount total phenolic content obtained by subcritical water extraction, solvent extraction, and solvent. At 130 °C, subcritical water extraction gave only slightly higher recovery of phenolic compounds compared with soxhlet extraction with water and MeOH, and with solvent extraction with methanol. The subcritical water extraction at 200 °C gave the highest amount total phenolic contents (>7 times that obtained with soxhlet extraction with water), while requiring smaller amount of solvent and shorter extraction time, that is, one hour for subcritical water extraction, compared with 4 hours and 2 hour, respectively, for soxhlet extraction and solvent extraction. The significantly large amount of compound extracted by SCW was possibly due to the fact that subcritical water caused the hydrolysis of larger phenolic based compounds such as larger phenolic compounds like tannic acid and sinapic acid. Besides these compounds,

lignin which is a component of plant cell walls could possibly be converted to phenolic compounds by hydrolysis and dealkylation reaction. The mechanism is schematically shown in Figure 4.9. Under subcritical water condition, hydrolysis takes place at ether and ester bounds in lignin. Hydrolysis reaction is accelerated by a high ion product of water. From figure 4.9 shown it can be seen that the dealkylation of lignin gives catechol, which was then hydrolysis into phenol (Goto et al., 2007). The results in this study thus clearly demonstrated that the subcritical water extraction is therefore considered a benign alternative for extraction of total phenolic from fruits of bitter melon.

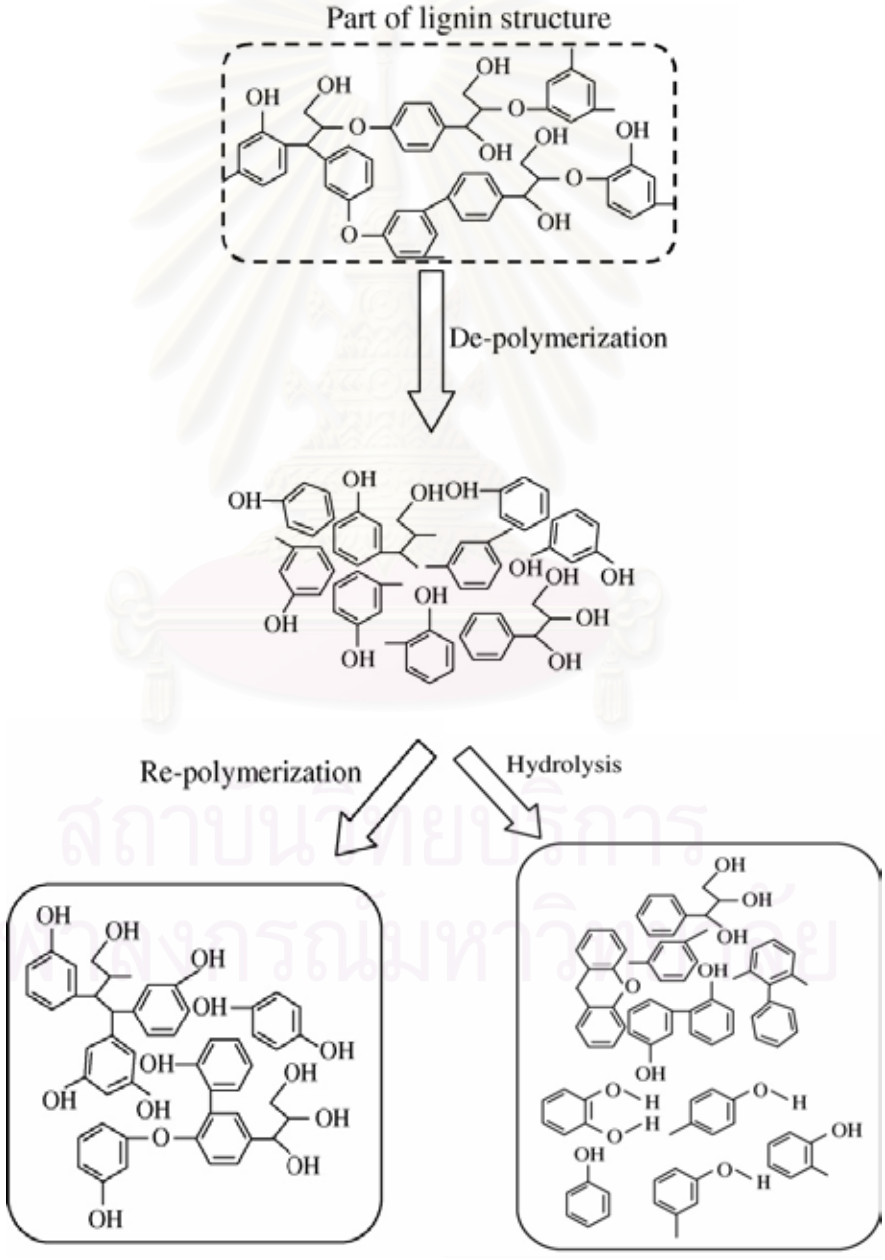


Figure 4.9 The scheme for degradation of lignin under subcritical water condition.

Table 4.2 Comparison of total phenolic contents for different extraction methods.

	SCWE	SCWE	Soxhlet extraction	Solvent extraction	Solvent extraction
Sample (g)	1	1	1	1	1
Extractio solvent	Water	Water	Water	MeOH	MeOH
Pressure (MPa)	10	10	ambient	ambient	ambient
Temperature (°C)	130	200	100	65	65
Flow rate (ml/min)	3	2	-	-	-
Time (hour)	1	1	4	2	2
Solvent volume (ml)	180	120	200	10	200
Total phenolic contents (mg/g DW)	7.69	52.63	6.68	5.37	6.00

In addition to the total phenolic content, the four main constituents of the phenolic acids were determined by HPLC analysis and the results are presented in Table 4.3 which correspond with the results of total phenolic, that is, the amount of phenolic acid constituent obtained by subcritical water extraction was higher than that obtained by soxhlet extraction and solvent extraction methods

Table 4.3 Phenolic acid constituents of extracts from bitter melon fruits obtained with subcritical water extraction, solvent extraction and soxhlet extraction (mg/g dry material).

Phenolic acid	Extraction Methods		
	SCWE [a]	Solvent extraction [b]	Soxhlet extraction [c]
Catechin	46.16	1.61	1.77
Gallic acid	0.51	0.02	0.05
Gentisic acid	5.89	0.42	0.74
Chlorogenic acid	0.14	0.08	0.03

[a] Temperature = 200 °C, Pressure = 10 MPa, Water flow rate = 2 ml/min, Extraction time = 1 hour

[b] Solvent = methanol, Temperature = 100 °C, Extraction time = 2 hours, Solvent volume = 200 ml

[c] Solvent = water, Temperature = 100 °C, Extraction time = 4 hours, Solvent volume = 200 ml

In addition, the antioxidant activities of various extracts obtained by different methods are shown in Table 4.4, presented in terms of IC_{50} values. The IC_{50} value of the extract obtained with SCWE at 200 °C for 2 h (5.49 µg/ml) was lower than that of the extract obtained with solvent extraction at 65 °C for 2 hr (17.34 µg/ml) and soxhlet water extraction for 4 h (15.62 µg/ml), indicating that subcritical water extraction gave the extracts with the highest antioxidant activity. The fact that the antioxidant activity in the extract derived by different technique corresponds with the amount of total phenolic contents implies that the phenolic compounds in bitter melon are key components in the plant that exhibit such activity. Nevertheless, it might also be case that other compounds could be extracted along with the phenolic cotents by subcritical water that also the antioxidation activity.

Table 4.4 Antioxidant activity of extracts obtained by different extraction method presented as IC_{50} values.

Extraction Method	IC_{50} (µg/ml)
Subcritical water extraction [a]	5.49 ± 0.54
Solvent extraction [b]	17.34 ± 2.45
Soxhlet extraction [c]	15.62 ± 1.47

[a] Temperature = 200 °C, Pressure = 10 MPa, Water flow rate = 5 ml/min, Extraction time = 1 hour

[b] Solvent = methanol, Temperature = 100 °C Extraction time = 2 hours, Solvent volume = 200 ml

[c] Solvent = water, Temperature = 100 °C Extraction time = 4 hours, Solvent volume = 200 ml

CHAPTER V

CONCLUSION AND RECOMMENDATION

5.1 Conclusion

1. Bitter melon is a good source of phenolic compounds which possess strong antioxidant activity.
2. The amount of total phenolic contents in the extracts increased as temperature increased.
3. Water is a suitable and environmentally benign solvent for the extraction of total phenolic.
4. In this study, the most suitable condition for subcritical water extraction of total phenolic was at extraction temperature of 150-200 °C, water flow rate of 2 ml/min, and pressure of 10 MPa for a hour.
5. The IC_{50} value of the extraction obtained by subcritical water extraction was found to be lower than the extracts obtained by soxhlet extraction and solvent extraction which means that the extract by subcritical water extraction had higher antioxidant activity than the extract obtained by soxhlet extraction and solvent extraction.
6. Antioxidant activity of the extraction obtained by subcritical water extraction decreased as the temperature increased. Thus, indicating that antioxidant activity was determined not only by their total phenolic contents but depended on other chemical substances in plants that was extracted along with the phenolic contents and that could be degraded with increasing temperature.
7. The extracts obtained by SCWE had higher total phenolic contents and antioxidant activity than that obtained by solvent extraction and soxhlet extraction.
8. The subcritical water extraction is a promising alternative for extraction of the antioxidative phenolic compounds from bitter melon.

5.2 Recommendation

1. For future studies, the bitter melon fruit sample of different particle sizes should be investigated to improve the extraction rate.
2. Extraction could be carried out sequentially with increasing temperatures, to selectively extract specific phenolic compounds.
3. Other phenolic acids in the fruit of bitter melon such as protocatechuic acid, vanillic acid, caffeic acid, ferulic acid and syringic acid could be analyzed and the correlation could be made for the amount of each phenolic acid and antioxidant activity of the extract.
4. To reduce the energy required for subcritical water extraction, the extraction temperature might be reduced employing a suitable surfactant.

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APPENDICES

APPENDIX A

EXPERIMENTAL AND DATA ANALYSIS

A-1 Standard calibration curve of gallic acid

Table A-1 Standard calibration curve data of gallic acid in water

Concentration of gallic acid (mg/ml)	Absorbance at 750 nm		
	Exp. 1	Exp. 2	Average
1.500	0.1.220	1.208	1.214
1.000	0.900	0.970	0.935
0.500	0.455	0.431	0.443
0.250	0.200	0.200	0.200
0.125	0.095	0.065	0.080
0.06250	0.060	0.054	0.058
0.03125	0.033	0.033	0.033
0.01563	0.024	0.026	0.025
0.00781	0.018	0.012	0.015
0.00391	0.015	0.011	0.013
0.00195	0.007	0.009	0.008
0.000976563	0.004	0.004	0.004

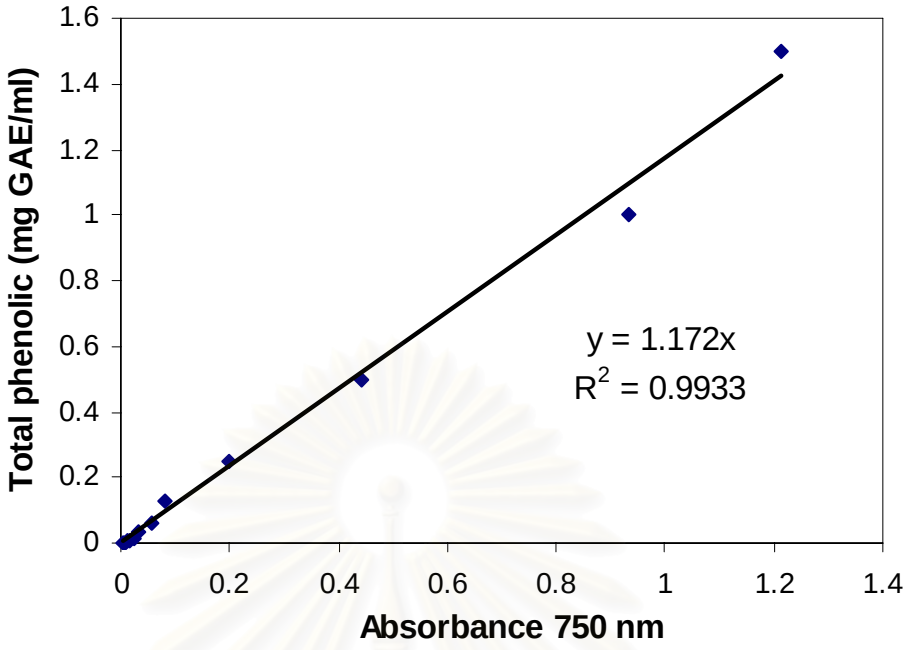

Figure A-1 Standard calibration curve of gallic acid in water

Table A-1 Standard calibration curve data of gallic acid in methanol

Concentration of gallic acid (mg/ml)	Absorbance at 750 nm		
	Exp. 1	Exp. 2	Average
0	0	0	0
0.05	0.054	0.050	0.052
0.1	0.095	0.97	0.096
0.15	0.159	0.159	0.159
0.25	0.258	0.258	0.258
0.5	0.548	0.546	0.547
0.75	0.804	0.814	0.809

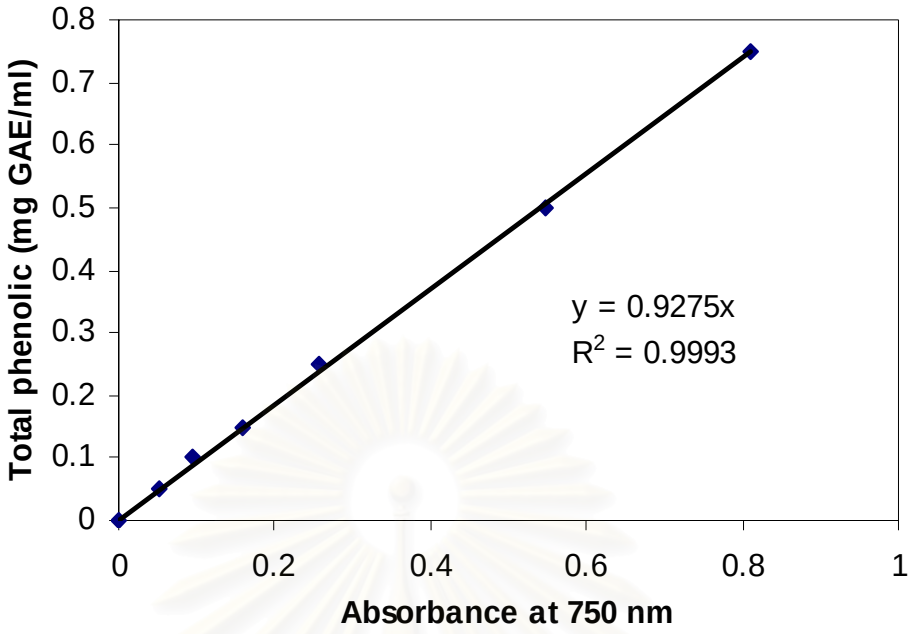

Figure A-1 Standard calibration curve of gallic acid in methanol

A-2 Standard calibration curve for HPLC analysis

Table: A-2.1 Standard calibration curve data of gallic acid

Concentration of gallic acid (mg/ml)	Peak Area (UV detector at 254 nm)
0.05	12503313
0.10	17583244
0.15	25347172
0.20	39651500
0.40	62022904

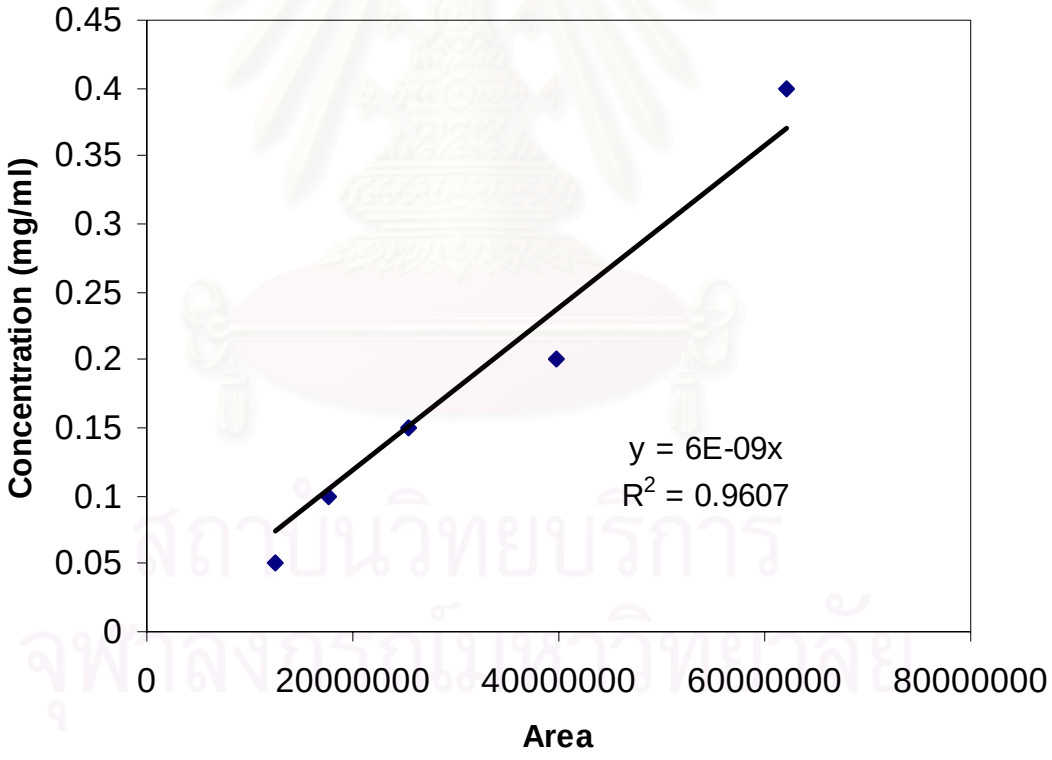

Figure A-2.1 Standard calibration curve of gallic acid

Table: A-2.2 Standard calibration curve data of catechin

Concentration of gallic acid (mg/ml)	Peak Area (UV detector at 254 nm)
0.05	85477
0.10	1122818
0.15	2157079
0.20	3610811
0.30	4400193
0.40	5603441
0.50	6476714
1.50	25630898
3.00	60328012
6.00	104821592

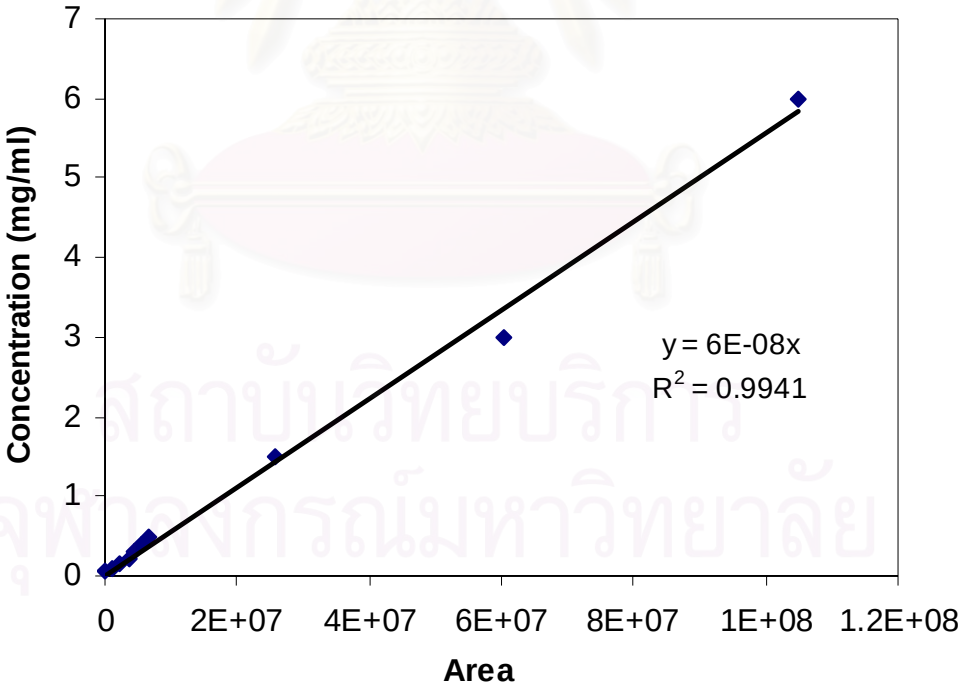

Figure A-2.2 Standard calibration curve of catechin

Table: A-2.3 Standard calibration curve data of gentisic acid

Concentration of gallic acid (mg/ml)	Peak Area (UV detector at 254 nm)
0.025	877265
0.050	1595757
0.075	2461043
0.100	3077180
0.150	4454046
0.200	5728469
0.500	15826421

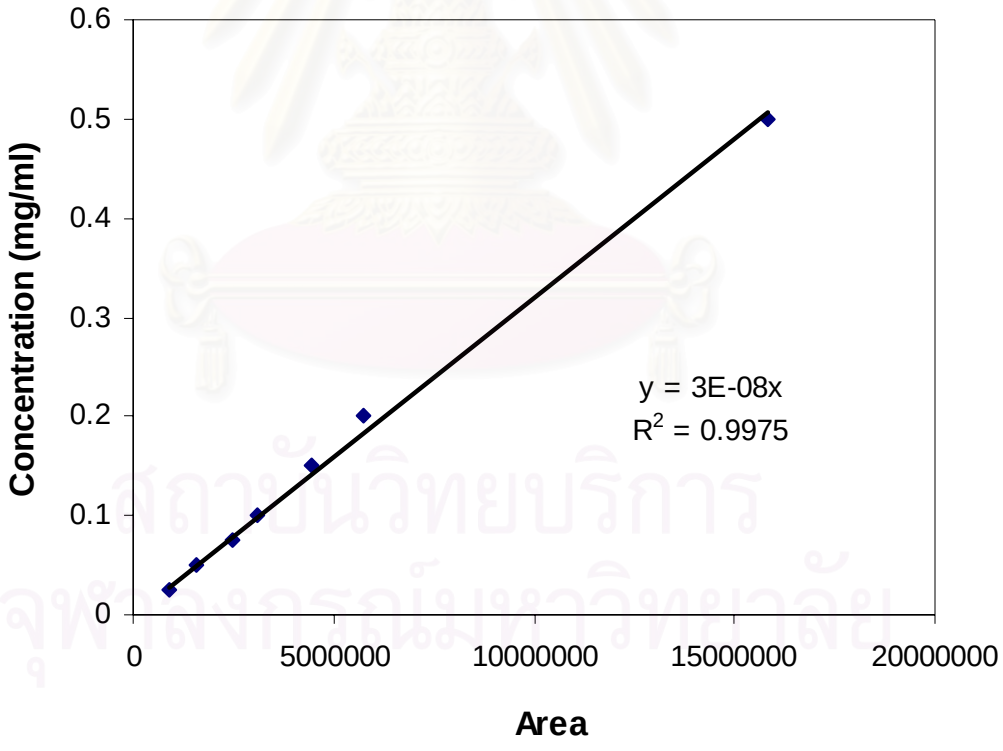

Figure A-2.3 Standard calibration curve of gentisic acid

Table: A-2.4 Standard calibration curve data of chlorogenic acid

Concentration of gallic acid (mg/ml)	Peak Area (UV detector at 254 nm)
0.025	2058119
0.050	3864985
0.100	8635273
0.150	12204195
0.200	16395547

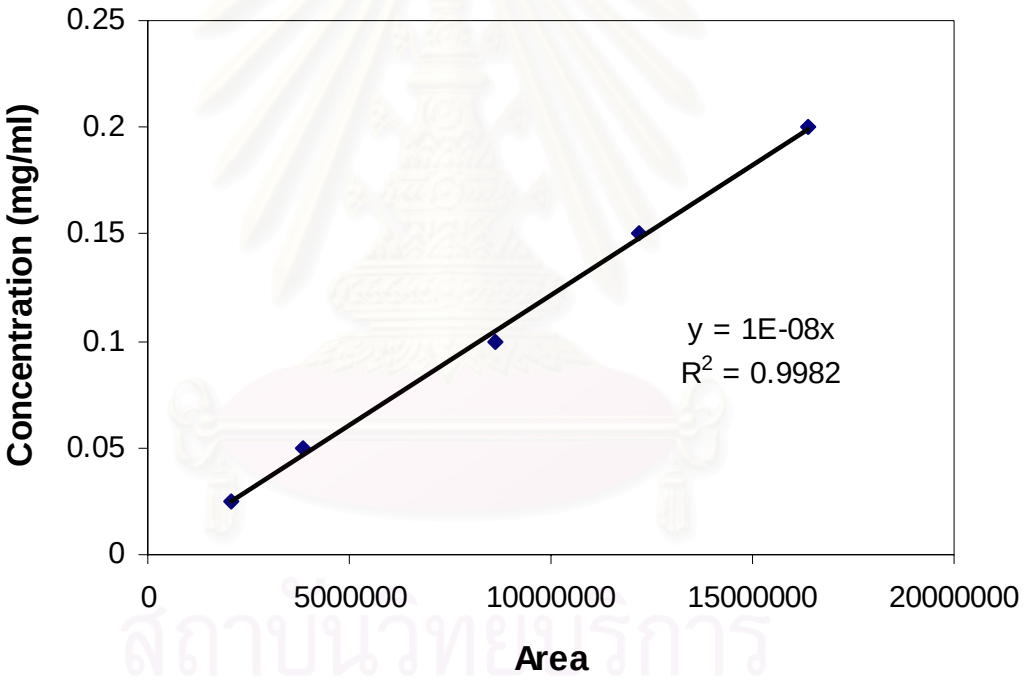

Figure A-2.4 Standard calibration curve of chlorogenic acid

APPENDIX B

EXPERIMENTAL DATA

B-1 Experimental data of total phenolic with subcritical water extraction

Effect of temperature

Table B-1.1 Total phenolic contents of subcritical water extraction at temperature = 130 °C, flow = 3 ml/min, pressure = 10 MPa, extraction time = 2 hours

Time (min)	Total phenolic contents (mg/g DW)			
	Exp. 1	Exp. 2	Average	Std.
10	1.888	1.792	1.840	0.068
20	3.399	3.399	3.399	0
30	4.470	4.483	4.477	0.009
40	5.668	5.699	5.684	0.022
50	6.852	6.812	6.832	0.028
60	7.675	7.700	7.688	0.017
80	9.011	8.919	8.965	0.065
100	9.983	9.962	9.973	0.0148
120	10.690	10.709	10.699	0.0140

Table B-1.2 Total phenolic contents of subcritical water extraction at temperature = 150 °C, flow = 3 ml/min, pressure = 10 MPa, extraction time = 2 hours

Time (min)	Total phenolic contents (mg/g DW)			
	Exp. 1	Exp. 2	Average	Std.
10	36.853	37.609	37.231	0.535
20	41.013	40.668	40.841	0.244
30	42.177	44.114	43.395	1.016
40	45.442	44.842	45.142	0.424
50	46.922	46.080	46.501	0.596
60	48.545	47.871	47.708	0.230
80	48.980	48.920	48.950	0.042
100	48.208	50.474	49.841	0.895
120	50.263	50.661	50.462	0.282

Table B-1.3 Total phenolic contents of subcritical water extraction at temperature = 180 °C, flow = 3 ml/min, pressure = 10 MPa, extraction time = 2 hours

Time (min)	Total phenolic contents (mg/g DW)			
	Exp. 1	Exp. 2	Average	Std.
10	21.057	22.022	21.540	0.683
20	32.887	32.014	32.286	0.3843
30	40.873	40.363	40.618	0.3613
40	45.112	46.002	45.657	0.488
50	48.794	49.201	48.997	0.288
60	51.890	51.097	51.493	0.560
80	52.443	53.543	52.993	0.778
100	54.745	53.937	54.341	0.572
120	55.525	55.525	55.525	0

Table B-1.4 Total phenolic contents of subcritical water extraction at temperature = 200 °C, flow = 3 ml/min, pressure = 10 MPa, extraction time = 2 hours

Time (min)	Total phenolic contents (mg/g DW)			
	Exp. 1	Exp. 2	Average	Std.
10	25.551	25.005	25.278	0.386
20	36.445	36.5875	36.516	0.101
30	44.777	44.9675	44.872	0.135
40	48.896	49.1225	49.009	0.160
50	53.248	52.810	53.028	0.310
60	54.675	55.203	54.939	0.374
80	58.600	59.082	58.841	0.341
100	61.115	60.740	60.927	0.265
120	62.988	62.921	62.954	0.048

Table B-1.5 Total phenolic contents of subcritical water extraction at temperature = 230 °C, flow = 3 ml/min, pressure = 10 MPa, extraction time = 2 hours

Time (min)	Total phenolic contents (mg/g DW)			
	Exp. 1	Exp. 2	Average	Std.
10	11.880	10.855	11.367	0.725
20	19.542	19.763	19.653	0.156
30	25.259	24.899	25.079	0.255
40	28.966	28.434	28.700	0.376
50	31.013	30.676	30.844	0.238
60	32.444	32.808	32.626	0.257
80	33.989	35.388	34.688	0.989
100	36.466	36.473	36.470	0.005
120	38.259	37.938	38.098	0.227

Effect of water flow rate

Table B-1.6 Total phenolic contents of subcritical water extraction at flow = 5 ml/min, temperature = 200 °C, pressure = 10 MPa, extraction time = 2 hours

Time (min)	Total phenolic contents (mg/g DW)			
	Exp. 1	Exp. 2	Average	Std.
10	17.743	18.446	18.094	0.497
20	28.008	29.227	28.618	0.862
30	37.102	38.368	37.735	0.895
40	42.153	43.372	42.763	0.862
50	46.478	47.720	47.099	0.878
60	50.591	51.857	51.224	0.895
80	56.134	57.447	56.790	0.928
100	60.177	61.536	60.857	0.961
120	64.068	65.497	64.783	1.011

Table B-1.7 Total phenolic contents of subcritical water extraction at flow = 4 ml/min, temperature = 200 °C, pressure = 10 MPa, extraction time = 2 hours 30 minutes

Time (min)	Total phenolic contents (mg/g DW)			
	Exp. 1	Exp. 2	Average	Std.
10	23.907	23.086	23.497	0.580
20	34.079	33.188	33.634	0.630
30	41.298	40.454	40.876	0.597
40	46.853	46.079	46.466	0.547
50	50.790	49.923	50.357	0.613
60	53.450	52.607	53.028	0.597
80	57.552	56.779	57.165	0.547
100	60.927	59.966	60.447	0.679
120	63.622	62.732	63.177	0.630
150	66.236	65.345	65.790	0.630

Table B-1.8 Total phenolic contents of subcritical water extraction at flow = 3 ml/min, temperature = 200 °C, pressure = 10 MPa, extraction time = 3 hours 20 minutes

Time (min)	Total phenolic contents (mg/g DW)			
	Exp. 1	Exp. 2	Average	Std.
10	24.961	25.594	25.278	0.447
20	36.153	36.880	36.516	0.514
30	44.474	45.270	44.872	0.563
40	48.634	49.384	49.009	0.530
50	52.607	53.450	53.028	0.597
60	54.470	55.407	54.939	0.663
80	58.372	59.310	58.841	0.663
100	60.318	61.536	60.927	0.862
120	62.404	63.505	62.954	0.779
150	63.728	64.853	64.290	0.796
180	64.888	65.919	65.404	0.729
200	65.861	66.822	66.341	0.679

Table B-1.9 Total phenolic contents of subcritical water extraction at flow = 2 ml/min, temperature = 200 °C, pressure = 10 MPa, extraction time = 5 hours

Time (min)	Total phenolic contents (mg/g DW)			
	Exp. 1	Exp. 2	Average	Std.
10	28.067	28.887	28.477	0.580
20	37.278	38.098	37.688	0.580
30	44.638	45.411	45.024	0.547
40	47.72	48.470	48.095	0.530
50	50.855	52.015	51.435	0.580
60	52.971	54.165	53.568	0.597
80	54.833	55.56	55.196	0.514
100	56.919	57.646	57.282	0.514
120	58.595	59.228	58.911	0.447
150	60.529	61.138	60.833	0.431
180	62.076	62.661	62.369	0.414
210	63.505	64.044	63.775	0.381
240	64.677	65.333	65.005	0.464
270	65.615	66.294	65.955	0.481
300	66.505	67.208	66.857	0.497

B-2 Experimental data of phenolic acid constituents with subcritical water extraction

Effect of temperature

Table B-2.1 Phenolic acid constituents of subcritical water extraction at various temperature, flow = 3 ml/min, pressure = 10 MPa

Temperature (°C)	Time (h)	Phenolic acid constituents (mg/g DW)			
		Catechin	Gentisic acid	Gallic acid	Chlorogenic acid
130	1	3.332	0.900	0.021	0
	2	5.586	1.170	0.025	0
150	1	36.736	2.091	0.119	0.075
	2	38.005	2.907	0.119	0.075
180	1	38.372	2.859	0.446	0.092
	2	41.065	4.144	0.463	0.106
200	1	47.342	5.913	0.420	0.124
	2	52.219	5.913	0.473	0.151

Effect of water flow rate

Table B-2.2 Phenolic acid constituents of subcritical water extraction at various water flow rate, temperature = 200 °C, pressure = 10 MPa, volume extraction = 600 ml

Flow rate (ml/min)	Time (min)	Phenolic acid constituents (mg/g DW)			
		Catechin	Gentisic acid	Gallic acid	Chlorogenic acid
2	300	53.634	6.312	0.519	0.137
3	200	53.026	5.913	0.483	0.151
4	150	52.926	6.248	0.424	0.156
5	120	53.213	6.778	0.423	0.107

B-3 Experimental data of phenolic acid constituents with conventional method

Table B-3.1 Phenolic acid constituents of conventional method

Method	solvent	Time (h)	Phenolic acid constituents (mg/g DW)			
			Catechin	Gentisic acid	Gallic acid	Chlorogenic acid
Soxhlet extraction	Water	2	1.289	0.579	0.027	0
		4	1.772	0.735	0.051	0.025
		6	2.021	1.081	0.009	0
	Methanol	2	1.543	0.644	0.025	0.101
		4	0.489	1.025	1.421	0
		6	1.080	0.737	0.017	0.004
Solvent extraction	Methanol	2	1.611	0.421	0.014	0.076

B-4 Experimental data of antioxidant activity

Antioxidant activities were important properties of phenolic compound. These may be loss when extraction temperature was high or exposure when contacts to light and some organic solvents. Furthermore, antioxidant activities have related with extraction methods. Some methods may be make phenolic compound degradation. This experiment was investigated to compare the antioxidant activities at the various conditions in each method using ABTS method by 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical against free radicals. The $ABTS^{\bullet+}$ stock solution had absorbance of 0.70 ± 0.02 units at 734 nm using the spectrophotometer. The percentage inhibition (PI%) was calculated from reduction absorbance as reaction with ABTS in series at known concentration. The values of PI were calculated using the following equation:

$$PI\ (\%) = [1-(A_t / A_r)] \times 100$$

where A_t and A_r are absorbance of test sample and absorbance of the reference, respectively. For comparing the antioxidant activity of the extracts obtained at various conditions, concentration of sample producing 50% reduction of the radical absorbance (IC_{50}) was used as an index. The IC_{50} values for various extracts were found from the plots of percent inhibition (PI) versus the corresponding concentration of the sample.

For example, antioxidant activity using subcritical water extraction at 130°C, flow rate = 3 ml/min, extraction time = 60 min, pressure = 10 MPa.

Concentration (µg/ml)	Absorbance	PI%
7.78	0.071	88.47
3.89	0.298	51.62
1.94	0.423	31.33
0.97	0.511	17.05
IC_{50} = 4.16	Ref = 0.616	

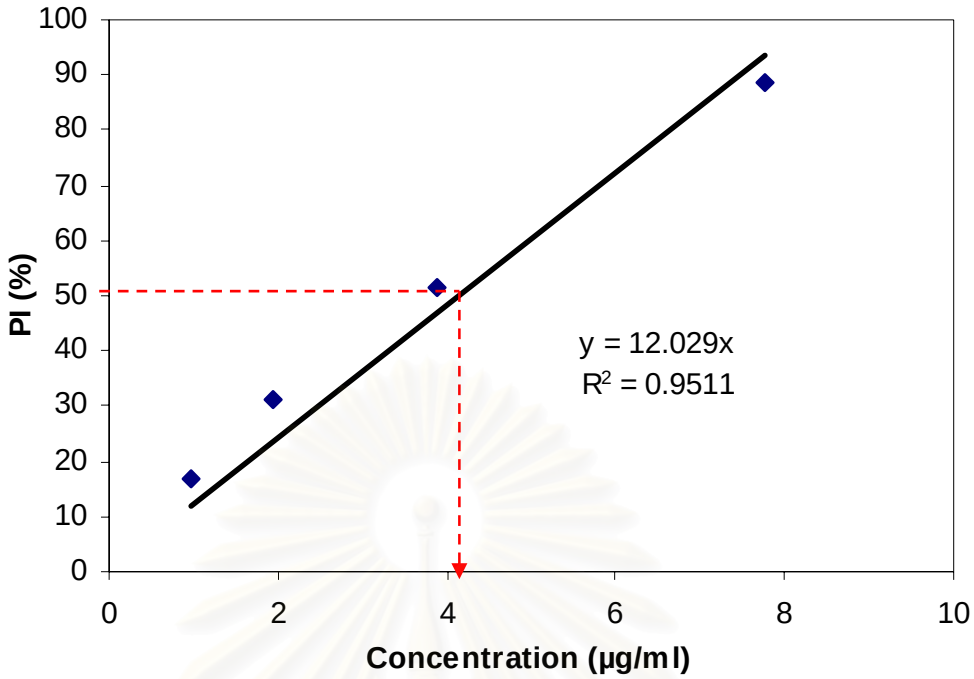

Figure B-4.1 Sample of IC_{50} determination

The plotting between PI (%) and concentration gives the fitted equation was y = 12.029 x which gives IC_{50} was 4.16 µg/ml

Table B-4.1 IC_{50} of subcritical water extraction of bitter melon at various temperature (water flow rate = 3 ml/min)

Time (min)	Temp. = 130 °C		Temp. = 150 °C		Temp. = 180 °C		Temp. = 200 °C	
	Linear equation	IC_{50} (µg/ml)	Linear equation	IC_{50} (µg/ml)	Linear equation	IC_{50} (µg/ml)	Linear equation	IC_{50} (µg/ml)
10	y = 11.284x	4.43	y = 16.174x	3.09	y = 15.525x	3.22	y = 10.521x	4.75
20	y = 12.592x	3.97	y = 12.117x	4.13	y = 13.156x	3.80	y = 9.161x	5.46
30	y = 10.567x	4.73	y = 15.418x	3.24	y = 8.953x	5.59	y = 9.899x	5.05
40	y = 13.560x	3.69	y = 12.590x	3.97	y = 7.987x	6.26	y = 9.049x	5.53
50	y = 9.571x	5.22	y = 13.951x	3.58	y = 7.659x	6.53	y = 9.517x	5.25
60	y = 12.029x	4.16	y = 9.313x	5.37	y = 7.038x	7.10	y = 9.013x	5.55
Average		4.48		3.97		5.42		5.26
SD		0.55		0.82		1.57		0.31

Table B-4.2 IC_{50} of subcritical water extraction of bitter melon at various water flow rate (temperature = 200 °C)

Time (min)	flow = 2 ml/min		flow = 3 ml/min		flow = 4 ml/min		flow = 5 ml/min	
	Linear equation	IC_{50} (µg/ml)	Linear equation	IC_{50} (µg/ml)	Linear equation	IC_{50} (µg/ml)	Linear equation	IC_{50} (µg/ml)
10	y = 9.092x	5.50	y = 10.521x	4.75	y = 10.948x	4.57	y = 7.182x	6.96
20	y = 6.194x	8.07	y = 9.161x	5.46	y = 7.490x	6.68	y = 5.642x	8.86
30	y = 5.398x	9.26	y = 9.899x	5.05	y = 6.032x	8.29	y = 5.652x	8.85
40	y = 4.410x	11.34	y = 9.049x	5.53	y = 5.095x	9.81	y = 5.886x	8.49
50	y = 3.423x	14.61	y = 9.517x	5.25	y = 5.753x	8.69	y = 6.891x	7.26
60	y = 3.879x	12.89	y = 9.013x	5.55	y = 3.981x	12.56	y = 5.398x	9.26
Average		10.28		5.26		8.43		8.28
SD		3.33		0.31		2.72		0.94

APPENDIX C

The 17 th Thailand Chemical Engineering and Applied Chemistry Conference

(TiCHE 17 th)

29-30 October 2007, The Empress Hotel, Chiang mai, Thailand

Extraction of Phenolic Compounds from Fruits of Bitter Melon (*Momordica charantia*) with Subcritical Water Extraction and Antioxidant Activities of These Extracts

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Contributed Paper

Extraction of Phenolic Compounds from Fruits of Bitter Melon (*Momordica charantia*) with Subcritical Water Extraction and Antioxidant Activities of These Extracts

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ABSTRACT

Bitter melon (Momordica charantia) is traditionally known for its medicinal properties such as antidiabetic, anticancer, anti-inflammation, antivirus, and cholesterol lowering effects. It contains many phenolic compounds that may have the potential as antioxidant and antimutagen. Although the value of bitter melon is realized, scientific information on phenolic composition of bitter melon and antioxidant and antimutagenic activities of its extracts from food grade solvents are limited. This study were investigated the total phenolic contents of bitter melon obtained by subcritical water extraction (SCWE) and antioxidant activities of these extracts. The effect of extraction temperature was considered and the results were compared with the extracts obtained by solvent extraction and soxhlet extraction. The total phenolic contents of bitter melon obtained by the SCWE at 130 °C, the SCWE at 150 °C, SCWE at 180 °C, the SCWE at 200°C, the solvent extraction, and soxhlet extraction were 10.571, 25.219, 42.915, 48.177, 7.743, and 4.992 mg gallic acid equivalents (GAE)/g dry weight (DW), respectively. Overall, the extracts obtained by SCWE were significantly higher than solvent extraction and soxhlet extraction. The

main phenolic acid contained in bitter melon was gallic acid. The phenolic acid was calculated from HPLC analysis of the extracts that the gallic acids of the extracts from bitter melon obtained by the SCWE at 130 °C, the SCWE at 150 °C, SCWE at 180 °C, the SCWE at 200 °C, the solvent extraction, and soxhlet extraction were 0.0913, 0.3169, 0.5502, 0.6462, 0.0271, and .0120 mg/g DW, respectively. Antioxidant was represented by IC50 index which the IC50 values of extract obtained with the SCWE at 130 °C, the SCWE at 150 °C, the SCWE at 180 °C, the SCWE at 200 °C, the solvent extraction, and soxhlet extraction were 4.480, 3.970, 5.757, 5.720, 8.426, and 6.107 μg/ml, respectively. The IC_{50} values of the SCWE at all temperatures were lower than that of solvent extraction and soxhlet extraction, which means SCWE gave the extracts with higher antioxidant activity.

Keywords: Subcritical water extraction, Bitter melon, Momordica charantia, Antioxidant, Phenolic.

1. INTRODUCTION

Phenolic compounds are categorized as secondary metabolites essential for growth and reproduction of plants. They are known as hydrophilic antioxidants, and are produced as a response for defending injured plants against pathogens. They potentially show antioxidant, antimutagen, antitumor, anti-inflammatory, and anticarcinogenic properties [1]. In general, deep–colored vegetables and fruits including bitter melon are good sources of phenolic compounds.

Bitter melon (Momordica charantia) or commonly as Ma-ra-khee-nok, is an herbal plant grown in Thailand and other tropical regions. It is traditionally known for its medicinal properties such as antidiabetic, antitumorous, anticancer, anti inflamatory, antiviral, and cholesterol lowering effects etc. [2, 3, 4]. The main constituents of bitter melon which are responsible for these effects are such as triterpene, proteid, steroid, alkaloid, inorganic, lipid, and phenolic compounds [3]. The protein in bitter melon including protein MAP-30, alpha–momorcharin, and beta–momorcharin were shown to have the ability for fighting against HIV [5].A steroid, charantin, contained mainly in the aerial parts, has been proven for its antidiabetic activity [6]. The phenolic compounds from bitter melon

extracted by solvent extraction were reported to exhibit antioxidant activity [7]. Recently, subcritical and pressurized fluids have become an interesting alternative in the extraction of herbal plants and the most benign and available solvent for pressurized solvent extraction is water. The subcritical fluid extraction is a technique for extraction of plants based on the use of solvent whose temperature lies between boiling and critical temperatures as an extractant, and at high pressure enough to maintain the liquid state [8]. The important advantages of this method are its simplicity, reduced extraction time, higher quality of the extract, lower cost of the extracting agent, and an environmentally friendly technique [9]. Extraction with subcritical fluid using water as a solvent has been shown to be effective for several compounds, such as essential oils from majoram [10], oregano [8], and coriander seeds [11] etc. Moreover, Jesada showed that charantin which is a fat soluble steroid could be successfully extracted benignly from fruit of bitter melon using polar solvents at subcritical condition such as acetone and ethanol [12]. Although the authors showed that subcritical water was not suitable for extraction of charantin from fruit of bitter melon, water is an adequately good solvent for extraction of phenolic compounds from bitter melon due to the higher solubility of phenolic compounds in water. In this study, we investigated the total phenolic contents of bitter melon obtained by subcritical water extraction. The effect of extraction temperature was considered and the results were compared with the extracts obtained by solvent extraction and soxhlet extraction. Moreover, the antioxidant activities of these extracts were determined.

2. MATERIALS AND METHODS

2.1 Materials and chemicals

The fruits of bitter melon were obtained from the market in Bangkok, Thailand. Gallic acid was obtained from Sigma Chemical Co. (St Louis, Mo, USA). Methanol was purchased from Fisher Scientific, UK. Water used in the experiments was distilled and deionized water.

2.2 Sample preparation

The fruits of bitter melon were cleaned and cut into small pieces, and then oven dried at 50 ^{o}C for a day. The dried sample was then pulverized into fine powder in a grinder, which was then stored at 4 ^{o}C until use.

2.3 Subcritical water extraction (SCWE)

The subcritical water extraction was carried out in a laboratory-built apparatus shown in Figure 1. The extraction system consisted of two HPLC pumps (PU 980, JASCO, Japan) used to deliver the water and solvent through the system at constant flow rates, a degassing instrument (ERC 3215, CE, Japan), an oven (D63450, HARAEUS, Germany), where the extraction vessel (10 ml, Thar Design, USA) was mounted, a pressure gauge, and a back pressure regulator valve (AKICO, Japan). All connections were made with stainless steel capillaries (1/16 inch inside diameter).

Water was passed through a degassing equipment to remove dissolved oxygen, the degassed water was then delivered to preheating coil, made from 3 m length stainless steel tubing, installed in the oven, and delivered through to the extraction vessel, which was preloaded with 1.0 g of sample. The back pressure regulator valve placed at the outlet of the extraction system was used to maintain the system pressure to ensure that the water was in liquid state at the temperatures tested. Before starting the extraction, all connections were checked for possible leakage. The second pump was then turned on to deliver ethanol at constant flow rate of 1 ml/min to wash off any residual product in the outlet line behind the extractor. The extract was cooled in a coil immersed in a water bath to prevent possible product degradation, and the extract was collected in fractions in sample collecting vials every 10 minutes in a first hour and every 20 minutes in the second hour. After extraction, the compound remained in the sample residue was extracted repeatedly in 30 ml methanol until the extract was clear. The samples were then evaporated under vacuum to remove the water and methanol until volume of the samples were remained about 10 ml and stored at 4 °C until analysis.

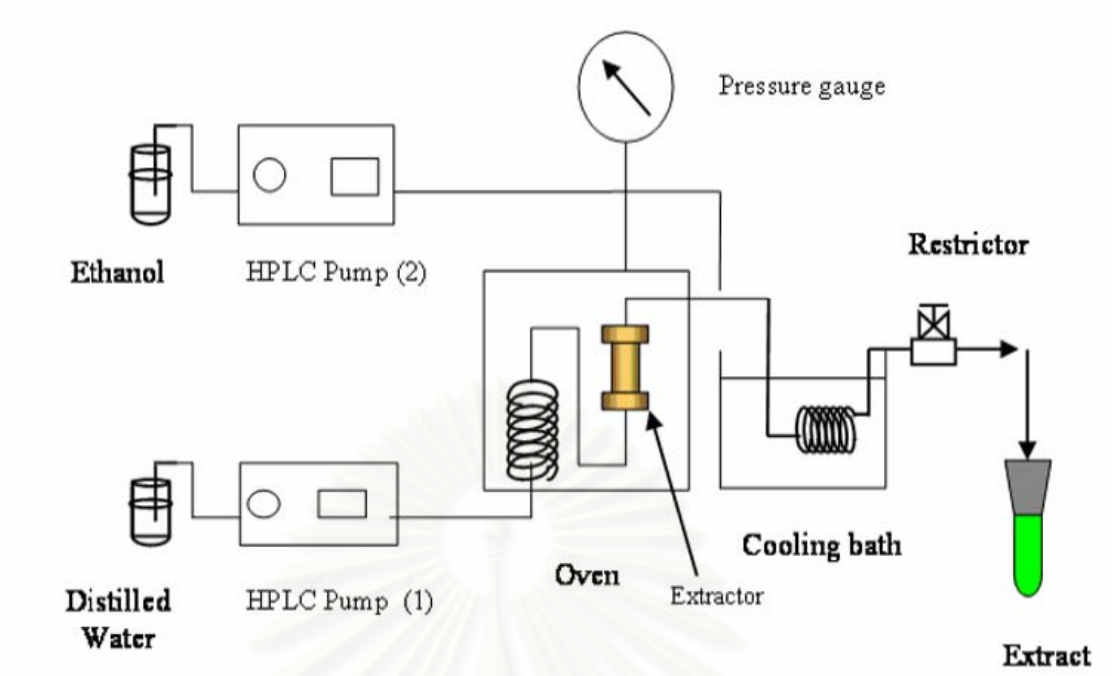

Figure 1 Diagram of experimental setup subcritical water extraction.

2.4 Solvent extraction

One gram of the fine ground sample was weighed into a test tube and 10 ml methanol was added and the sample was then extracted for 2 h in an ultrasonic bath (275DAG, Crest, Malaysia) at 65 ^{o}C. After extraction, the sample was cooled to room temperature and then centrifuged at 1500 rpm for 15 min. The sample residue was extracted repeatedly with 30 ml of methanol. The extract was filtered with filter paper (No.4, Whatman, England) and evaporated under vacuum to remove the methanol. Then the concentrated extract was stored at 4 ^{o}C until use.

2.5 Soxhlet extraction

One gram of the fine ground sample was weighed into a thimble and was extracted with 200 ml of methanol for 4 hr. The sample residue was removed from the thimble and extracted repeatedly with 30 ml of methanol using ultrasonication. The extract was filtered with filter paper (No.4, Whatman, England) and evaporated under vacuum to remove the methanol. Then the concentrated extract was stored at 4 ^{o}C until analysis.

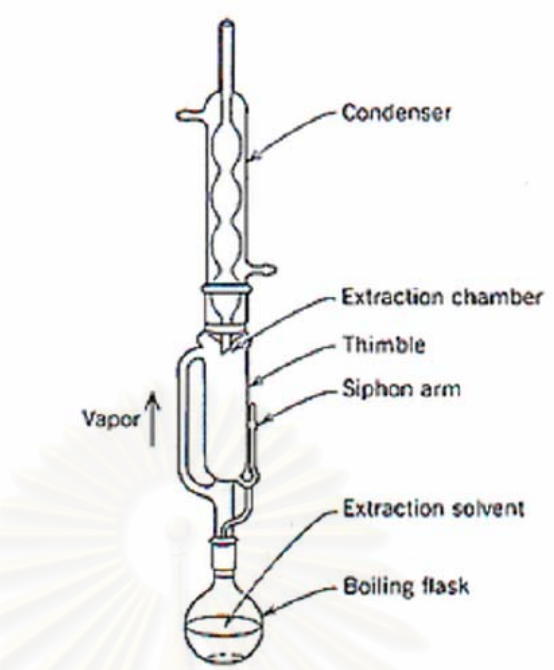

Figure 2 Diagram of soxhlet apparatus.

2.6 Total phenolic contents analysis

The total phenolic content was analyzedwith the Folin-Ciocalteau method modified from [13]. 0.1 ml of the extract was mixed with 2.8 ml of distilled water, 0.1 ml of 50% Folin-Ciocalteau reagent, and 2 ml of Na2CO3 (2 g/100ml). The mixture was incubated at room temperature for 30 minutes. The mixture absorbance was measured spectrophotometrically at wavelength 750 nm. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligrams per gram dry material. Chiang Mai J. Sci. 2008; 35(1) 127

2.7 Phenolic acid constituent analysis

The phenolic acid constituents of the extracts were determined using HPLC by modified from the method of Cai et al. [14]. HPLC were performed with a C-18 Inertsil ODS-3 column (5 µm particle, 4.6 x 250 mm ID) and equipped with UV detector. The UV detector absorbance was monitored at 254 nm. The mobile phases consisted of solvent A (0.1% trifluoroacetic acid in acetonitrile), solvent B (0.1% trifluoroacetic acid in HPLC grade water), and solvent C (100% methanol, HPLC grade). Flow rate was set at 1.0 ml/ min, and column temperature was maintained at 37 °C throughout of the

test. The initial solvent composition was 0% solvent A and 100% solvent B. A linear gradient was used to increase solvent A from 0% to 10% within 7 minutes. This solvent composition was maintained at an isocratic flow for 3 min. The solvent A was then increased from 10% to 40% using a 20-min linear gradient. This composition was maintained for 2 min and returned to the initial composition in 3 min. Solvent C was used for washing the column after each run. The sample injection volume was 10 µl. The concentrations of phenolic acids in the sample were calculated from standard curves, from a plot of peak areas versus concentrations for a series of standardsolutions.

2.8 Antioxidant activity determination

Antioxidatant activity was detemined using ABTS (2, 2'-azinobis-(3 EthylbenzoThiazoline 6-sulfonic acid) radical scavenging assay which was carried out following the method of Re et al. [15] with some modifications. The extract was diluted in series in water and each diluted samples were added with the ABTSo+ stock solution ,which included 7mM ABTS and 2.45 mM potassium persulfate, with the volume ratio of 1:10 (sample solution: ABTSo+ stock solution). The ABTSo+ stock solution had absorbance of 0.70 0.02 units at 734 nm using the spectrophotometer. The solutions were mixed using a vortex and the mixtures were incubated at room temperature for 10 minutes, and then the absorbance was taken at 734 nm using the spectrophotometer.

For comparing the antioxidant activity of the extracts obtained at various conditions, concentration of sample producing 50% reduction of the radical absorbance (IC_{50}) was used as an index. The IC_{50} values for various extracts were found from the plots of percent inhibition (PI) versus the corresponding concentration of the sample. The values of PI were calculated using the following equation:

$$PI\ (\%) = [1-(At\ /Ar)] \times 100 \qquad (1)$$

Where At and Ar are absorbance of test sample and absorbance of the reference, respectively.

3. RESULTS AND DISCUSSION

3.1 Total phenolic and phenolic acid contents analysis

The total phenolic contents of the extracts obtained by the SCWE at 130 °C, the SCWE at 150 °C, SCWE at 180 °C, the SCWE at 200 °C, the solvent extraction, and soxhlet

extraction were 10.571, 25.219, 42.915, 48.177, 7.743, and 4.992 mg GAE/g dry weight (DW), respectively. Overall, the extracts obtained by SCWE were significantly higher than solvent extraction and soxhlet extraction as shown in Fig. 3. Temperature is expected to have a significant effect on extraction efficiency. The amount of the total phenolic contents of the extracts obtained by the SCWE increased when the temperature increased and the total phenolic contents of each collected samples were found the most among in 10 min as shown in Figure 4.

The main phenolic acid contained inbitter melon was gallic acid. The phenolic acid was calculated from HPLC analysis of the extracted that this results indicated the extracts obtained by SCWE for 2 hr contained higher amount of gallic acids were than that obtained by solvent extraction at 65 °C for 2 hr and soxhlet extraction for 4 h. The gallic acids of the extracts from bitter melon obtained by the SCWE at 130 °C, the SCWE at 150 °C, SCWE at 180 °C, the SCWE at 200 °C, the solvent extraction, and soxhlet extraction were 0.0913, 0.3169, 0.5502, 0.6462, 0.0271, and 0.0120 mg/g DW, respectively.

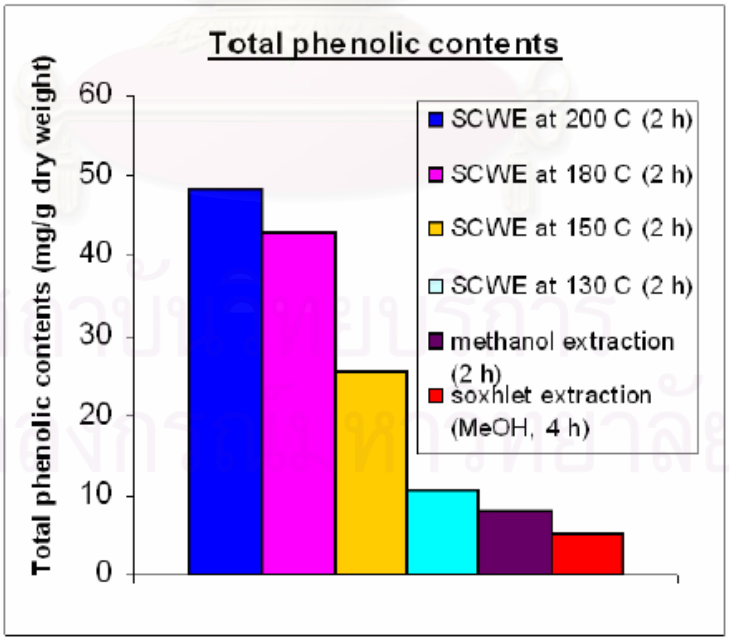

Figure 3 Total phenolic contents of bitter melon extracts by the SCWE at 130 °C, the SCWE at 150 °C, the SCWE at 180 °C, the SCWE at 200 °C, the solvent extraction and soxhlet extraction, respectively.

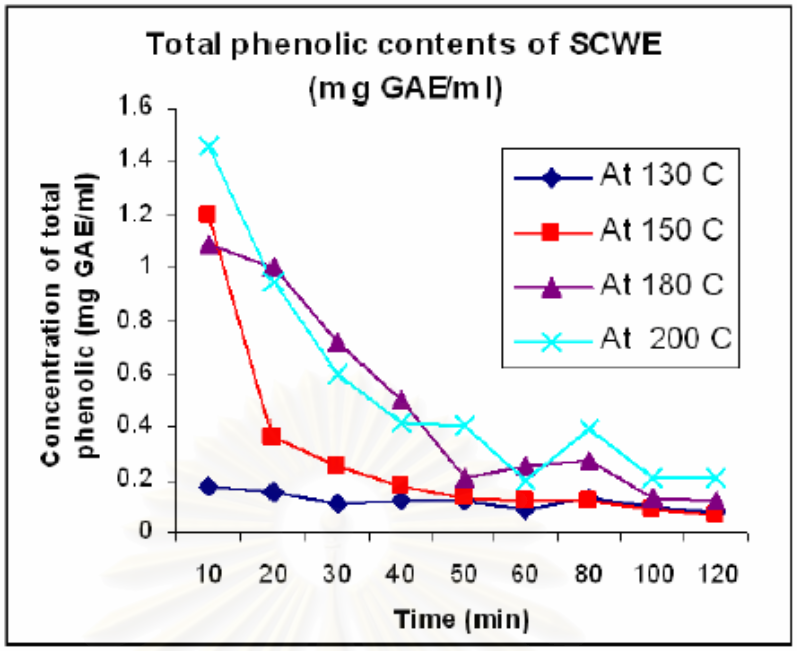

Figure 4 Effect of temperature on extraction efficiency of SCWE.

3.2 Antioxidant activity

Antioxidant was represented by IC_{50} index which is the concentration of sample producing 50% reduction of the radical absorbance. The IC_{50} values of each collected sample of the SCWE at 130 °C, at 150 °C, at 180 °C, at 200 °C for 2 h and IC50 values of the extracts obtained with solvent extraction at 65 °C for 2 hr and soxhlet extraction for 4 h were 4.480, 3.970, 5.757, 5.720, 8.426, and 6.107 µg/ml, respectively as shown in Figure 5.

The IC_{50} values of the SCWE at all temperatures were lower than that of solvent extraction and soxhlet extraction, which means SCWE gave the extracts with higherantioxidant activity. There was no significant difference in the antioxidant activities of the SCWE from bitter extracted with different extraction time but the extraction temperature gave significantly different results as shown in Figure 6. Even though the total phenolic contents of the extracts obtained by different extraction methods and extraction temperatures were significantly different, their different antioxidant activities indicated that antioxidant activity was determined not only by their total phenolic contents but also by other compounds extracted from the sample.

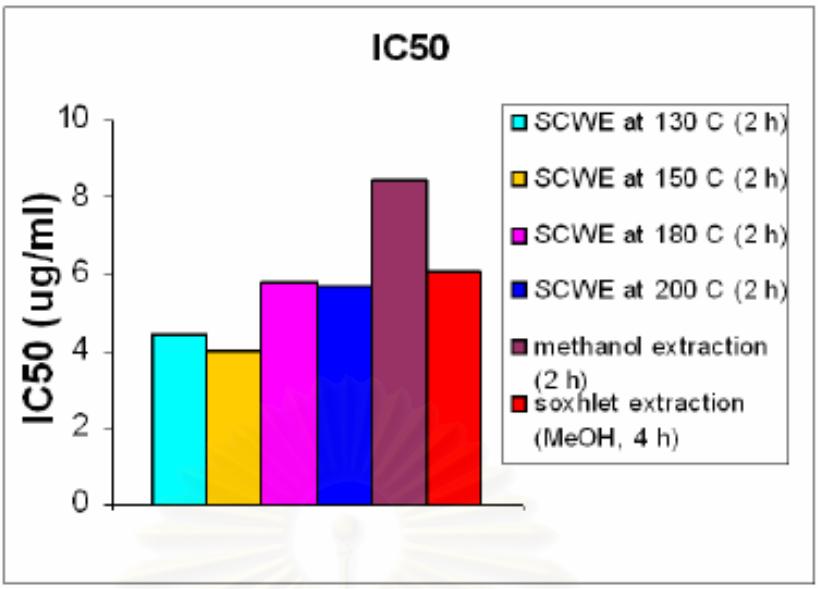

Figure 5 Antioxidant activity (IC_{50}) of the bitter melon extracts by the SCWE at 130 °C, the SCWE at 150 °C, the SCWE at 180 °C, the SCWE at 200 °C, the solvent extraction and soxhlet extraction, respectively.

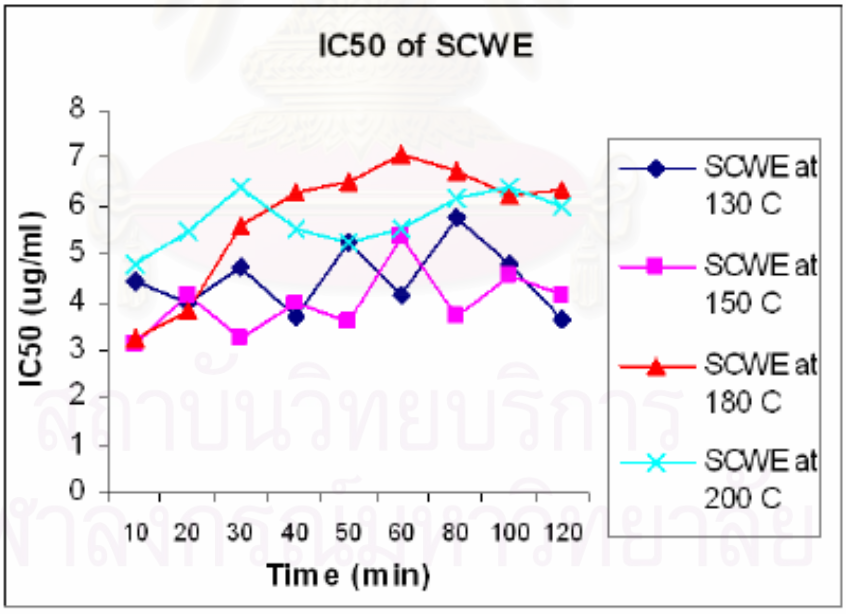

Figure 6 Effect of temperature on antioxidant activity (IC_{50}) of the bitter melon extracts by the SCWE.

4. CONCLUSIONS

Bitter melon is a good source of phenolic compounds which possess potent antioxidant activity. The extracts obtained by SCWE had higher total phenolic contents and antioxidant activity than that obtained by solvent extraction and soxhlet extraction. And these results indicated that the SCWE is a promising alternative for extraction of the antioxidative phenolic compounds from bitter melon.

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