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SUBCRITICAL WATER EXTRACTION OF POLYPHENOLIC COMPOUNDS FROM *TERMINALIA CHEBULA* FRUITS

Miss Phattarakorn Rangsriwong

A Thesis Submitted in Partial Fulfillment of the Requirements
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สมอไทยเป็นพืชที่มีสรรพคุณในการบำบัดรักษาโรคได้มากมาย โดยส่วนที่นิยมนำมาใช้คือส่วนผลเนื่องจากมีสารพอลิฟีนอลิกเป็นองค์ประกอบหลักโดยเฉพาะกรดแกลลิค กรดแอลลาจิก และคอริลาจิน สารเหล่านี้มีคุณสมบัติหลายประการที่เป็นประโยชน์ต่อการนำไปใช้เช่น ประสิทธิภาพในการรักษาโรคมะเร็ง และยังเป็นสารที่มีคุณสมบัติในการต่อต้านอนุมูลอิสระและต่อต้านเชื้อจุลินทรีย์ได้อีกด้วย งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อทำการทดสอบหาสภาวะที่เหมาะสมของอุณหภูมิในช่วง 120-220 องศาเซลเซียส และอัตราการไหลของน้ำที่ใช้สกัด 2-4 มิลลิลิตร/นาที ที่ความดันคงที่ 4 เมกะปาสคาลในการสกัดสารพอลิฟีนอลิกได้แก่ กรดแกลลิค กรดแอลลาจิก และคอริลาจิน จากผลสมอไทยโดยใช้น้ำกึ่งวิกฤติ และทำการเปรียบเทียบประสิทธิภาพของวิธีการสกัดด้วยน้ำกึ่งวิกฤติกับวิธีการสกัดแบบซอคเลต และการสกัดโดยใช้น้ำที่ความดันปกติ รวมไปถึงวิเคราะห์หาปริมาณสารพอลิฟีนอลิกและทดสอบคุณภาพของสารสกัดในรูปของคุณสมบัติในการต่อต้านอนุมูลอิสระ พบว่าความสามารถในการสกัดกรดแกลลิคและกรดแอลลาจิกสูงขึ้นเมื่ออุณหภูมิเพิ่มขึ้นและได้ปริมาณสูงสุดที่ 180 องศาเซลเซียส หลังจากนั้นสารจะเกิดการสลายตัว ส่วนคอริลาจินจะเกิดการสลายตัวที่อุณหภูมิสูงกว่า 150 องศาเซลเซียส เมื่อพิจารณาผลของอัตราการไหลของน้ำที่ใช้สกัดที่อุณหภูมิคงที่ 180 องศาเซลเซียส เห็นได้ว่าการสกัดกรดแกลลิคและกรดแอลลาจิกเพิ่มขึ้นตามอัตราการไหล โดยมีค่าสูงสุดที่ 4 มิลลิลิตร/นาที และคอริลาจินสูงสุดที่ 3 มิลลิลิตร/นาที ซึ่งเมื่อเปรียบเทียบกับวิธีการสกัดแบบทั่วไปพบว่าอุณหภูมิของน้ำกึ่งวิกฤติที่เพิ่มสูงขึ้นส่งผลให้ปริมาณสารพอลิฟีนอลิกโดยรวมลดลง แต่ไม่ส่งผลต่อความสามารถในการต่อต้านอนุมูลอิสระของสารสกัด นอกจากนี้สารสกัดที่ได้ยังมีปริมาณมากกว่าวิธีการสกัดแบบทั่วไปอีกด้วย ดังนั้นสรุปได้ว่าน้ำสภาวะกึ่งวิกฤติมีประสิทธิภาพในการสกัดสารฟีนอลิกจากผลสมอไทยทั้งในแง่ของปริมาณและคุณภาพของสารที่สกัดได้ โดยสภาวะการสกัดที่เหมาะสมคือที่อุณหภูมิ 180 องศาเซลเซียส ที่อัตราการไหล 4 มิลลิลิตร/นาที

ภาควิชา.....วิศวกรรมเคมี.....ลายมือชื่อนิสิต.....ภัทรกร รังษีวงศ์.....

สาขาวิชา.....วิศวกรรมเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....อาทิวรรณ โชติพฤกษ์.....

ปีการศึกษา.....2550.....

4970495421 : MAJOR CHEMICAL ENGINEERING DEPARTMENT
KEY WORD: SUBCRITICAL WATER / *TERMINALIA CHEBULA* / GALLIC ACID / ELLAGIC ACID / CORILAGIN / ANTIOXIDANT

PHATTARAKORN RANGSRIWONG : SUBCRITICAL WATER EXTRACTION OF POLYPHENOLIC COMPOUNDS FROM *TERMINALIA CHEBULA* FRUITS. THESIS ADVISOR : ASST. PROF. ARTIWAN SHOTIPRUK, Ph.D., 71 pp.

Fresh or dried fruit of *Terminalia chebula* Retz. is commonly used as herbal medicine as it contains various phytochemicals including gallic acid (GA), ellagic acid (EA), and corilagin (CG). These polyphenolic compounds also exhibited therapeutic properties such as antioxidant, anticarcinogenic, and antimicrobial activities. This study investigated the extraction of polyphenolic compounds such as gallic acid, ellagic acid, and corilagin from *T. chebula* fruits by subcritical water extraction (SWE). We examined the effect of extraction temperature (120-220°C) and water flow rates (2-4 ml/min) at the pressure of 4 MPa on the amounts of compounds extracted and determined the suitable conditions for SWE of these compounds. In addition, the total phenolic contents and antioxidant activities of the extracts were analyzed and compared to those obtained by water extraction and soxhlet extraction. The results showed that the amount of GA and EA increased with an increase in temperature up to 180°C, where the maximum amounts were obtained. The temperature higher than 180°C caused the loss of these products due to thermal degradation. For CG, the degradation was also observed and it occurred at even lower temperature (>150°C). In addition, flow rate was found to affect the extraction behavior. At a fixed temperature of 180°C, gallic acid and ellagic acid increased with an increase in volumetric flow rate up to 4 ml/min. For corilagin, the highest amount of this extract is obtained at 3 ml/min. Compared to other conventional extraction methods, SWE could extract higher amounts of products. Moreover, although higher temperature of SWE caused lower total phenolic contents, the extracts obtained by SWE indeed had higher antioxidant activities than those obtained with conventional methods. It can therefore be concluded that SWE could effectively extract a considerable amount of phenolic compounds from *T. chebula* fruits with high selectivity and quality, and the suitable condition was at temperature of 180°C and water flow rate of 4 ml/min.

Department.....Chemical Engineering.....Student's signature.....
Field of study...Chemical Engineering.....Advisor's signature.....
Academic year.....2007.....

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CHAPTER I

INTRODUCTION

1.1 Rationale

Terminalia chebula Retz. is a native plant in India and Southeast Asia. In Thailand, the plant is commonly known as *Samor thai*. There has been considerable interest in this plant in the field of herbal medicine due to its numerous phytochemicals contained in different parts of the plant. The most important part of *Samor thai* is the fruit, either fresh or dried, which has been reported to have the strongest antioxidant capacity and the highest phenolic contents over 133 Indian medicinal plants (Surveswarun et al., 2007). The fruits of *T. chebula* contain several polyphenolic compounds including gallic acid (GA), ellagic acid (EA), and corilagin (CG) (Worasuttayangkurn, 2001). These compounds are associated with a lower risk of various chronic diseases. For example, gallic acid helped to promote apoptosis, attenuated G0/G1 to the S phase, and COX in HL-60 leukemia cells (Madlener et al., 2006), caused growth inhibition and apoptotic death of human DU-145 prostate cancer cells (Veluri et al., 2006), and induced apoptotic cell death in cancer cell lines including human stomach cancer (KATO III) and human colon adenocarcinoma (COLO 205) (Yoshioka et al., 2000). The compound was also found to show moderate *in vitro* cytotoxicity against cultured human tumor cell lines including A-549, SK-OV-3, SK-MEL-2, XF-389, and HCT-15 (Lee et al., 1995). Ellagic acid helped to decrease the cell viability, inhibited cell proliferation, and induced cell death of several malignant cell lines (MCF-7, S115, HOS-1, PC-3, and PNT1A) (Saleem et al., 2002). It was also shown to be able to induce p53/p21 expression, G1 arrest and apoptosis in bladder cancer cells (Li et al., 2005) and reduced pancreatic stellate cell inflammation (Masamune et al., 2005). In addition, the compound was found to have antimicrobial effects on bacterial human pathogen (*C. accolans*) as well as on human pathogenic yeast (*C. albicans*) (Fogliani et al., 2005). Corilagin is a phenolic compound found to be protective against GalN/LPS-induced liver injury through suppression of oxidative stress and apoptosis (Kinoshita et al., 2007). The compound could potently inhibit HIV-1 replication in HeLa $CD4^+$ cells (Notka et al.,

2003), and exhibited strong antioxidative activity as active as epigallocatechin gallate (EGCG), a strong antioxidant in green tea. (Tabata et al., 2008). In general, for the medicinal compounds in herbal fruits such as *T. chebula* to take the effect, a large amount of the fruits must be consumed. For these reasons, extraction and concentration of these phytochemicals are often necessary.

Phenolic compounds such as gallic acid, ellagic acid, and corilagin were reported to be effectively extracted by organic solvents such as ethanol, ethyl acetate (Kaur et al., 1998), ether (Malekzadeh et al., 2001), and 70% methanol (Saleem et al., 2002). However, if these solvents were not properly removed from the extract, they would be harmful to the consumers' health. Although previous research showed that these phenolic compounds could also be extracted more benignly with hot water (70-80°C) (Naik et al., 2004), the process took a long time due to the low solubility of the compounds in water. To increase the solubility of the compounds, the temperature should be increased. In addition, by increasing the pressure, the water temperature can be further increased beyond its normal boiling point (at 100°C). At such conditions water is called pressurized hot water (PHW) or subcritical water and its ability to extract various organic compound increases.

Water at subcritical conditions usually refers to water in liquid state with temperature between boiling (100°C) and critical temperature (374°C), temperature at critical point. Practically, the use of subcritical water extraction (SWE) provides a number of advantages over conventional extraction techniques such as lowering extraction time, lowering the cost of the extracting agent, and being environmental friendly solvent. Examples of compounds that have been extracted from plants by subcritical water were such as catechins and proanthocyanidins from grape seeds (García-Marino et al., 2006) and hydrolysable tannins from *Phyllanthus niruri* (Markom et al., 2007). Despite these several advantages, high temperature operation might cause thermal degradation of the compounds. Anekpankul et al. (2005) reported that at the operating temperature above 200°C, the degradation of damnacanthal from roots of *Morinda citrifolia* was pronounced.

In this study, we proposed to investigate the suitable conditions for subcritical water extraction of gallic acid, ellagic acid, and corilagin from *Samor thai* fruits by

considering the effect of extraction temperature and water flow rates. Furthermore, comparison was made for this extraction method and the soxhlet extraction with water and ethanol as solvents. Moreover, the antioxidant activities of the subcritical water extracts would be measured and compared to that of the extracts obtained by the conventional water and solvent extraction techniques.

1.2 Objectives

1.2.1 To investigate suitable conditions for subcritical water extraction of gallic acid, ellagic acid, and corilagin from *Terminalia chebula* fruits.

1.2.2 To compare the efficiency of subcritical water extraction with conventional extraction methods.

1.2.3 To evaluate the antioxidant activities of the extracts by using ABTS assay.

1.3 Working scopes

1.3.1 Evaluation of the suitable conditions for subcritical water extraction of gallic acid, ellagic acid, and corilagin by determining two effects of temperatures (120-220 °C), and flow rate (2-4 ml/min) at a fixed pressure of 4 MPa on the amounts of the extracted compounds, and the extraction rate.

1.3.2 Comparison of the extraction efficiency of subcritical water extraction with that of soxhlet extraction and hot water extraction (100°C).

1.4 Expected benefits

1.4.1 Provide an efficient alternative method for extraction of plant derived phytochemical bioactive compounds.

1.4.2 Provide fundamental information useful for an industrial scale extraction process.

1.4.3 Extracts can be developed further to use as standardized extracts or as reference for herbal or natural products for pharmacological studies.

CHAPTER II

BACKGROUND AND LITERATURE REVIEWS

Background

2.1 *Terminalia chebula* Retz.

Terminalia chebula Retz. (Figure 2.1) is also known as Myrobalan or black Myrobalan or, in Thai, as Samor thai (or Samor upphaya (Central), Maa-na (North), Maa-nae (Karen-Chiang Mai), Maak-nae (Karen-Mae Hong Son)). It is a native plant in India and Southeast Asia which belongs to the Combretaceae family and is widely grown in deciduous forest and areas of light rainfall including Thailand. The plant is a medium to large tropical perennial plant that can reach a height of 20 meters. The fruits of Samor thai are simple fruit, ellipsoid, dry drupe, five ridge, and greenish-yellow with spindle-shaped seed.

Samor thai is a popular folk medicine for treatment of several illnesses including anticancer, antidiabetic, and antibacterial. Different parts of Samor thai are used such as bark, leaf, flower, and fruit, which contain various phytochemicals that exhibit various medicinal properties. The part mostly consumed is the fruits, which are taken either as fresh fruits or are processed into products such as juice or dried fruits. Recent study has reported that *T. chebula* fruit extract had the strongest antioxidant capacity and the highest phenolic content over 133 Indian medicinal plants (Surveswarun et al., 2007). Major bioactive constituents contained in the fruit are such as gallic acid, ellagic acid, and corilagin (Worasuttayangkurn, 2001).

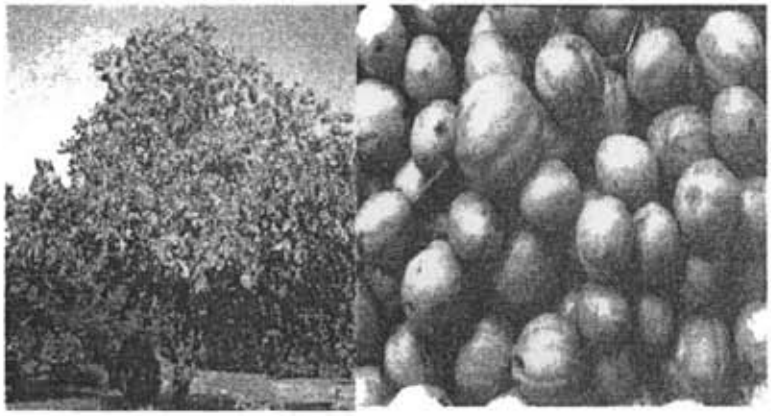


Figure 2.1 *Terminalia chebula* Retz.

2.2 Polyphenolic compounds

Phenolic compounds are a group of chemicals whose structures comprise of at least one benzene ring and one hydroxyl group. This basic structure of phenolic compounds is known as phenol, which contains only one benzene ring and hydroxyl group. Differences between other complicated phenolic compounds exist in various chemical groups at the three different positions of benzene ring; orto, meta, and para. Based on the number of the phenol rings in the compound's structures, phenolic compounds can be divided in to three groups: monocyclic, dicyclic, and polycyclic phenols or polyphenolics.

Polyphenolic compounds are phytochemicals which play a major role in the protection of chronic diseases and oxidative process. They are mostly present as the main constituents in fruits and vegetables including *T. chebula*. Fruits of *T. chebula* contain three major components identified as gallic acid, ellagic acid, and corilagin. Their chemical structures and molecular weights are shown in Figure 2.2. These compounds have been proven to exhibit several therapeutic effects especially against some chronic diseases including various forms of cancer and cardiovascular diseases (Lin et al., 1993; Constantinou et al., 1995; Festa et al., 2001; Kawada et al., 2001; Yilmaz and Toledo, 2004) and exhibited antioxidant activities (Rangkadilok et al., 2007).

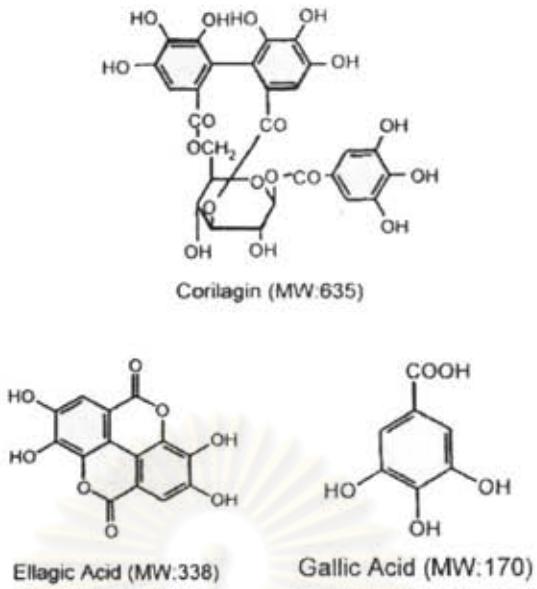


Figure 2.2 Chemical structures and molecular weights of corilagin, ellagic acid, and gallic acid. (Markom et al., 2007)

2.3 Sub and supercritical technology and natural product extraction

Sub and supercritical fluid technology is currently expanding into a wide range of applications. Supercritical fluids are defined as fluid at the temperatures and pressures above the critical values. These fluids can no longer be classified as a liquid or a gas (Figure 2.3). The most often used supercritical fluid is CO_2, particularly for analytical and process-scale extractions of natural products. CO_2 has low critical temperature, thus the operating temperature is desirably low. Furthermore, the process leaves no toxic residues in the final products. Pure supercritical CO_2 can be used to extract a wide variety of solutes of low-polarity from natural materials. However, in many cases, the polarity of pure CO_2 is too low to quantitatively extract polar solute without the need to add polar organic modifiers or to increase the extraction temperature.

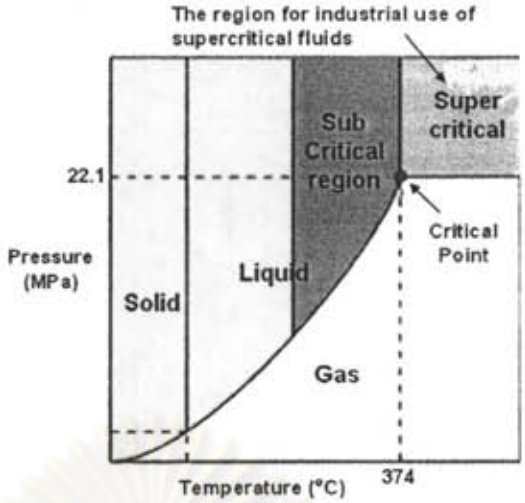


Figure 2.3 Theoretical Pressure – Temperature phase diagram for pure compound.

For slightly polar compound, water is an alternative environment-friendly solvent. It has additional advantages of being readily available at low cost, however, in its "natural" state, water is not a good solvent for most organics. When the water temperature rises nevertheless, it can quantitatively extract a wide variety of organic solutes from many different matrixes. Water at elevated temperature (typically between its boiling point temperature (100°C) and its critical temperature (374°C) and at a pressure high enough to maintain the liquid state) is called "subcritical water" or "superheated water", or "pressurized hot water". An increase in the temperature of water results in a decrease in its dielectric constant and an increase in ionization constant (K_W). The significance of the changes in these two properties for subcritical water is described as follows.

The dielectric constant

The breakdown of the hydrogen-bonded structure at such high temperature also causes water dielectric constant to fall, making it possible to dissolve organic compound. For instance, pure water at ambient condition has a dielectric constant of 79, as is shown in the Figure 2.4 Increasing the temperature to 250°C at a pressure of 5 MPa (necessary to maintain the liquid state) results in a significant reduction of this value to about 27. At

this condition, water has the polarity similar to that of ethanol at 25°C and 0.1 MPa (Clifford et al., 2002; Smith, 2002).

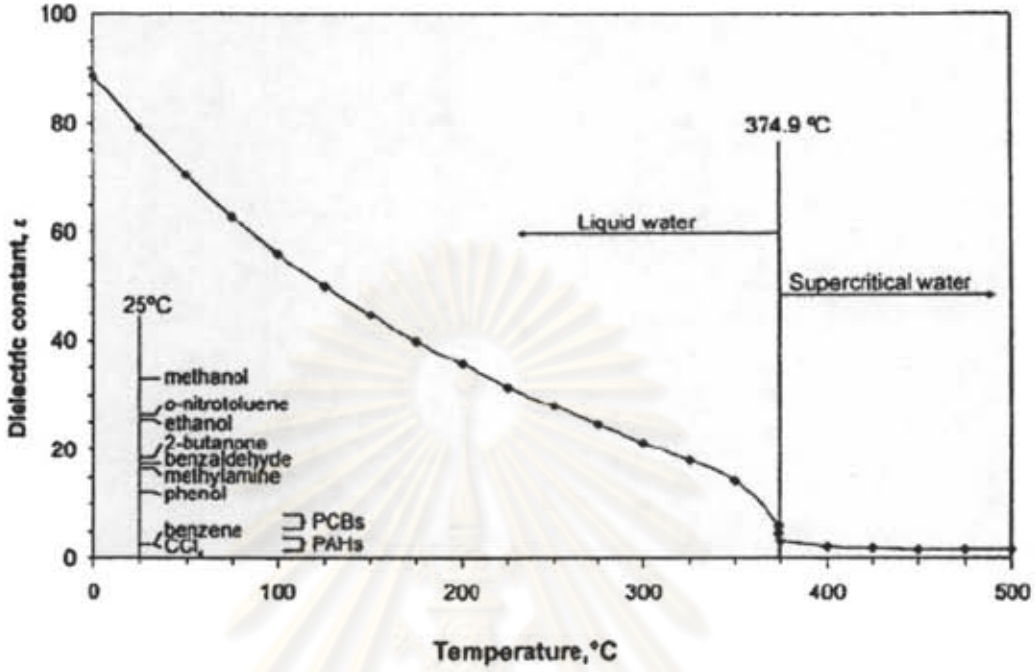


Figure 2.4 Dielectric constant of water versus temperature. (King, 2004)

Ionization Constant (K_w)

Naturally, the molecule of water endothermically ionizes or dissociates into hydrogen [H^+] and hydroxide [OH^-] ion. This is due to electric field fluctuations caused by nearby dipole libration (Geissler *et al.*, 2001). The reaction equation can be written as:

$$H_2O \rightleftharpoons H^+ + OH^-$$

As a result of the instability of hydrogen ion, it is hydrated with water itself which results in the formation of hydronium ion [H_3O^+]. The above equation is better written as:

$$2H_2O \rightleftharpoons H_3O^+ + OH^-$$

The product of ionic concentrations (hydronium and hydroxide ion) is defined as ion product constant, K_W (also called ionization constant, dissociation constant). The relation is expressed as:

$$K_w = [H_3O^+][OH^-]$$

Although the extent of ionization is tiny ($[H^+]/[H_2O] = 2.8 \times 10^{-9}$ at 37°C), the ionization and changes in the tiny concentrations of hydrogen ions have absolute importance in several chemical processes.

Since a change in temperature causes a change in chemical equilibrium, therefore K_W is also temperature dependent; that is it increases with increasing temperature up to about 250°C and then decreases onwards. Figure 2.5 illustrates the relationship of the K_W with water temperature.

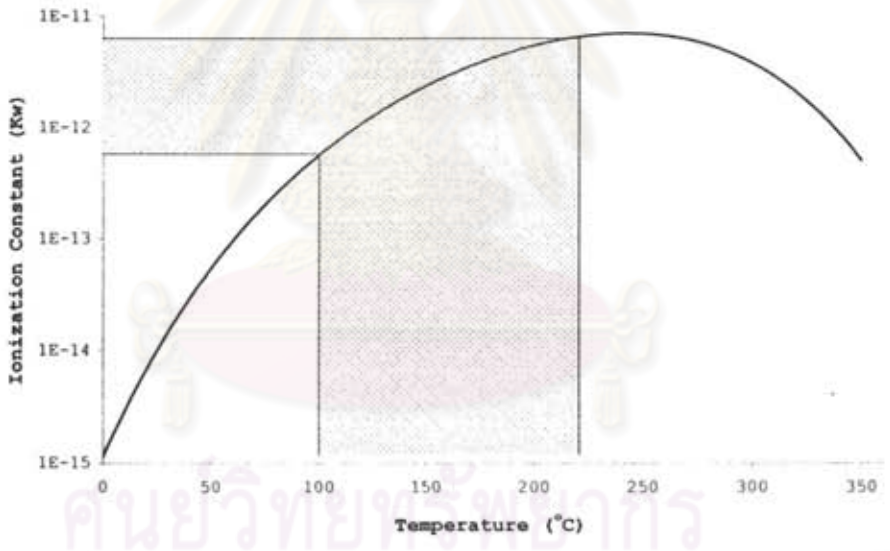


Figure 2.5 The ionization constant of water at various temperatures.

Source: Marczewski (2002), redrawn by the authors.

2.4 Antioxidant activity measurement by ABTS method

Various methods have been used to assay the antioxidant activity of natural products. These methods give varying results depending on the specificity of the free radical being used as the reactant. The common methods used for measuring the radical-scavenging activity of antioxidants against free radicals are 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$), 2,2-Diphenyl-1-picrylhydrazyl (DPPH), the superoxide anion radical (O_2) such as the xanthine/xanthin oxidase (AC/XO) assay, and oxygen radical absorbance capacity (ORAC). Of these methods, the most rapid and inexpensive method is the use of the ABTS radical cation. This method is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors, and to evaluate antioxidant activity of natural products. $ABTS^{\bullet+}$ can be generated from oxidation of ABTS with MnO_2, potassium persulfate or peroxide radicals as shown in Figure 2.4. The odd electron in the ABTS free radical gives a strong absorption maximum at 414, 645, 734 and 815 nm and its color is blue/green. When the odd electron of $ABTS^{\bullet+}$ becomes paired with hydrogen from a free radical scavenging antioxidant to form the reduced ABTS, the resulting decolorization is stoichiometric with respect to the number of electrons. Thus measuring the decolorization simply with spectrophotometer quantifies the antioxidant activity of the compound.

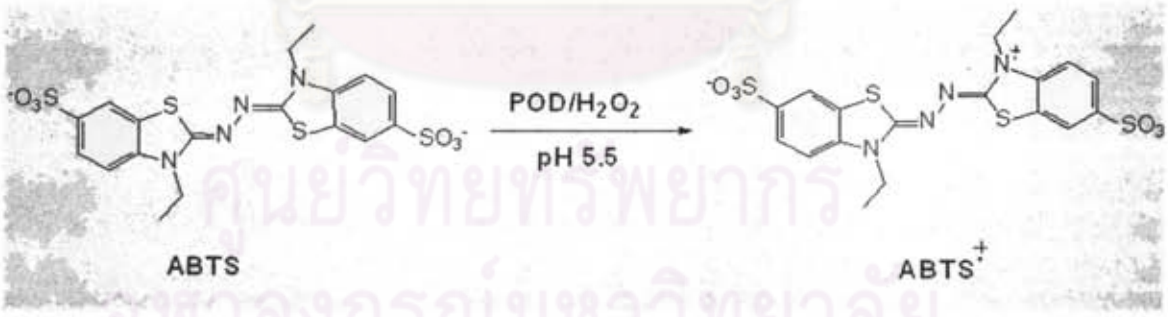


Figure 2.6 The formation of $ABTS^{\bullet+}$ with hydrogen peroxide

Source: http://www.aktuelle-wochenschau.de/2005/woche7/wochenschau7.html

Literature reviews

It is commonly known that plants including fruits, vegetables, and herbal plants contain various phytochemicals, several of which exhibiting various pharmacological properties. These secondary metabolites are generally found in a small amount, and for the compounds to take the effect, large amount of fruits, vegetables and herbal plants must be consumed. For these reasons, extraction and concentration of these phytochemicals are often necessary. One of the most of widely grown and popular used medically herbal plant in Thailand is *Terminalia chebula*, which has the strongest antioxidant capacity and the highest phenolic content over 133 Indian medicinal plants (Surveswarun et al., 2007). It is usually taken fresh or as processed products such as juice or dried fruit because the fruit either fresh or dried contains many polyphenolic compounds including gallic acid, ellagic acid, and corilagin (Worasuttayangkurn, 2001). These compounds also exhibited cancer cell growth inhibitory (Saleem et al., 2002). Recent study has confirmative reported that methanolic extract from *T. chebula* fruit could be used as therapeutic agent for cancer prevention which they blocked or suppressed the events associated with chemical carcinogenesis due to the presence of phenolics (Prasad et al., 2006). In addition, Malekzadeh et al (2001) compared the antibacterial activity of various myrobalan extracted with in three solvents (ether, ethanol and water) and concluded that aqueous extracts of the plant were more active than other extracts by determining diameter of inhibition zone of a bacteria, *H. pylori*. Moreover, the bioactive components in dried fruits of *T. chebula* were extracted with distilled water at about 70-80°C and the extract was evaluated for the antioxidant activities and a probable radioprotector (Naik et al., 2004). The analysis showed that the aqueous extract can be used as an effective antioxidant and a radioprotector due to the presence of gallic acid and ellagic acid. The reviews of the studies on *T. chebula* fruit are summarized in Table 2.1.

Subcritical water extraction is a current technology used for the extraction of environmental pollutants such as organic (Hawthorne et al., 1994; Yang et al., 1995) and inorganic metals (Priego-López et al., 2002). Reviews of subcritical water extraction of environmental samples are summarized in Table 2.2. Recently, this technique becomes frequently used for the isolation of natural product for the production of fragrances,

flavors, and pharmaceuticals because it is nontoxic and leaves no harmful residues. Successful cases have been reported for essential oils from majoram (Jiménez-Carmona et al., 2002), savory and peppermint (Kubátová et al., 2001), and oregano (Ayala, et al., 2001). In addition to essential oils, other bioactive compounds have been extracted by this technique. They are hypericin and pseudohypericin from St. John's wort (Mannila et al., *2002)*, iridoid glycosides from *Veronica lonifolia* (Suomi et al., 2000), kava lactones from kava root (Kubátová et al., 2001), anthraquinones from roots of *Morinda citrifolia* (Shotipruk et al., 2004). In addition, subcritical water has been applied for extraction of phenolic compounds such as catechin, epicatechin and gallic acid from grape seeds (García-Marino et al., 2006) and hydrolysable tannins (gallic acid, ellagic acid and corilagin) from *Phyllanthus niruri* (Markom et al., 2007). Reviews of subcritical water extraction of natural product are summarized in Table 2.3. In comparison with conventional solvent extraction method, most studies described this technique as environmental friendly, inexpensive, and that it requires short extraction time.

Although the results from previous studies suggest the possibility of using hot water and/or subcritical water for extraction of these phenolic compounds, the quantitative study on the effects of extraction conditions on the amount of extracted compounds from *T. chebula* fruits is still limited. In this study, we will focus on extraction of corilagin, gallic acid, and ellagic acid from *T. chebula* fruits. The suitable condition would be determined to maximize the extraction efficiency. Nevertheless, the effective extraction condition should not only mean those that yield the high amount of the three compounds, but the extract should also still be biologically active. Since it is known that high temperature may cause degradation of antioxidant activities of natural compounds (Rogalinski et al., 2002, Anekpankul, 2006), thus in this study the extract should be assayed for any possible degradation under different extraction temperature.

Several assays frequently have been used to estimate antioxidant activities of fruits and vegetables and other food products. Some of these assays are scavenging activity of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) (Re et al., 1998), 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Brand-Williams et al.,1995; Gil et al., 2002), superoxide radical formation by xanthine/xanthin oxidase (AC/XOD) (Prakash, 2001), and the oxygen radical absorption capacity (ORAC) (Cao et

al., 1993; Ou et al.,2001; Prior et al., 2003). Of these free radical scavenging assays, the simplest and widely used method is the ABTS assay owing to its rapidly reaction with the extracts and the commercial peroxidase used that does not need previous purifications. Therefore, in this study, the effect of subcritical water temperature on the possible degradation of the antioxidant activity of the extract will be determined using ABTS assay. The materials and methods used in this study are described in chapter 3.

Table 2.1: Reviews on the studies of *T. chebula* fruit.

Author	*Parts used*	*Solvent*	*Product*	*Condition*		*Objective*
1. Worrasutta-yangkurn, 2001	dried fruit pulp	Deionized water	N/A	Temperature Pressure Time	100 °C 0.1 MPa 30 min	To evaluate the toxicity of powder and water extract of *T.chebula* in mice.
2. Saleem et al., 2002	dried fruits	Methanol (70%)	Gallic acid, and Ellagic acid	Temperature Pressure Time	25 °C 0.1 MPa 1 hr	To study the effects of the extract on the inhibition of cancer cell growth.
3. Naik et al., 2004	dried fruits	Distilled Water	Gallic acid, and Ellagic acid	Temperature Pressure Time	70-80 °C 0.1 MPa 2 hr	To study on the aqueous extract of *T.chebula* as a potent antioxidant and a probable radioprotector.

Table 2.2: Reviews on investigation of subcritical water extraction of pollutants from soil.

Author	*Contaminant*	*Sample size*	*Condition*		*Objective*
1. Miller et al., 1998	Polycyclic aromatic hydrocarbons (PAHs)	N/A	Flow rate Temperature Pressure Time	0.1 ml/ min 25-225°C 3-6 MPa 30 min	To determine the PAH and pesticide by SWE in compost.
2. Hawthorne et al., 2000	Polycyclic aromatic hydrocarbons (PAHs)	< 6 mm	Flow rate Temperature Pressure Time	1 ml/ min 250, 300°c 5 MPa 30, 60 min	To compare soxhlet extraction, PLE, SFE, and SWE for extraction of PAH from ore.
3. Krieger et al., 2000	Cloransulam-methyl	N/A	Flow rate Temperature Pressure Time	0.4 to 3.5 ml/ min 50, 100, 150°C 6.5, 13.5, 50 MPa 30 min	To study the effect of SWE of triazolopyrimidine sulfonanilide herbicides from soil.

Author	*Contaminant*	*Sample size*	*Condition*	*Objective*
4. Mcgowin et al., 2001	PAHs & pesticide	< 2 mm	Flow rate 1 ml/ min Temperature - PAH 110, 150, 250, 350°C - Pesticide 110, 130, 150, 250°C Time 20 min	To determine the PAH and pesticide by SWE in compost.
5. Dadkhah et al., 2002	PAHs	< 4 mm	Flow rate 1 ml/ min Temperature 230, 250, 270°C Pressure 4 MPa Time 45, 90 min	To examine the effects of small-scale batch extraction of soils polluted with PAHs by using SWE.
6. Richter et al., 2003	Pesticides	< 2 mm	Flow rate 2 ml/ min Temperature 50 to 300°C Pressure 1200 psi Time 25 min	To evaluate efficiency of water at subcritical region to extract from soils a group of typical pesticides used in agriculture.

Author	*Contaminant*	*Sample size*	*Condition*		*Objective*
7. Hashimoto et al., 2004	Dioxins	< 0.1 mm	Flow rate Temperature Pressure Time	2 ml/ min 125, 150, 300, 350°C 0.2 MPa 30 min	To understand of behavior of dioxins during SWE and optimize their efficiency.

Table 2.3: Reviews on investigation of subcritical water extraction of natural products.

Author	*Plants*	*Parts used*	*Product*	*Condition*		*Application*
1. Basile et al., 1998	*Rosmarinus officinalis*	Leaves	α-Pinene ,Camphor Camphene, Borneol, Limonene, Verbenone, 1, 8-Cineole, Isobornyl acetate	Flow rate Temperature Pressure Time	1, 2, 4 ml/ min 125-175°C 2 MPa 200 min	Fragrance and flavor

Author	*Plants*	*Parts used*	*Product*	*Condition*	*Application*
2. Pawlowski et al., 1998	Agriculture commodities e.g. banana, lemon, etc.	Fruit pulp	Thiabendazole (TBZ), Carbendazim (MBC)	Flow rate 2-20 ml/ min Temperature 50, 75°C Pressure 5 MPa Time 20 min	Food
3. Clifford et al., 1999	*Syzygium aromaticum*	Bud	Eugenol, Eugenyl acetate, Caryophyllene	Flow rate 2 ml/ min Temperature 150°C Pressure N/A Time 100 min	Essential oil
4. Jiménez-Carmona et al., 1999	*Thymus mastichina*	Leaves	α-Pinene, β-Pinene Linalool, Geraniol, etc.	Flow rate 2 ml/ min Temperature 150°C Pressure N/A Time 100 min	Essential oil

Author	*Plants*	*Parts used*	*Product*	*Condition*	*Application*
5. Miller et al., 2000	N/A	N/A	*d*-Limonene, Carvone, Eugenol, Nerol, 1,8-Cineole1	Flow rate 0.1 ml/ min Temperature 25 to 200°C Pressure 7 MPa	Fragrance and flavor
6. Fernández-Perez et al., 2000	*Laurel*	Leaves	1, 8 Cineole1, α-Phellandrene, β-Pinene, etc.	Flow rate 2 ml/ min Temperature 150°C Pressure 5 MPa Time 30 min	Essential oil
7. Gámiz-Gracia et al., 2000	*Foeniculum vulgare*	Fennel	α Pinene, Limonene, β Pinene, Comphor, β Mircene, Linalyl propanoate	Flow rate 0.5-3.0 ml/ min Temperature 150°C Pressure 5 MPa Time 50 min	Essential oil

Author	*Plants*	*Parts used*	*Product*	*Condition*	*Application*
8. Kubátová et al., 2001	Satureja hortensis and Menthe piperita	N/A	Cymene, Thymol , Borneol, Linalool, etc.	Flow rate 1 ml/ min Temperature 100, 150,175°C Pressure 6.5 MPa Time 30 min	Fragrance and flavor
9. Kubátová et al., 2001	*Piper methysticum*	root	Dihydrokawain, Kawain, Yangonin, etc.	Flow rate 1 ml/ min Temperature 175°C Pressure 6 MPa Time 20 min	Fragrance
10. Ayala et al., 2001	*Lippia graveolens*	Leaves	1, 3-Cyclohexadien, α-Phellandrene, 3-Carene, etc.	Flow rate 1-4 ml/ min Temperature 100-175°C Pressure 1.0-5.1 MPa Time 24 min	Essential oil

Author	*Plants*	*Parts used*	*Product*	*Condition*		*Application*
11. Ollanketo et al., 2002	*Salvia officinalis*	N/A	Rosmarinic acid, Carnosal, Carnosic acid, Methyl carnosate	Flow rate Temperature Pressure Time	1 ml/ min 70, 100, 150°C 100 kg/cm^2 60 min	Essential oil
12. Eng Shi Ong et al., 2003	*Coptidis, Glycyrrhizae* and *Scutellariae radix*	Root	Glycyrrhizin, Baicalein	Flow rate Temperature Pressure Time	1 ml/ min 95-140 °C 1-2 MPa 40 min	Essential oil
13. Ozel et al., 2003	*Thymbra spicata*	Leaves	Carvacrol ,p-Cymene, Thymol, Caryophyllene, E-3-caren-2-ol	Flow rate Temperature Pressure Time	2 ml/ min 100, 125, 150, 175°C 2, 6, 9 MPa 40 min	Essential oil

Author	*Plants*	*Parts used*	*Product*	*Condition*	*Application*
14. Shotipruk et al., 2004	*Morinda citrifolia*	Root	Anthraquinones (Alizarin)	Flow rate 2,4,6 ml/ min Temperature 110, 170,220°C Pressure 7 MPa	Medicin
15. García-Marino et al., 2006	*Vitis vinifera*	Seed	Cathechins and Proanthocyanidins (gallic acid)	Flow rate 1 ml/ min Temperature 50, 100, 150°C Pressure 6-7 MPa Time 30 min	Medicin
16. Markom et ai., 2007	*Phyllanthus niruri* Linn.	N/A	Gallic acid, Ellagic acid and Corilagin	Flow rate 1.5 and 3 ml/ min Temperature 60 and 100°C Pressure 10-15 MPa Time 60 min	Medicin

CHAPTER III

MATERIALS AND METHODS

3.1 Plant Materials and Chemicals

The dried fruits of *T. chebula* were obtained from Chulabhorn Research Institute and then crushed into fine powder using pestle and mortar or blender. The reference standards (gallic acid and ellagic acid) and Folin-Ciocalteau reagent were purchased from Sigma-Aldrich Chemicals (Missouri, USA). Standard corilagin was purified and identified using TLC, UV spectrum, Mass Spectrophotometer and NMR by the Laboratory of Pharmacology, Chulabhorn Research Institute, Bangkok. Methanol, HPLC grade, and formic acid were obtained from Merck (Darmstadt, FR Germany). Water used in the experiments was distilled and deionized water.

3.2 Methods

3.2.1 Subcritical water extraction

Subcritical water extraction was performed using an apparatus shown in Figure 3.1. The extraction system consisted of two HPLC pumps (PU 980, JASCO, Japan) used for delivering water and solvent, a degassing instrument (ERC 3215, CE, Japan), an oven (D63450, HARAEUS, Germany), in which the extraction vessel (10 ml, Thar Design, USA) was mounted, a pressure gauge, and a back pressure regulator valve (AKICO, Japan). All connections are made with stainless steel capillaries (1/16 inch inside diameter).

Distilled water was passed through a degassing equipment to remove dissolved oxygen. The degassed water was then delivered, at a constant flow rate with the first HPLC pump, to a 3-m preheating section installed in the oven to heat it to the required temperature, which then passed through the extraction vessel, preloaded with 1 g of ground *T. chebula* fruits. The pressure of the system was adjusted to the desired condition (4 MPa) by using the back-pressure regulator valve at the outlet coil to ensure that water

was in liquid state at the temperatures tested. Before heating the extraction system, all connections were checked for possible leakage. The oven was turned on and the temperature was set at the desired operating condition. When the temperature reached the set point, the extraction started. 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. The extract was cooled in a coil immersed in a water bath to prevent possible product degradation, and was then collected in fractions in collecting flasks. After that, the extract was concentrated by evaporating off water under vacuum and then analyzed by HPLC with UV detection at the wavelength of 270 nm.

Several extraction experiments were carried out to determine the effect of temperature and water flow rates on the product yield and quality. The conditions tested are summarized in Table 3.1.

Table 3.1: Extraction process variables to be studied

Variables	**Condition**
Temperature	120-220°C
Flow rate	2-4 ml/min
Pressure	4 MPa

After each extraction, the amounts of polyphenolic compounds remained in the fruits residue were determined by solvent extraction with distilled water at room temperature. The fruit residues were taken out of the extractor and placed into a 100 ml Erlenmeyer flask, containing 30 ml of distilled water. It was then allowed to release the products into the solvent overnight. The solution was then replaced with 30 ml of fresh distilled water daily for 3 days.

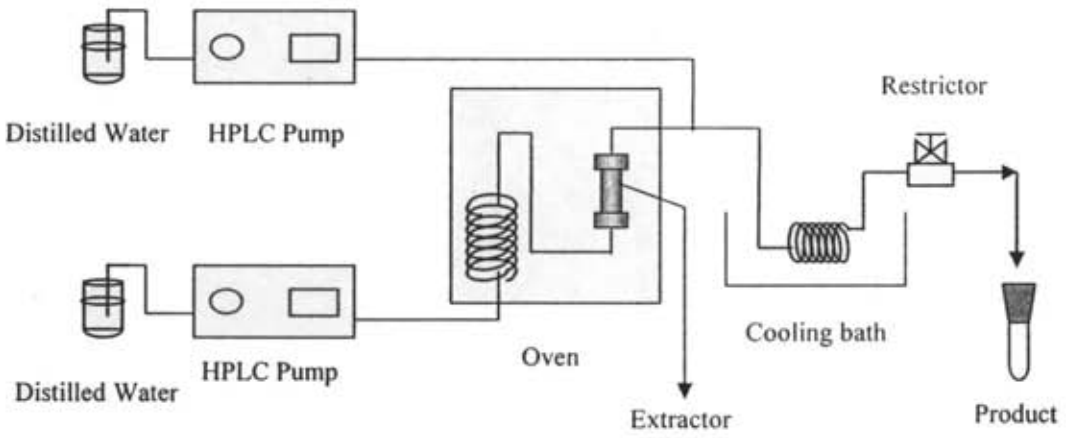


Figure 3.1 Diagram of experimental setup subcritical water extraction.

3.2.2 Soxhlet extraction

One gram of ground *T. chebula* fruits was placed into a thimble of a soxhlet apparatus and extracted with 150 ml of distilled water or 100% ethanol. Extraction was carried out for 2 hours and the extract was then analysed for the concentrations of polyphenolic compounds using HPLC.

3.2.3 Water extraction

One gram of ground *T. chebula* fruits was extracted with 150 ml of water at room temperature and hot water (70° and 100°C) in a stirred vessel. The extraction at room temperature was carried out for 24 h. Those extracted with hot water were carried out for 2 h. The extracts were analyzed by using HPLC.

3.2.4 HPLC analysis of *T. chebula* extracts

The HPLC analysis was performed using an HP1100 HPLC system with a thermostatically controlled column oven, a binary pump, and a diode-array detector (Hewlett Packard, USA). A 150 × 3.9 mm i.d., 5 μm reversed phase column, Symmetry® C18 was used for the analysis of the active compounds in *T. chebula* samples (Waters Corporation, Milford, Massachusetts). The sample injection volume was 10 μL and the

compounds were eluted with a gradient system of 0.1% formic acid (solvent A): methanol (solvent B) at a flow rate of 1 ml/min at the constant column temperature of 25°C, with the UV detection at 270 nm. The gradient system started with 4% solvent B at 0 min and was changed to 80% solvent B in 27 min, with the total run time of 30 min. Retention times of gallic acid, corilagin, and ellagic acid were 5.08, 16.97, and 24.69 min, respectively, and the chromatogram of the *T. chebula* extract is shown in Figure 3.2.

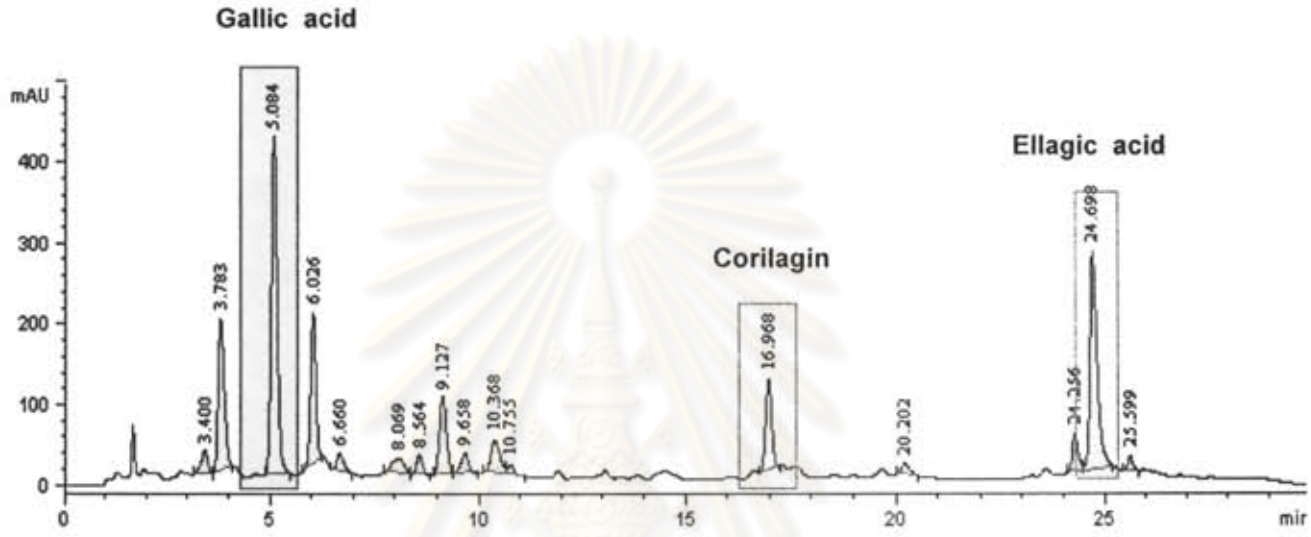


Figure 3.2 HPLC chromatogram of *T. chebula* extracted.

3.2.5 Determination of total phenolic content

The determination of the total phenolic content using Folin-Ciocalteau method modified from that described in previous study (Rodríguez-Meizoso et al., 2006). 0.1 ml of the concentrated extracts from subcritical water extraction and other conventional methods were dissolved in distilled water 2.8 ml and each mixture was added with 2 ml of 2% aqueous sodium carbonate solution. After 3 min, 0.1 ml of 50% Folin- Ciocalteau reagent was added to the mixtures and left at room temperature for 30 min, after which the absorbance was measured at 750 nm using distilled water as a reference. The content of total phenolic was calculated on the basis of calibration curve of gallic acid.

3.2.6 Antioxidant activity measurement

Antioxidant activity of the extracts from subcritical water extraction and other conventional methods was tested using ABTS method modified from that of Re et al., 1999. For the purposes of comparing the antioxidant activity of various extracts, the concentration of sample producing 50% reduction of the radial absorbance (IC_{50}) was used as an index. To find this value, the concentrated extract was diluted in series with distilled water and each diluted extract was added into $ABTS^{\bullet+}$ solution (aqueous solution of 7 mM ABTS and 2.45 mM potassium persulfate having absorbance of 0.70 ± 0.02 at 734 nm) with the volume ratio of 1:10 (sample solution:ABTS solution). The solutions were mixed using a vortex and the mixtures were incubated in the dark at room temperature for 10 min, after which the absorbance was measured at 734 nm using distilled water as a reference.

The value of percent inhibition (PI) was calculated using the following equation:

$$PI(\%) = \left[1 - \left(A_t / A_r\right)\right] \times 100 \qquad (3.1)$$

A_t and A_r are absorbance of test sample and absorbance of the ABTS reference, respectively. These values were plotted against sample concentration and linear regression of the data were made and used to determine the value of IC_{50}.

CHAPTER IV

RESULTS AND DISCUSSION

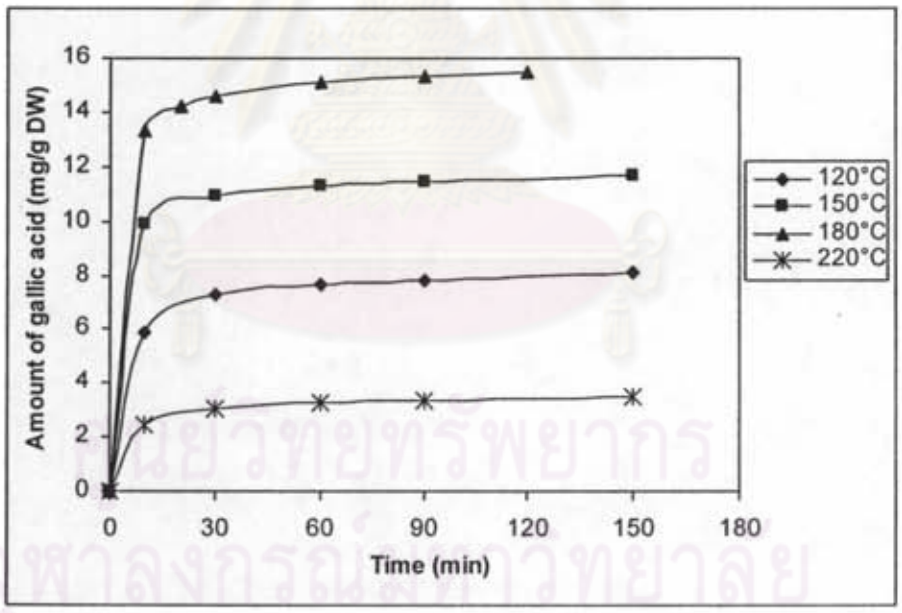
This chapter presents the experimental results dealing with extraction of phenolic compounds (gallic acid, ellagic acid, and corilagin) using subcritical water. Firstly, the effects of temperatures and water flow rates on the amounts of these compounds were investigated to determine the most suitable extraction condition. In addition, the performance of subcritical water extraction was compared to that of other conventional extraction methods such as hot water extraction and soxhlet ethanol extraction. Moreover, the contents of the total phenolic compounds in *T. chebula* extracts were also determined. Finally, the antioxidant activities of the extracts obtained by subcritical water extraction were compared with those of the extracts obtained by conventional extraction methods.

4.1 Effect of temperatures

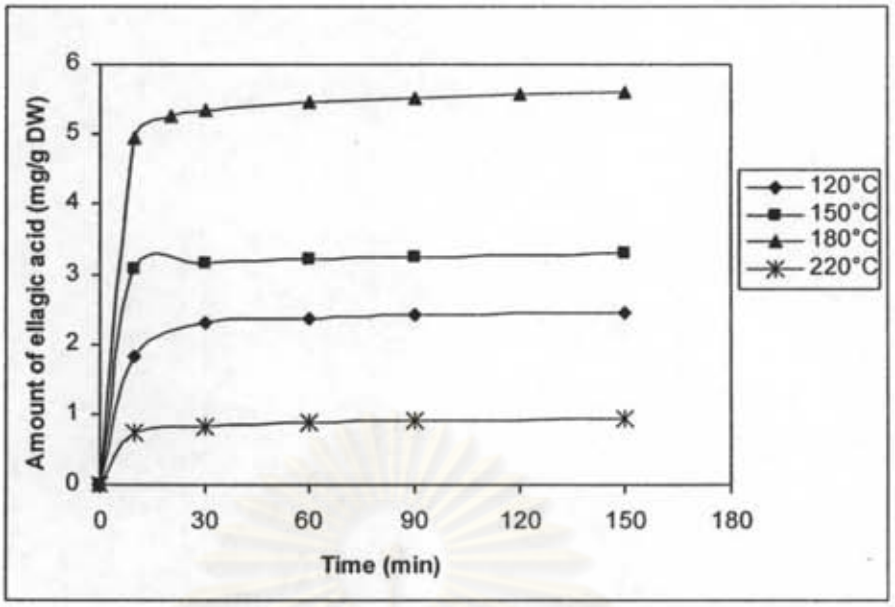
For subcritical water extraction, the ability to extract various compounds depends largely on extraction temperatures employed. In the present work, the effect of subcritical water extraction temperatures in the range of 120-220°C on the amounts of gallic acid, ellagic acid, and corilagin were investigated at a fixed pressure of 4 MPa and a fixed flow rate of 4 ml/min. As shown in the results of Fig. 4, extraction of the phenolic compounds could be completed within the first 10 minutes, that is, at 150°C for example, 84.77%, 92.93%, and 87.21% for gallic acid, ellagic acid, and corilagin were extracted.

As shown in Fig.4.1a and 4.1b, the amounts of gallic acid and ellagic acid increased as the temperature increased up to 180°C. At the high water temperature, the solubility of the compounds in water could be increased due to the decrease in water polarity at higher temperature. At 180°C, the amounts of gallic acid and ellagic acid were the highest, 15.473 and 5.588 mg/g dry weight, respectively. At 220°C, the amounts of both extracted compounds decreased due to the thermal degradation of the products. The

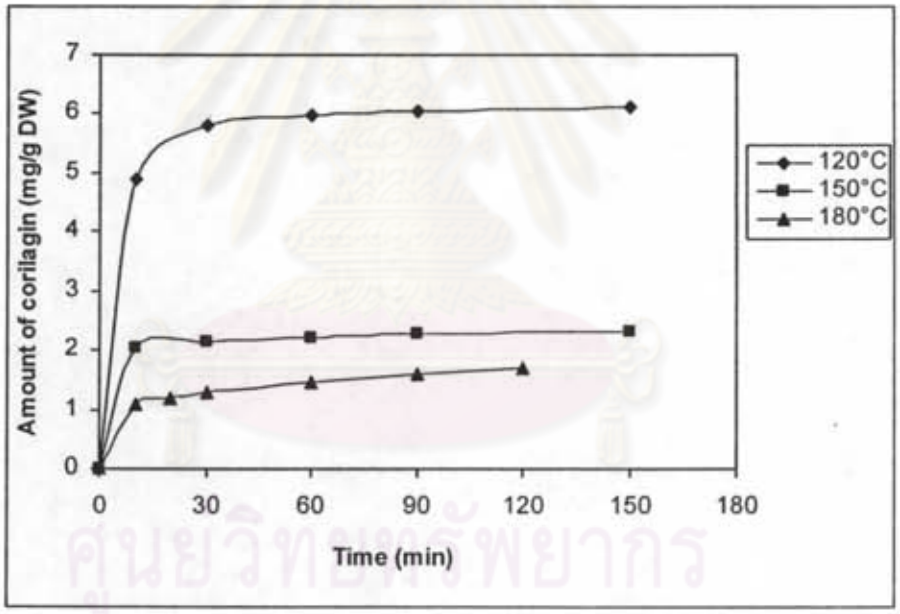
degradation of these compounds was also confirmed by the HPLC analysis, which showed that no gallic acid or ellagic acid remained in the sample residues. For subcritical water extraction of corilagin, the result in Fig. 4.1c showed that the compound was more easily decomposed than other compounds as the temperature increased. The highest quantity of corilagin (6.108 mg/g dry weight) was obtained at 120°C, and the amount of the compound extracted decreased at the higher extraction temperatures. At 220°C, the compound decomposed completely. At such high temperature, water ionization constant (K_w) increased. Consequently, water ionizes to hydronium ion (H_3O^+) and hydroxide ions (OH^-), which play an important role in hydrolysis reaction. Thus, at this temperature, corilagin, which is a phenolic compound of larger molecular size, could be readily hydrolyzed into its smaller phenolic constituents such as gallic acid and ellagic acid. The mechanism of the hydrolysis reaction is schematically shown in Fig. 4.2 (Fogliani et al., 2005). The decomposition of colliragin possibly explained the increase in gallic acid and ellagic acid contents at 180°C.



(a)



(b)



(c)

Figure 4.1 Effect of subcritical water extraction temperature on the amount of (a) gallic acid, (b) ellagic acid, and (c) corilagin at a fixed flow rate of 4 ml/min and pressure 4 MPa for 150 min.

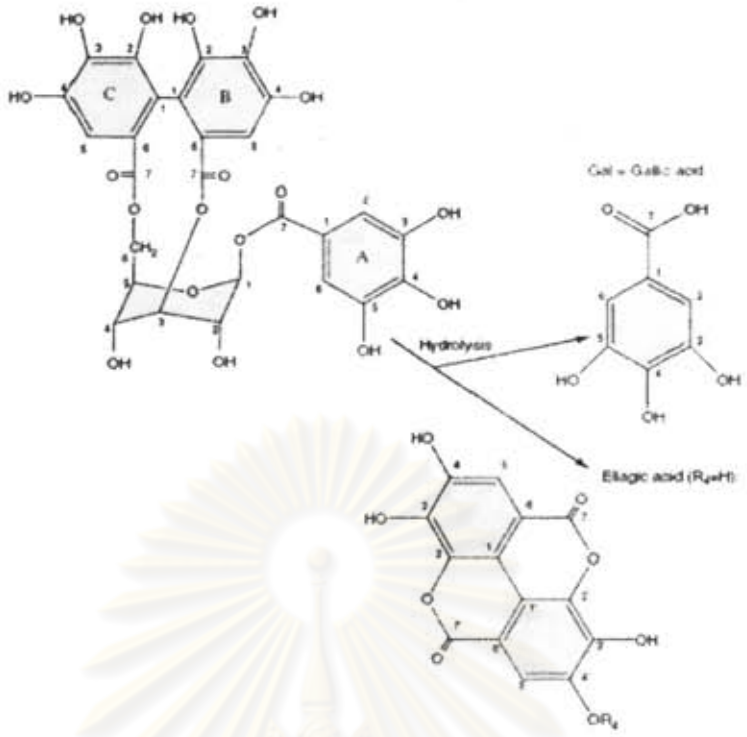
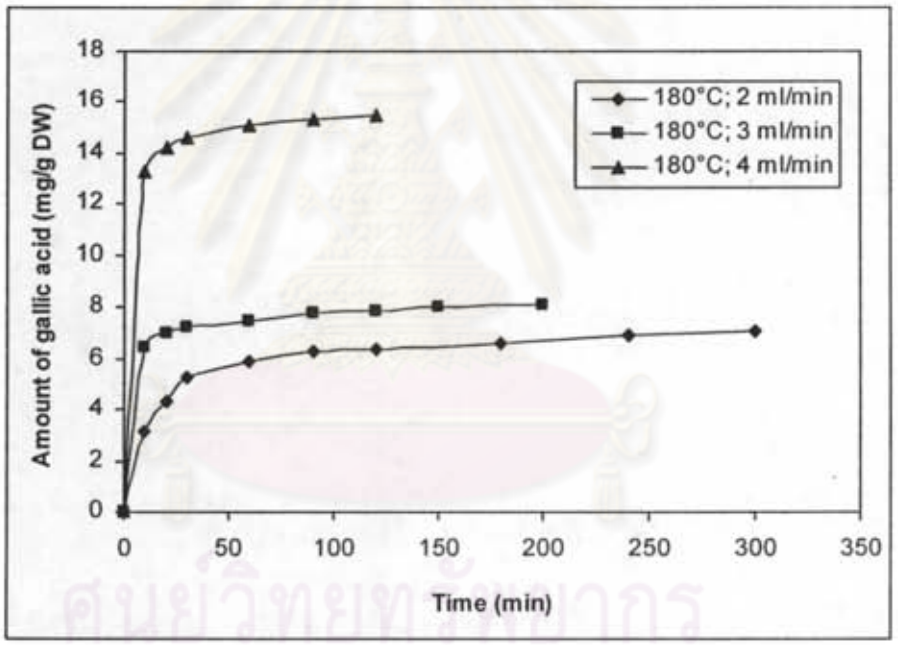


Figure 4.2 The hydrolysis reaction of corilagin.

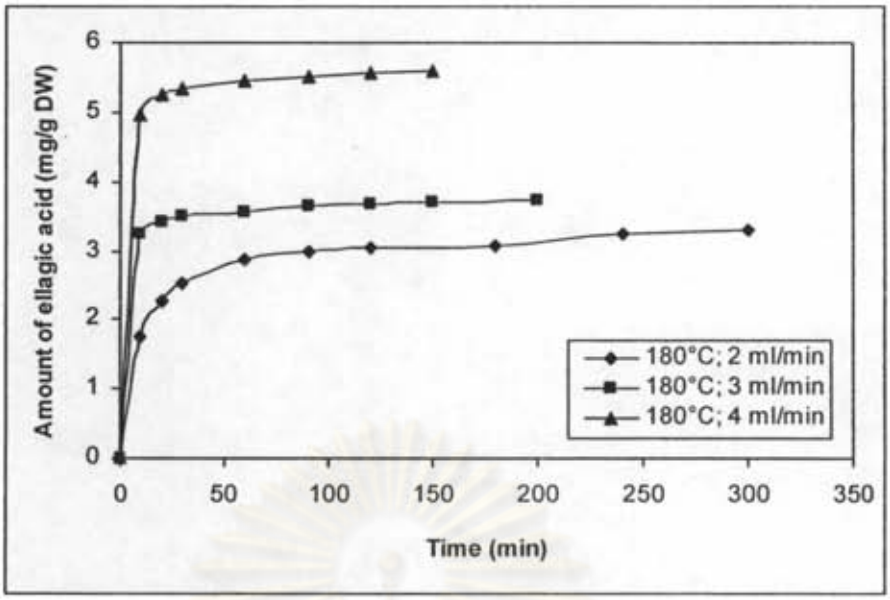
4.2 Effect of water flow rates on subcritical water extraction

The effects of water flow rates of 2, 3, and 4 ml/min were determined for extraction at the temperature of 180°C at a fixed pressure at 4 MPa for 600 ml of water. The results for the amounts of gallic acid, ellagic acid, and corilagin extracted versus time are shown in Fig.4.3a, 4.3b, and 4.3c, respectively. Fig. 4.3a and 4.3b demonstrated that the amounts of the desired compounds increased with an increase in volumetric flow rate up to 4 ml/min for gallic acid and ellagic acid. This means that the extraction rate at such early time was influenced by external mass transfer. On the other hand, the amount of corilagin extracted at 4ml/min was however lower than that obtained at the lower flow rate (Fig. 4.3c). Generally, an increase in flow rate should affect the extraction rate but not the total amounts of the compounds extracted in the process. The reason for the different amounts of gallic acid and ellagic acid obtained in this study was possibly due to the fact that at the low flow rate, the residence time of the extraction was higher. This exposure of the compounds to such high temperature conditions could cause the product

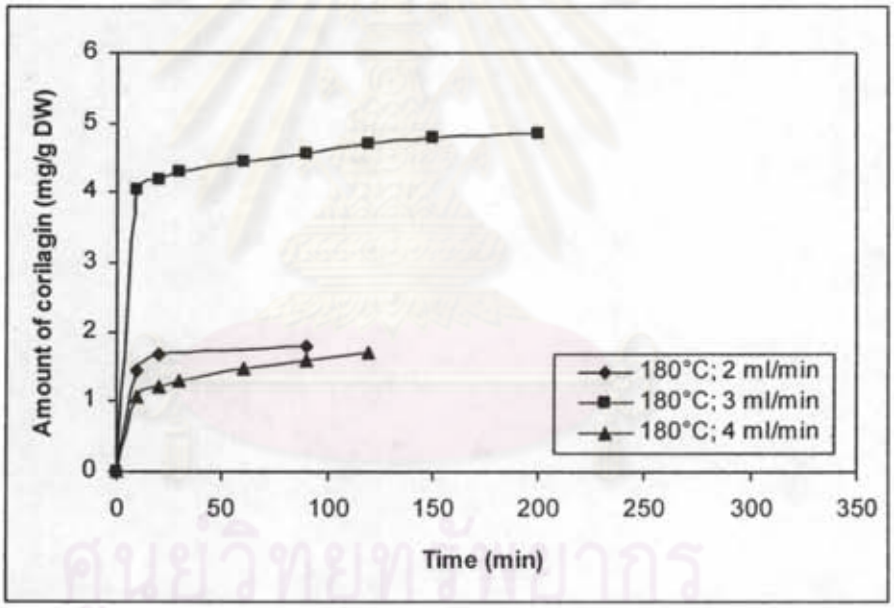
decomposition. At this point, the extraction process was no longer limited by external mass transfer, but instead was limited by the decomposition that took place. Since the extraction of phenolic compounds from *T. chebula* by subcritical water in this study involved large degree of compound degradation, the extraction behavior was therefore more complex than the system of extraction without degradation, in which only solubility and mass transfer play major roles. For corilagin however, the highest amount extracted at the flow rate of 3 ml/min in stead of 4 ml/min. Although at 4 ml/min, the residence time was the smallest, the higher degree of corilagin decomposition could possibly resulted from the fact that at this flow rate, higher amount of water passed the plant sample. Higher degree of hydrolysis could take place due to the larger amount of hydronium ion (H_3O^+) and hydroxide ion (OH^-) at the subcritical water condition.



(a)



(b)



(c)

Figure 4.3 Effect of water flow rate on the amount of (a) gallic acid, (b) ellagic acid, and (c) corilagin versus time for SWE at 180°C and pressure 4 MPa for 600 ml of water used.

4.3 Comparison of subcritical water extraction and conventional extraction methods

In the present study, the comparison was made between the subcritical water extraction and the conventional extraction methods such as hot water extraction at 100°C, soxhlet water, and soxhlet ethanol extraction. All results shown in Table 4.1 were obtained from extractions with the fixed volume (150 ml) of solvent. For subcritical water extraction, the data presented in the table was taken from the experiment operated at 4 ml/min at 120°C where the degradation of corilagin started to be observed, and at 180°C, before gallic acid and ellagic acid started to be observed. From the experimental results, subcritical water extraction was found to be less time consuming as only 37.5 min was required to extract the three compounds in the amounts specified in the Table 4.1. For other extraction methods, the maximum possible total phenolic contents were obtained during the first 2 h of extraction, and longer extraction times would decrease the amounts of the compounds (as shown in Appendix A).

Table 4.1 Comparison of solvent used, extraction time, and component contents based on fixed volume of 150 ml of solvent used for different extraction methods.

Extraction Method	Temperature (°C)	Extraction Time (min)	Component content (mg/g DW)			
			GA	EA	CG	Overall
Subcritical water extraction	120°C	37.5	7.398	2.315	5.855	15.568
	180°C	37.5	14.717	5.376	1.335	21.428
Hot water extraction	100°C	120	7.041	1.482	7.435	15.958
Soxhlet water extraction	100°C	120	6.368	2.266	5.366	14.000
	100°C	240	7.592	2.138	6.225	15.955
Soxhlet ethanol extraction	78.3°C	120	4.916	3.005	2.000	9.922
	78.3°C	240	3.329	2.352	3.001	8.682

Table 4.1 shows that, in case of extraction time comparison, 240-minute water extraction in a soxhlet apparatus gave slightly higher amount of the phenolic compounds of interest than the 120-minute one. For ethanol extraction in a soxhlet apparatus, slightly higher amount of compound was obtained with 120 minute extraction. Therefore, it could be concluded that the extraction time of 120 minute would be suitable for soxhlet extractions. The comparison of the extraction results obtained with ethanol and water after 120 min indicated that water was more preferable for extraction of the three phenolics of interest. The amounts of the compounds extracted with soxhlet water extraction were however slightly lower than that obtained with water extraction in a stirred vessel for the same extraction time of 120 min. The higher extraction efficiency in the boiling water extraction could be attributed to the turbulence caused by boiling water in such system, compared with soxhlet water extraction. According to various methods shown in Table 4.1, subcritical water extraction at flow rate of 4 ml/min, 180°C, could extract higher amounts of gallic acid and ellagic acid than other conventional extraction methods while requiring the least extraction time. At this condition, the polarity of water is reduced to approach that property of these organic components. For corilagin, the degradation took place significantly at 180°C, nevertheless at 120°C, the amount of corilagin extracted was only slightly lower than hot water extraction at 100°C. Compared with different extraction methods, it can be concluded that subcritical water extraction could effectively be used to extract the phenolic compounds from T. chebula fruits.

4.4 Determination of total phenolic contents

In this section, the quantification of the contents of total phenolic was performed on the basis of a standard curve with gallic acid, which is the major component in *T. chebula* fruit. Total phenolic contents were measured for the extracts obtained with subcritical water extraction at flow rate of 4 ml/min at 120°C, 150°C, 180°C and 220°C and 4 MPa for the first 10 min of extraction, during which the total phenolic contents could mostly be extracted. These results were compared with conventional extraction methods such as hot water extraction at 100°C and soxhlet water, and ethanol extractions, for the same extraction time of 2 hours. The results in Fig. 4.4 showed that the increase in

subcritical water temperature reduced the total amounts of total phenolic compounds extracted. These results were similar to those obtained in the previous work with oregano leaves (Rodríguez-Meizoso et al., 2006) where total antioxidant compounds extracted by subcritical water at several temperatures decreased at higher temperatures. Furthermore, the total phenolic contents of the subcritical water extracts were lower than those in the extracts obtained by hot water extraction at 100°C either in a stirred vessel or by using soxhlet water extraction (Fig.4.4). Despite these results for the total phenolic contents, the amounts of gallic acid and ellagic acid analyzed by HPLC (Fig.4.1a and 4.1b) suggested that the greater the temperature the higher amounts of both compounds were extracted. In addition, it should be noted from Table 4.1 that the subcritical water could extract higher amounts of gallic acid and ellagic acid over the conventional extraction methods, in contrary to the amounts of total phenolic compounds. This indicated that the selectivity of subcritical water extraction for gallic acid and ellagic acid was greater with subcritical water extraction.

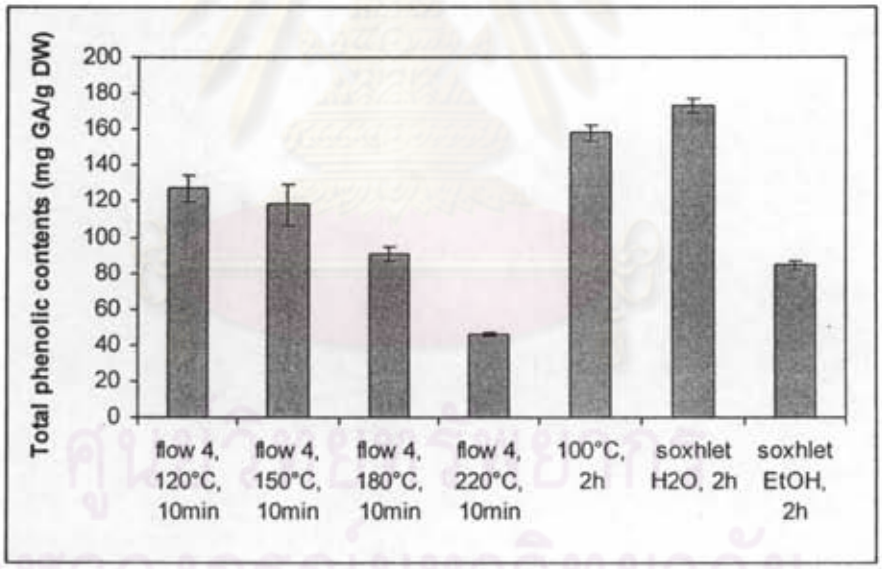


Figure 4.4 Total phenolic contents in extracts obtained by SWE at flow rate of 4 ml/min, hot water extraction at 100°C, and soxhlet water, and ethanol extraction.

4.5 Antioxidant activity

The previous discussion indicated that considerable amounts of phenolic contents in *T.* chebula fruit could be extracted at high temperature conditions as the water polarity is reduced to values similar to those of the extracts. Nevertheless, extraction under high temperature with pressurized conditions could cause product degradation, and therefore the quality of the extracts should be examined. In the present study, the extracts of *T. chebula* fruits produced by various extraction methods were analyzed for the antioxidant activities by using the ABTS procedure as describe in chapter 3. Antioxidant activity was represented by IC_{50} index which was the concentration of the sample solution producing a 50 % reduction in the radical absorbance, thus the higher IC_{50}, the lower antioxidant activity. First, for each of the extraction condition, the extracts were collected at different time, and each of the collected fractions was measured for the antioxidant activity.

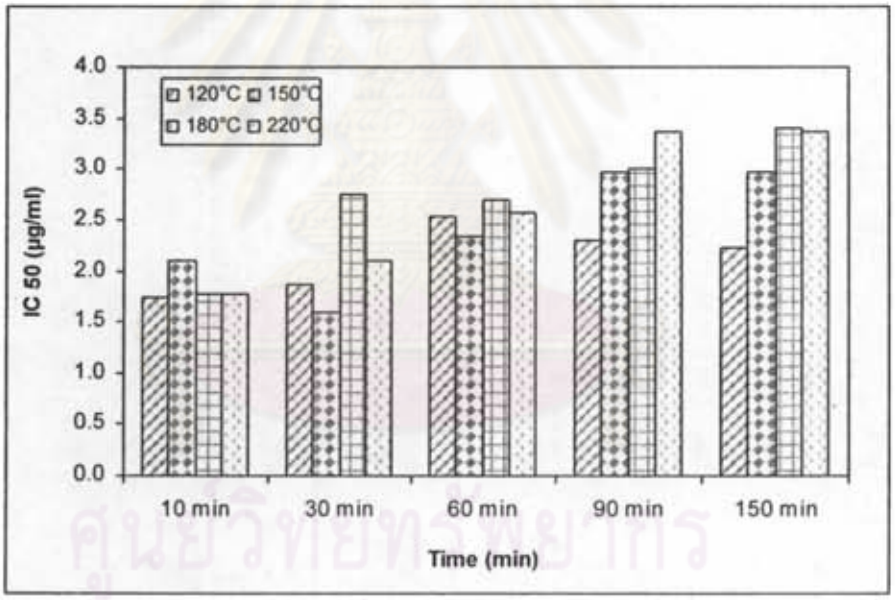


Figure 4.5 Effect of time and temperature on antioxidant activity (IC_{50}) of the *T. chebula* extracts by subcritical water extraction at flow rate of 4 ml/min and 4 MPa.

The result in Fig. 4.5 shows that IC_{50} value was generally the highest for the first fractions collected after 10 min of extraction. The amounts of the phenolic compounds of

interest were also obtained at high quantity in this fraction. Although the extract obtained with subcritical water at temperature 220°C contained the lowest total phenolic compounds, its antioxidant activity was similar to that of the extract obtained at temperature 120°C-180°C, under which higher products were obtained. It is possible that the temperatures of the subcritical water extraction did not seem to have great effect on the antioxidant activity of the early extracts and at higher temperature the extract composition differs from that at lower temperature and may contain higher percentages of more active phenolic compounds or nonphenolic compounds which show high antioxidant activity such as flavonoids. As the extraction proceeded, some antioxidative compounds might be degraded, thus the activity decreased. The low IC_{50} value for extracts obtained in the first 10 minutes of subcritical water extraction was lower than the values obtained with hot water at 100°C and ethanol extraction for 2 hours (Fig.4.6).

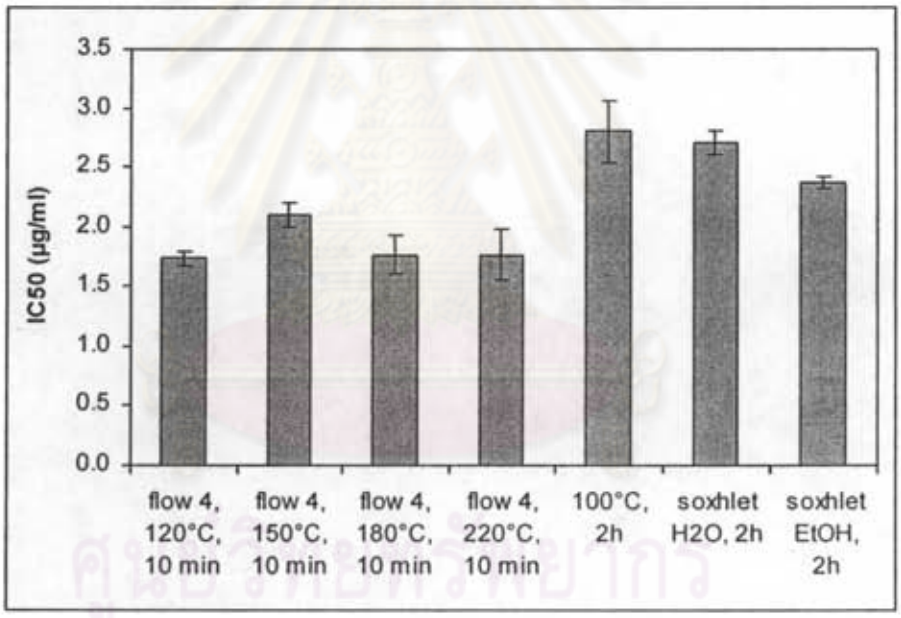


Figure 4.6 Antioxidant activities (IC_{50}) of *T. chebula* extracted by SWE at flow rate of 4 ml/min at 120°C, 150°C, 180°C and 220°C and 4 MPa for first 10 min, and by conventional methods (hot water extraction at 100°C and soxhlet water and ethanol extraction for 2 h).

Figure 4.6 shows the IC_{50} values of the extracts obtained after the first 10 min at various subcritical water conditions at flow rate of 4 ml/min at 120°C, 150°C, 180°C and 220°C and 4 MPa were 1.73, 2.10, 1.77, 1.77 μg/ml, respectively. It should be noted that all the IC_{50} values for extracts obtained with subcritical water were lower than those of the extracts obtained with hot 2 h water extraction at 100°C and soxhlet water and ethanol extraction which were 2.80, 2.70, and 2.367 μg/ml, respectively. This result indicated that the antioxidant activity of the subcritical water extracts was higher than that of the extracts obtained with conventional extraction methods. Moreover, the IC_{50} of *T. chebula* juice product from Chao Phraya Apaipubet hospital was also measured to be 2.53 μg/ml (see Appendix A), which was higher than those obtained with subcritical water extraction. These results demonstrated that subcritical water extraction could more effectively extract considerable amounts of phenolic compounds with high selectivity, and their overall antioxidant activities of these extracts were also higher than those obtained with the conventional extraction methods.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

1. The amounts of gallic acid and ellagic acid increased as temperatures increased up to 180°C. On the other hand, corilagin was more easily decomposed as the temperature increased.
2. At higher temperature than 180°C, the degradation of gallic acid and ellagic acid occurred.
3. At a fixed temperature of 180°C, gallic acid and ellagic acid increased with an increase in volumetric flow rate up to 4 ml/min. For corilagin, the highest amount of this extract was obtained at 3 ml/min.
4. The suitable condition for subcritical water extraction of polyphenolic compounds from *T. chebula* fruits was found to be at the temperature of 180°C and the flow rate of 4 ml/min.
5. With the best condition of subcritical water extraction (180°C, 4 ml/min), the higher amounts of gallic acid and ellagic acid could be extracted in a shorter operation time compared to the conventional methods.
6. Hot water extraction at 100°C was the most suitable method for extraction corilagin.
7. From the experimental studies, the temperature of subcritical water did not have a significant effect on the antioxidant activity of early extracted products.
8. Subcritical water was found to be an effective solvent for extraction of phenolic components from *T. chebula* fruits, resulting in the extracts with higher amounts of the compounds, higher selectivity, and higher antioxidant activities, compared to the extracts obtained by other conventional methods. The advantages and disadvantages of subcritical water extraction are summarized in Table 5.1.

Table 5.1 The advantages and disadvantages of subcritical water extraction.

Advantage	Disadvantage
1. High amounts of the commercial compounds extracted	1.High cost of equipments
2. High selectivity of compounds extracted	2. Product degradation caused by high temperature.
3. High antioxidant activities of the extracts	
4. Short extraction time	
5. Small solvent volume required	

5.2 Recommendations

1. For further study, the factors that affected the extraction efficiency such as particle size and solvent to sample ratio should be considered to improve the efficiency of the method.
2. The possibility of lowering the subcritical water extraction temperature should be considered by coupling water extraction with a benign solubilizing surfactant agent. One example of potential surfactant is propylene glycol which is safe for human consumption, and already used currently as a preservative in pharmaceutical products. The amounts of phenolic compounds and the antioxidant activity of the extracts obtained by the extraction with propylene glycol solution at various conditions should be determined to access the feasibility of such system.
3. Although the extract obtained with subcritical water at temperature 220°C contained the lowest total phenolic compounds, its antioxidant activity was similar to that of the extract obtained at temperature 120°C-180°C, under which higher products were obtained. It is possible that at higher temperature the extract composition differs from that at lower temperature

and may contain higher percentages of more active phenolic compounds or nonphenolic compounds that show high antioxidant activity. More extraction experiments and analysis of the extract composition with HPLC are recommended to confirm this interesting situation.

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APPENDICES

APPENDIX A
EXPERIMENTAL DATA

A-1 Standard calibration curve for HPLC analysis of gallic acid and ellagic acid

Table A-1.1 Standard calibration curve data of gallic acid.

Concentration of gallic acid (mg/ml)	Peak Area (UV detector at 270 nm)
0.027	573.05
0.054	1502.34
0.108	3040.70
0.216	6127.14

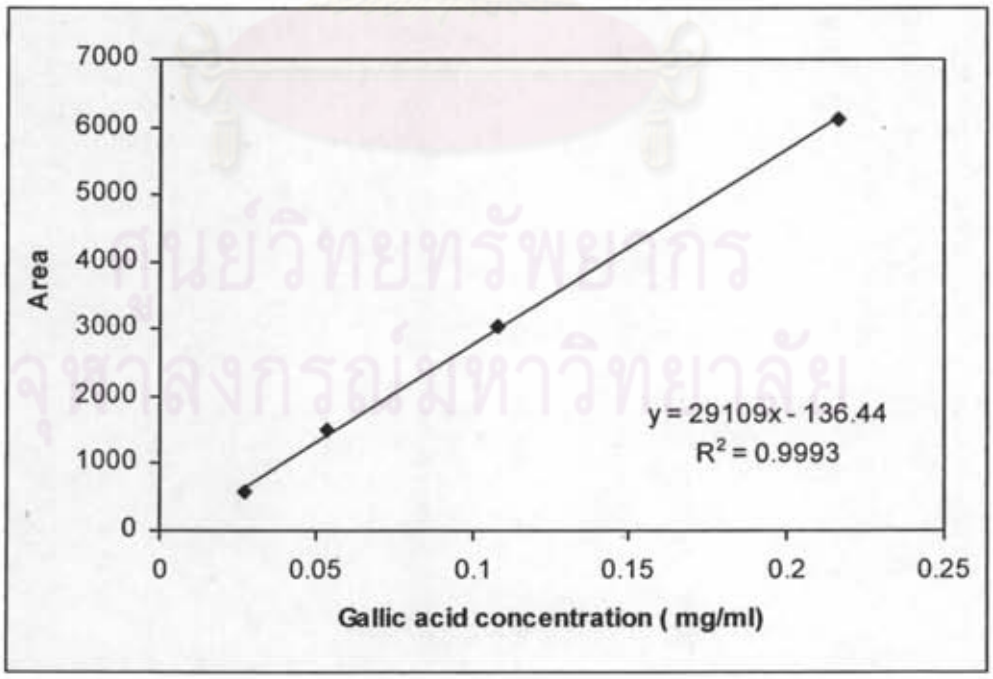

Figure A-1.1 Standard calibration curve of gallic acid.

Table A-1.2 Standard calibration curve data of ellagic acid.

Concentration of ellagic acid (mg/ml)	Peak Area (UV detector at 270 nm)
0.0128	800.71
0.0256	1651.85
0.0512	3425.55
0.1024	6840.90

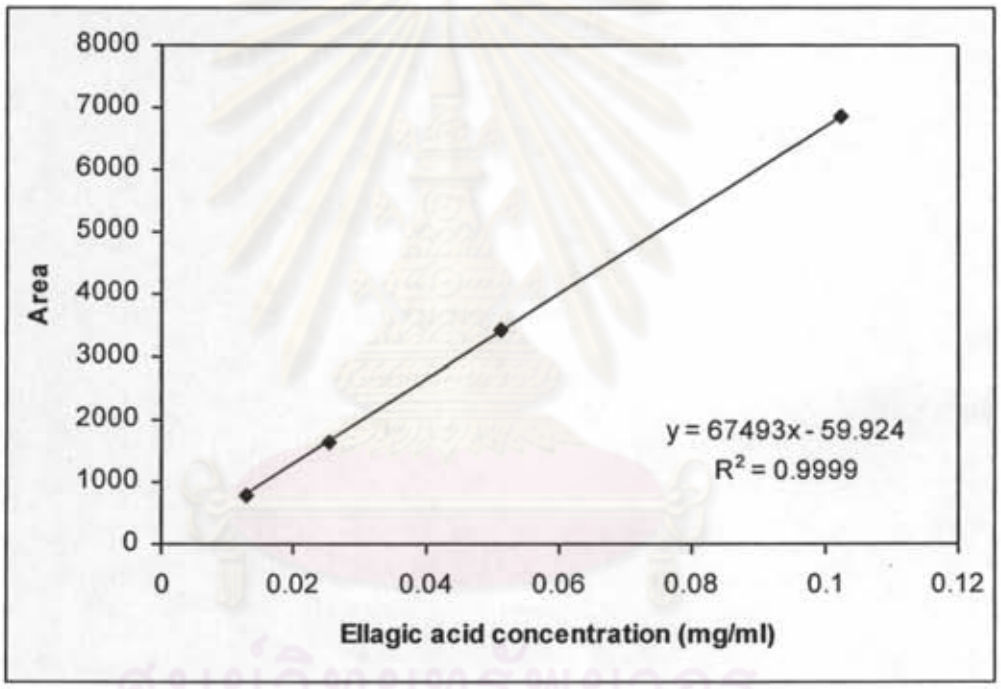

Figure A-1.2 Standard calibration curve of ellagic acid.

A-2 Standard calibration curve of gallic acid

Table A-2.1 Standard calibration curve data.

Concentration of Gallic acid (mg/ml)	Absorbance at 750 nm.			
	No.1	No.2	No.3	Average
1.00000	0.978	0.892	0.934	0.935
0.50000	0.448	0.410	0.472	0.443
0.25000	0.215	0.169	0.217	0.200
0.12500	0.082	0.081	0.076	0.080
0.06250	0.063	0.052	0.059	0.058
0.03125	0.034	0.031	0.035	0.033
0.01563	0.024	0.023	0.027	0.025
0.00781	0.015	0.013	0.017	0.015
0.00391	0.012	0.010	0.016	0.013
0.00195	0.005	0.006	0.012	0.008
0.00098	0.004	0.004	0.003	0.004

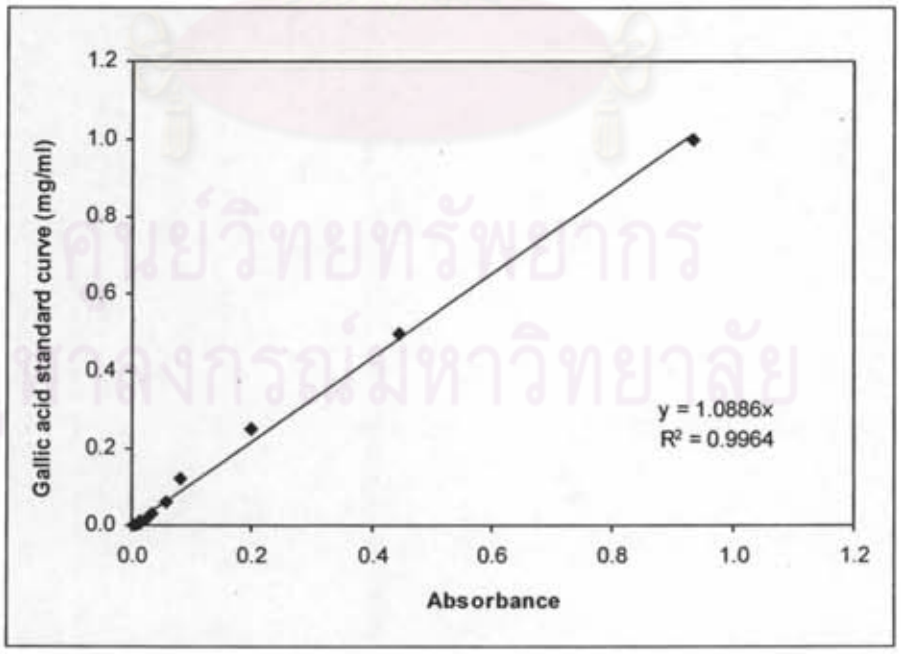

Figure A-2.1 Standard calibration curve of gallic acid (average).

A-3 Experimental data for HPLC analysis of gallic acid, ellagic acid, and corilagin

Table A-3.1 Component contents of subcritical water extraction at a fixed pressure of 4 MPa.

Flow rate (ml/min)	Temperature (°C)	Time (min)	Component contents (mg/g DW)			
			GA	EA	CG	Overall
2	180	300	7.08	3.31	1.79	12.18
3	180	200	8.06	3.73	4.86	16.65
4	120	150	8.09	2.45	6.11	16.65
	150	150	11.67	3.30	2.32	17.29
	180	150	15.47	5.59	1.71	22.77
	220	150	11.51	0.94	0.00	12.45

Table A-3.2 Component contents of conventional extraction methods.

Method	Temperature (°C)	Time (min)	Component contents (mg/g DW)			
			GA	EA	CG	Overall
Hot water extraction	30	1440	5.09	0.47	2.84	8.40
	70	120	5.14	1.06	4.87	11.07
	100	120	7.04	1.48	7.44	15.96
Soxhlet water extraction	100	120	6.37	2.27	5.37	14.00
		240	7.59	2.14	6.23	15.96
Soxhlet ethanol extraction	78.3	120	4.92	3.01	2.00	9.92
		240	3.33	2.35	3.00	8.68

A-4 Experimental data of total phenolic contents

Table A-4.1 Total phenolic contents of hot water extraction (100°C) at various times.

Temperature (°C)	Time (min)	Total phenolic contents (mg GA/g DW)			Std.
		No.1	No.2	Average	
100	60	175.37	172.11	173.74	2.31
	120	191.38	186.48	188.93	3.46
	180	175.70	174.39	175.05	0.92
	240	133.90	131.29	132.59	1.85
	300	144.35	139.45	141.90	3.46
	360	151.04	153.49	152.27	1.73
	420	144.19	146.14	145.16	1.39
	480	147.61	151.70	149.66	2.89
	540	143.04	145.82	144.43	1.96
	600	143.70	145.82	144.76	1.50

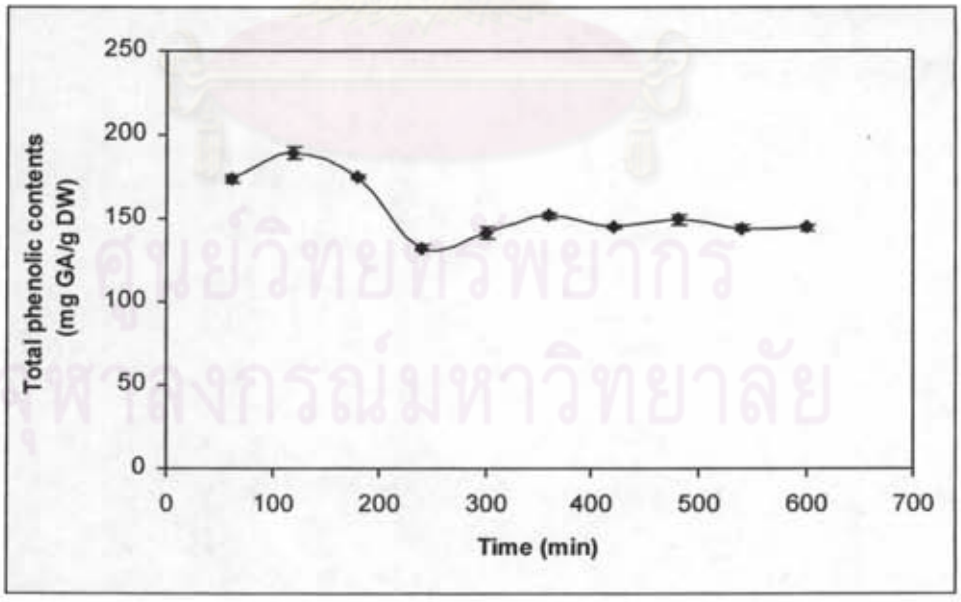

Figure A-4.1 Total phenolic contents at various times of hot water extraction (100°C).

Table A-4.2 Total phenolic contents of subcritical water extraction at temperature = 120 °C, flow = 4 ml/min, pressure = 4 MPa.

Time (min)	Volume (ml)	Total phenolic contents (mg GA/g DW)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	40	135.28	121.77	123.62	126.89	7.32
30	120	29.98	27.33	28.67	28.66	1.32
60	240	9.20	8.81	8.82	8.94	0.22
90	360	3.79	3.46	3.52	3.59	0.17
150	600	5.95	5.44	5.78	5.73	0.26
SUM		184.20	166.82	170.41	173.81	

Table A-4.3 Total phenolic contents of subcritical water extraction at temperature = 150 °C, flow = 4 ml/min, pressure = 4 MPa.

Time (min)	Volume (ml)	Total phenolic contents (mg GA/g DW)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	40	130.65	114.92	108.45	118.01	11.42
30	120	11.28	9.69	11.89	10.95	1.14
60	240	6.65	6.44	6.66	6.59	0.12
90	360	3.43	3.17	3.16	3.25	0.15
150	600	4.16	4.01	4.27	4.14	0.13
SUM		156.17	138.23	134.42	142.94	

Table A-4.4 Total phenolic contents of subcritical water extraction at temperature = 180 °C, flow = 4 ml/min, pressure = 4 MPa.

Time (min)	Volume (ml)	Total phenolic contents (mg GA/g DW)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	40	85.73	93.57	91.61	90.30	4.08
30	120	14.36	13.18	13.20	13.58	0.67
60	240	6.11	6.26	6.29	6.22	0.10
90	360	4.11	3.29	3.27	3.56	0.48
150	600	4.54	3.95	3.82	4.10	0.38
SUM		114.85	120.25	118.19	117.76	

Table A-4.5 Total phenolic contents of subcritical water extraction at temperature = 220 °C, flow = 4 ml/min, pressure = 4 MPa.

Time (min)	Volume (ml)	Total phenolic contents (mg GA/g DW)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	40	47.19	45.18	44.90	45.76	1.25
30	120	15.59	16.20	14.28	15.36	0.98
60	240	7.25	6.42	6.72	6.80	0.42
90	360	4.20	4.02	4.00	4.07	0.11
150	600	5.78	5.40	5.68	5.62	0.20
SUM		80.01	77.21	75.58	77.60	

Table A-4.6 Total phenolic contents of subcritical water extraction at flow = 3 ml/min, temperature = 180 °C, pressure = 4 MPa.

Time (min)	Volume (ml)	Total phenolic contents (mg GA/g DW)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	30	85.43	75.24	78.67	79.78	5.18
20	60	9.06	8.34	8.51	8.64	0.37
30	90	4.67	4.60	4.74	4.67	0.07
60	180	6.63	6.44	6.78	6.62	0.17
90	270	6.43	6.72	6.53	6.56	0.14
120	360	2.97	2.87	2.86	2.90	0.06
150	450	2.24	2.13	2.22	2.20	0.06
200	600	2.42	2.01	2.06	2.16	0.22
SUM		119.85	108.37	112.38	113.53	

Table A-4.7 Total phenolic contents of subcritical water extraction at flow = 2 ml/min, temperature = 180 °C, pressure = 4 MPa.

Time (min)	Volume (ml)	Total phenolic contents (mg GA/g DW)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	20	53.34	49.20	47.35	49.97	3.07
20	40	20.71	18.59	18.14	19.14	1.37
30	60	9.86	8.63	8.23	8.91	0.85
60	120	9.91	8.86	8.16	8.98	0.88
90	180	4.91	5.50	5.45	5.29	0.33
120	240	2.94	3.60	3.83	3.46	0.46
180	360	3.58	4.15	4.27	4.00	0.37
240	480	5.39	5.26	5.95	5.53	0.37
300	600	2.63	3.74	3.39	3.25	0.57
SUM		113.27	107.54	104.78	108.53	

Table A-4.8 Total phenolic contents of conventional methods.

Method	Temperature (°C)	Time (min)	Total phenolic contents (mg GA/g DW)				Std.
			No.1	No.2	No.3	Average	
Hot water extraction	30	1440	82.30	76.42	73.58	77.43	4.45
	70	120	87.35	79.26	75.15	80.59	6.21
	100	120	161.19	158.97	153.05	157.73	4.21
Soxhlet water extraction	100	120	174.33	176.92	168.96	173.40	4.06
		240	151.38	153.42	150.09	151.63	1.68
Soxhlet ethanol extraction	78.3	120	83.18	87.20	82.98	84.45	2.38
		240	82.89	70.74	74.66	76.09	6.20

A-5 Experimental data of antioxidant activity

Table A-5.1 IC_{50} of subcritical water extraction at temperature = 120 °C, flow = 4 ml/min, pressure = 4 MPa.

Time (min)	Volume (ml)	IC50 (µg/ml)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	40	1.70	1.70	1.80	1.73	0.06
30	120	2.00	1.90	1.70	1.87	0.15
60	240	2.80	2.50	2.30	2.53	0.25
90	360	2.50	2.00	2.40	2.30	0.26
150	600	2.40	2.00	2.30	2.23	0.21

Table A-5.2 IC_{50} of subcritical water extraction at temperature = 150 °C, flow = 4 ml/min, pressure = 4 MPa.

Time (min)	Volume (ml)	IC50 (µg/ml)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	40	2.20	2.00	2.10	2.10	0.10
30	120	1.70	1.50	1.60	1.60	0.10
60	240	2.50	2.20	2.30	2.33	0.15
90	360	3.10	3.00	2.80	2.97	0.15
150	600	3.20	2.70	3.00	2.97	0.25

Table A-5.3 IC_{50} of subcritical water extraction at temperature = 180 °C, flow = 4 ml/min, pressure = 4 MPa.

Time (min)	Volume (ml)	IC50 (µg/ml)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	40	1.60	1.80	1.90	1.77	0.15
30	120	2.60	2.95	2.70	2.75	0.26
60	240	2.40	2.80	2.90	2.70	0.26
90	360	2.80	3.20	3.00	3.00	0.20
150	600	3.05	3.55	3.60	3.40	0.31

Table A-5.4 IC_{50} of subcritical water extraction at temperature = 220 °C, flow = 4 ml/min, pressure = 4 MPa.

Time (min)	Volume (ml)	IC50 (µg/ml)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	40	2.00	1.70	1.60	1.77	0.21
30	120	2.30	1.90	2.10	2.10	0.20
60	240	2.60	2.60	2.50	2.57	0.06
90	360	3.40	3.40	3.30	3.37	0.06
150	600	3.40	3.10	3.60	3.37	0.25

Table A-5.5 IC_{50} of subcritical water extraction at temperature = 180 °C, flow = 3 ml/min, pressure = 4 MPa.

Time (min)	Volume (ml)	IC50 (µg/ml)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	30	1.40	1.70	1.60	1.57	0.15
20	60	2.00	2.30	2.00	2.10	0.17
30	90	2.30	2.50	2.60	2.47	0.15
60	180	1.90	2.20	2.20	2.10	0.17
90	270	1.70	1.90	1.80	1.80	0.10
120	360	2.60	2.70	3.00	2.77	0.21
150	450	3.00	3.20	3.20	3.13	0.12
200	600	3.30	3.30	3.40	3.33	0.06

Table A-5.6: IC_{50} of subcritical water extraction at temperature = 180 °C, flow = 2 ml/min, pressure = 4 MPa.

Time (min)	Volume (ml)	IC50 (µg/ml)				Std.
		No.1	No.2	No.3	Average	
0	0	0	0	0	0	0
10	20	1.70	1.80	1.80	1.77	0.06
20	40	1.80	2.00	1.80	1.87	0.12
30	60	1.40	1.30	1.50	1.40	0.10
60	120	1.20	1.50	1.40	1.37	0.15
90	180	2.10	2.20	2.30	2.20	0.10
120	240	2.10	2.40	2.50	2.33	0.21
180	360	2.10	2.40	2.50	2.33	0.21
240	480	1.20	1.40	1.30	1.30	0.10
300	600	2.00	2.40	2.40	2.27	0.23

Table A-5.7 IC_{50} of conventional methods and Samor thai juice.

Method	Temperature (°C)	Time (min)	IC50 (µg/ml)				Std.
			No.1	No.2	No.3	Average	
Hot water extraction	30°C	1440	2.60	2.90	2.80	2.77	0.15
	70°C	120	1.60	2.00	1.90	1.83	0.21
	100°C	120	2.50	3.00	2.90	2.80	0.26
Soxhlet water extraction	100°C	120	2.70	2.60	2.80	2.70	0.10
		240	2.60	3.00	2.90	2.83	0.21
Soxhlet ethanol extraction	78.3°C	120	2.30	2.40	2.40	2.37	0.06
		240	1.90	2.40	2.20	2.17	0.25
Juice	-	-	2.30	2.50	2.80	2.53	0.25

APPENDIX B

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Subcritical Water Extraction of Polyphenolic Compounds from *Terminalia chebula* Fruits

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Subcritical Water Extraction of Polyphenolic Compounds from *Terminalia chebula* Fruits

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Abstract

Fresh or dried fruit of *Terminalia chebula* Retz. is commonly used as herbal medicine as it contains various phytochemicals including gallic acid (GA), ellagic acid (EA), and corilagin (CG). These polyphenolic compounds also exhibited therapeutic properties such as antioxidant, antihyperlipidemia and anticarcinogenic activities. This study investigated the separation of polyphenolic compounds such as gallic acid and ellagic acid from *T. chebula* fruits by subcritical water extraction (SWE). We considered the effect of extraction temperature (120-220°C) and water flow rates (2-4 ml/min) at the pressure of 4-6 MPa on the amount of compounds extracted and determine the suitable conditions for subcritical water extraction of these compounds. The results showed that the amount of GA and EA extracted increased when the extraction temperature increased and they were the highest at 180°C beyond which the products decreased due to thermal degradation. At a fixed temperature of 180°C, the effect of water flow rate on the amount of desirable compounds indicated that the increase in water flow rate gave higher amount of GA and EA extracted. The suitable condition for subcritical water extraction of gallic acid

and ellagic acid from *T. chebula* fruits is at temperature of 180°C and water flow rate of 4 ml/min.

Keywords: Subcritical water extraction, Terminalia chebula, Samor thai, Polyphenolic, Gallic acid, Ellagic acid

1. Introduction

Terminalia chebula Retz. is a native plant in India and Southeast Asia. In Thailand, it is known as *Samor thai*. This herbal plant can be used to treat several illnesses due to its various phytochemicals that exhibit various medicinal properties depending on the part used. The most important part of the plants used is the fruit either fresh or dried *T. chebula* which contains many polyphenolic compounds including gallic acid (GA), ellagic acid (EA), and corilagin (CG) [1]. Their chemical structures and molecular weights are shown in Fig.1. These compounds have several therapeutic activities especially against some chronic diseases including cancer and cardiovascular diseases and antioxidant activities [2].

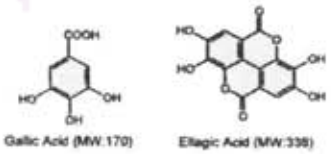

Fig.1 Chemical structures and molecular weights of gallic acid and ellagic acid

Previously, these phenolic antioxidative components in *T. chebula* were extracted by organic solvents such as ethanol, ethyl acetate [3], ether [4], and 70% methanol [5], but organic solvents would be harmful to the consumer's health if it is not properly removed from the extract. Although phenolic compounds can be extracted with hot water (70-80°C) [6], the process takes a long time due to the low solubility of the compound in water. Alternatively, subcritical water extraction (SWE) can be used, which is the extraction with water at the temperature between boiling (100°C) and critical (374°C) temperature, under high enough pressure to maintain water in the liquid state. At this condition, water polarity decreases, thus increases the solubility of several organic compounds in water.

Therefore, this study investigated the suitable conditions for subcritical water extraction of polyphenolic compounds such as gallic acid and ellagic acid from *T. chebula* fruits by

considering the effect of extraction temperature and water flow rates.

2. Materials and Methods

2.1 Materials

Water used in the experiments was distilled and deionized. The dried fruits of *T. chebula* were obtained from the Chulabhorn Research Institute and then crushed into fine powder using a blender.

2.2 Methods

The apparatus of subcritical water extraction is shown in Fig.2. The system consisted of two HPLC pumps (PU 980, JASCO, Japan) used for delivering water and solvent, a degassing instrument (ERC 3215, CE, Japan), an oven (D63450, HARAEUS, Germany), in which 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).

Before heating the extraction system, all connections were checked for possible leakage. The oven was preloaded with 1 g of ground *T. chebula* fruits and the temperature was set to the desired operating condition (120-220°C). When the temperature reached the set point, the extraction started. The degassed water (distilled water without dissolved oxygen) was then delivered at a constant flow rate (2-5 ml/min) with the first HPLC pump to a 3-m preheating section installed in the oven to heat it to the required temperature before passing through the extraction vessel. The pressure of the system was adjusted to the desired condition using the back-pressure regulator valve at the outlet coil. Because pressure has no effect on the water polarity [7], thus in this study, we used the pressure of 4-6 MPa to ensure that water was in liquid state at the temperatures tested. 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. The extract was cooled in a coil immersed in a water bath to prevent possible product degradation, and was then collected in fractions in collecting flasks. After that, the extract was analyzed by HPLC with UV detection at the wavelength of 270 nm.

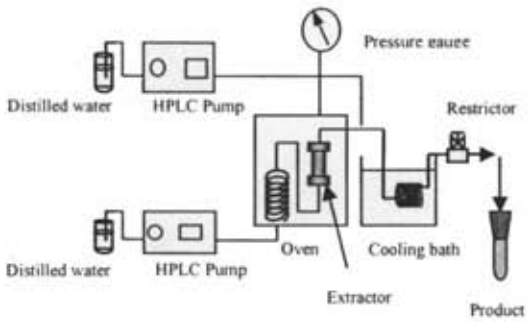

Fig.2 Diagram of experimental setup subcritical water extraction

After each extraction, the amount of polyphenolic compounds remaining in the fruit residue was determined by solvent extraction with distilled water. The fruit residue was taken out of the extractor and placed into a 100 ml Erlenmeyer flask, containing 30 ml of distilled water. It was then allowed to release the products into the solvent overnight. The solution was then replaced with 10 ml of fresh distilled water daily for 5 days.

3. Results and Discussion

3.1 Effect of extraction temperature

In this work, the effect of subcritical water extraction temperature in the range 120-220°C on the amount of gallic acid and ellagic acid at a fixed pressure of 6 MPa and a fixed flow rate of 5 ml/min was determined. To get the maximum possible product, we operated this extraction for 4 hr.

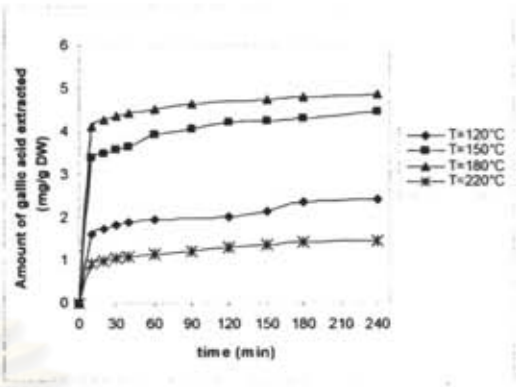

Fig.3 Effect of subcritical water extraction temperature on the amount of gallic acid extracted

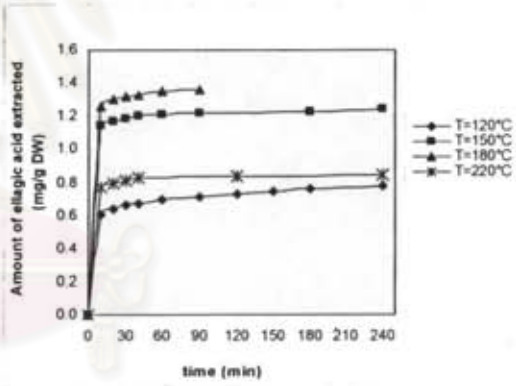

Fig.4 Effect of subcritical water extraction temperature on the amount of ellagic acid extracted

As shown in Fig.3 and Fig.4, as expected the results showed that increasing temperature caused increasing product solubility. In addition, the increased solubility could be also be caused by the decreasing

water polarity at higher temperature. At 180°C, the amount of GA and EA extracted was the highest. However, at 220°C, thermal degradation of the product occurred and this result was confirmed by HPLC analysis which showed that no GA or EA remained in the sample residue. Moreover, it can be seen that the volume of water used and the extraction time about 2 hr were enough for the extraction process.

3.2 Effect of water flow rates

The effect of water flow rate (2-4 ml/min) was determined for extraction at 180°C and at 4 MPa. The results for the amount of gallic acid and ellagic acid extracted versus volume of water used are shown in Fig.5 and Fig.6, respectively. It can be seen from these figures that the amount of the desired compounds increased with an increase in volumetric flow rate up to 4 ml/min. The reason is possibly because at low flow rate, the residence time of the extract in the extractor was higher and thus caused the decomposition of products. The amount of the GA and EA extracted at 4ml/min at 4 MPa was actually higher than that obtained with water at the flow rate of 5 ml/min and at the the pressure of 6 MPa as previously reported. Possible reasons for this should further be investigated.

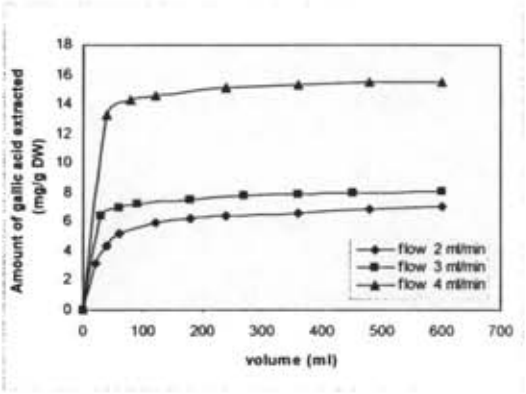

Fig.5 Effect of water flow rate on the amount of gallic acid extracted versus volume of water for SWE at 180°C

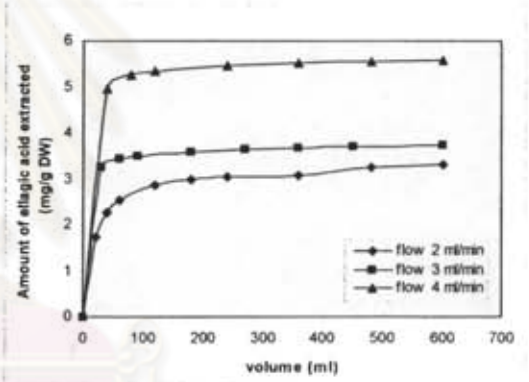

Fig.6 Effect of water flow rate on the amount of ellagic acid extracted versus volume of water for SWE at 180°C

4. Conclusions

The results in the present study showed that the higher the temperature, the greater the amount of phenolic compounds extracted, especially at the temperature of 180°C which gave the highest amount of the

extracts. However, the possible degradation of gallic acid and ellagic acid occurred at high extraction temperature. In the same way, increasing water flow rate increased the products. These results could be concluded that the suitable condition for subcritical water extraction of gallic acid and ellagic acid from *T. chebula* fruits is at temperature of 180°C and water flow rate of 4 ml/min. From these results, it could be suggested that subcritical water extraction is an alternative method for extraction of polyphenolic compounds from *T. chebula* fruits.

5. References

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