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# EXTRACTION AND CONCENTRATION OF PHENOLIC COMPOUNDS FROM *TERMINALIA CHEBULA* FRIUTS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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จันทร์พร ทับทิมดี : การสกัดและการทำให้เข้มข้นของสารประกอบฟีนอลิกจากผลสมอไทย. (EXTRACTION AND CONCENTRATION OF PHENOLIC COMPOUNDS FROM *TERMINALIA CHEBULA* FRIUTS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร. อาทิวรรณ โชติพฤกษ์, 67 หน้า.

งานวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาสภาวะที่เหมาะสมในการสกัดสารฟีนอลิกจากผลสมอไทย และการทำให้สารสกัดเข้มข้นขึ้นด้วยน้ำตาล โดยในกระบวนการสกัดได้ทำการศึกษาระบบตัวทำละลาย น้ำ-เอทานอล และ น้ำ-พรอพิลลีนไกลคอล ซึ่งปัจจัยที่ศึกษาในกระบวนการสกัดคือ อุณหภูมิ ความเข้มข้นของเอทานอล ความเข้มข้นของพรอพิลลีนไกลคอล และ เวลาที่ใช้ในการสกัด โดยใช้การออกแบบการทดลองเข้ามาช่วยหาสภาวะที่เหมาะสมในการสกัด พบว่าสภาวะที่เหมาะสมที่สุดในการสกัดสำหรับระบบน้ำ-เอทานอลคือที่อุณหภูมิ 76 องศาเซลเซียส ความเข้มข้นเอทานอล 76.4 % โดยปริมาตรและเวลาที่ใช้ในการสกัด 82 นาที ในระบบน้ำ-พรอพิลลีนไกลคอล สภาวะที่เหมาะสมที่สุดคืออุณหภูมิ 57 องศาเซลเซียส ความเข้มข้นพรอพิลลีนไกลคอล 36% โดยปริมาตรและเวลาที่ใช้ในการสกัด 23 นาที หลังจากนั้นในส่วนของการทำให้สารสกัดเข้มข้นขึ้นได้นำสารสกัดจากทั้งสองระบบตัวทำละลายที่ให้ปริมาณฟีนอลิกออกมามากที่สุดมาทำเข้มข้นด้วยกระบวนการการทำเข้มข้นด้วยน้ำตาลโดยใช้น้ำตาลกลูโคส พบว่าที่ปริมาณน้ำตาล 200กรัมต่อลิตรสารสกัด ทำให้ได้อัตราส่วนสถานะสารตัวอย่างที่ต้องการของทั้งสองระบบ และได้ทำการศึกษาสัมประสิทธิ์การกระจายตัวของกรดแกลลิก และกรดแอลลาจิกที่ความเข้มข้นน้ำตาลเดียวกันนี้ พบว่าอัตราส่วนสถานะสารตัวอย่างในระบบน้ำ-เอทานอลมีค่า 1.94 และระบบน้ำ-พรอพิลลีนไกลคอลมีค่า 1.85 โดยสัมประสิทธิ์การกระจายตัวของฟีนอลิกในระบบน้ำ-เอทานอลและน้ำ-พรอพิลลีนไกลคอลมีค่าเท่ากันคือ 1.98 ในส่วนของสัมประสิทธิ์การกระจายตัวของกรดแกลลิก และกรดแอลลาจิกในระบบน้ำ-เอทานอล มีค่า4.89 และ 3.52 ตามลำดับ ในระบบน้ำ-พรอพิลลีนไกลคอล ค่าสัมประสิทธิ์การกระจายตัวของกรดแกลลิก และกรดแอลลาจิกมีค่า 4.34 และ 3.22 ตามลำดับ สุดท้ายได้ทำการวัดประสิทธิภาพการต่อต้านอนุมูลอิสระของสารสกัดเข้มข้นพบว่ามีประสิทธิภาพดีกว่าสารสกัดที่ไม่ได้ผ่านกระบวนการทำเข้มข้น นอกจากนี้สารสกัดในระบบน้ำ-พรอพิลลีนไกลยังมีประสิทธิภาพในการต่อต้านอนุมูลอิสระดีกว่าสารสกัดในระบบน้ำเอทานอลและสารสกัดในระบบน้ำอีกด้วย

ภาควิชา.....วิศวกรรมเคมี.....**.....**ลายมือชื่อนิสิต**.....**

สาขาวิชา.....วิศวกรรมเคมี.....**.....**ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก**.....**

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# # 5070661721: MAJOR CHEMICAL ENGINEERING
KEY WORDS: WATER PROPYLENE GLYCOL / *TERMINALIA CHEBULA* / GALLIC ACID / ELLAGIC ACID / SUGARING OUT/ ANTIOXIDANT

CHANTAPORN TUBTIMDEE : EXTRACTION AND CONCENTRATION OF PHENOLIC COMPOUNDS FROM TERMINALIA CHEBULA FRIUTS. THESIS ADVISOR : ASSOC. PROF. ARTIWAN SHOTIPRUK, Ph.D., 67 pp.

This study aimed to extract phenolic compounds from *T.chebula* fruits and concentrate the extract using sugaring out concentration process. The water-ethanol (EtOH) and water- propylene glycol (PG) solvent systems were used in extraction process and the design of experiment (DOE) was employed to determine the optimal extraction conditions (temperature, concentration of EtOH and PG and extraction time). The results showed the optimal condition for water-EtOH was obtained at the temperature of 76°C and concentration of EtOH 76.4 %v/v and 82 min. For water-PG system, the optimal extraction condition was at temperature of 57°C, PG concentration of 36%v/v and extraction time of 23 min. In sugaring out concentration of the extracts, glucose was added to the extracts that contained highest phenolic compounds in both solvent systems from the extraction experiment. The concentration of glucose that gave the desired phase separation ratio as well as the distribution coefficients of gallic acid (GA) and ellagic acid (EA), two major phenolic compounds in *T. chbula*, was determined 200 g/L for both water-EtOH and water-PG systems. The phase ratios for water-EtOH and water-PG were 1.94 and 1.85, respectively. The distribution coefficients of total phenolic for both solvent systems were the same, which was 1.98. Those of GA and EA were 4.89 and 3.52 for water-EtOH, and 4.34 and 3.22 for water-PG system, indicating that sugaring out concentration is a potentially effective method for pheonic compound concentration. Finally, the concentrated extracts show higher antioxidant activities than the nonconcentrated extracts and that antioxidant activity of extract in water-PG was higher than those of the water or water-EtOH extracts.

Department:……Chemical Engineering…...Student’s signature……
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# CHAPTER I

## 1.1 Motivation and significant

The plant *Terminalia chebula* Retzius is a genus of *Terminalia* family and is widely distributed in tropical areas of the world. The fruits of *T. chebula* are commonly known as black Myroblans (Saleem et al., 2002), or in Thailand as Samor Thai. The color of the unripe fruits is green and of the ripe fruits is brown. The taste of the ripe fruits is generally bitter and sour. Traditionally, the dried ripe fruits of *Terminalia chebula* Retzius have been used in the treatment of asthma, sore throat, vomiting, hiccough, diarrhoea, bleeding piles, gout and heart and bladder diseases (Malekzadeh et al., 2001). In Thailand, the plant is commonly known as Samor Thai. The fruits of Samor thai, both fresh and dried, have been reported to have high content of phenolic compounds, which are known to deliver health benefits. In fact, different functional properties of phenolic compounds are dependent on different structures or levels. Two major components in Samor thai are ellagic acid (EA) and gallic acid (GA) (Rangsriwong et al., 2009; Sahelian, 2005). These compounds have strong antioxidant capacity as well as anticancer, antimicrobial, and anti-inflammatory activities. Ellagic acid (EA) exhibits both antibacterial and antioxidant activities by preventing or slowing down the deterioration of cells. It has exhibited not only antioxidant activity, as an inhibitor of in vitro lipid peroxidation, but can also be applied in the food industry as food additive. Gallic acid (GA) has been found to be a pharmacologically active antioxidant, antimutagenic and anticarcinogenic agent. It is capable of inhibiting the growth of cells from several types of tumor.

To extract bioactive compounds in *T.chebula* fruits, different solvents have been investigated, for example, 95% ethyl acetate (Kaur et al., 1998), hot water (Naik et al., 2004), 70% methanol (Gao et al., 2007), and 95% ethanol (Bhattacharya et al., 2009). Subcritical water extraction has also been applied as a benign solvent to extract dried ripe fruits of *T.chebula* Retz. The result showed that the amounts of extracted GA and EA increased with an increase in temperature up to 180°C but when temperature was increased to 220°C, the amount of GA and EA recovery decreased, probably due to the degradation at high temperature (Rangsriwong et al., 2009). To avoid the degradation of products, water-cosolvent systems have been considered to improve the solubility of compounds at relatively low temperature.

Water-Ethanol (Water-EtOH) mixture system, hydroalcoholic, is safe and acceptable for human consumption. The polarity of solvent mixture can continuously be increased by adding water to ethanol. This solvent system can be used for extraction of various organic compounds at lower temperatures compared with subcritical water extraction. It has been successfully used to extract phytochemical compounds from many plants such as phenolics and essential oil from dried sage (Durling et al., 2007) and isoflavones from soybean sprout cotyledon (Cho et al., 2009).

Another interesting water-cosolvent system for extraction is water-propylene glycol. Propylene glycol is a cosolvent and a cosurfactant normally used in pharmacology and as a food additive since it is nontoxic and is generally recognized as safe (GRAS) by the U.S. Food and Drug Administration (FDA). It can alter the polarity of water when the concentration is increased. In pharmaceuticals, water-propylene glycol is used to enhance the solubility of lipophilic drug such as valdecoxib (Liu et al., 2005) and paracetamol (Jouyban et al., 2006). It can also be used as preservative in food ingredients. To the best of our knowledge, few studies have been done using propylene glycol to extract phenolic compounds from plants.

For successful extraction, suitable operating conditions must be determined. One way to achieve this is by using an experimental design and response surface methodology. Central composite design (CCD) is one of the experimental designs, which has been widely used. In addition, response surface methodology (RSM) can be applied to determine the optimal set of experimental parameters that produce a maximum or minimum value of response. Examples of the studies in which CCD and RSM were used to optimize the process include the optimization of dehydration of alcohol–water system by inorganic membrane based pervaporation separation (Wee et al., 2010), the optimization of ethanol extraction and further purification of isoflavones from soybean sprout cotyledon (Cho et al., 2009), as well as the modeling and optimization of a multi-gravity separator for chromite concentration (Aslan., 2008).

After extraction, the extract is usually concentrated. Aqueous two phase system is the current method used to purify natural compounds using short chain alcohol/salt system. The process is simple and requires low energy, and therefore is economical. Previous studies demonstrated that 2,3-butanediol (BD) can be efficiently

separated from fermentation broths by Ethanol/$K_2HPO_4$ system (Jiang et al. 2009) and isopropanol/ammonium sulfate (Sun et al. 2008). However, the process (sometimes called salting out concentration) could cause some unwanted chemical reactions since it requires high concentration of salt. Alternatively, sugaring out effect concentration using monosaccharide or diasaccharide can be carried out to concentrate biomolecules. In a related study, the mixture of acetronitrile-water could be separated into two phases by glucose and xylose (Wang et al., 2008).

The objective of this study was to extract phenolic compounds from the dried ripe fruit of Samor Thai and concentrate the products after extraction. We proposed to use the experimental design and statistical analysis for the investigation of the suitable conditions for extraction with water-ethanol (Water-EtOH) and water-propylene glycol (Water-PG) systems. The effects of temperature of extraction, concentration of ethanol and propylene glycol and extraction time on extraction of phenolic compounds were determined. Furthermore, the extract obtained from the most suitable condition was further concentrated by sugaring out concentration using glucose. Here, the suitable amount of sugar added in to the extracts was determined to obtain phase separation and concentration of total phenolic compounds as well as specific compounds such as ellagic acid (EA) and gallic acid (GA). In addition, the antioxidant activity of concentrated extracts and original extracts in both solvent systems were measured.

## 1.2 Objectives

1.2.1 To extract the phenolic compounds form Terminalia chebula fruits by water-EtOH and water-PG systems.

1.2.2 To find the optimal conditions for extraction using experimental design considering extraction temperature, concentration of EtOH and PG and extraction time.

1.2.3 To concentrate the extract by sugaring out effects using glucose.

1.2.4 To compare the antioxidant activity of concentrated extracts by sugaring out effects with the original extract.

## 1.3 Working scopes

1.3.1 Study of the suitable conditions for extraction of phenolic compounds by determining the effects of temperatures (29-81°C), concentration of ethanol (0-80 %v/v) and propylene glycol (13-47 %v/v) and extraction time (19-71 min) using central composite design of experiment.

1.3.2 Concentrate the extract which contained the highest phenolic compounds in water-EtOH and water-PG solvent systems using sugaring out effect by glucose (concentration of glucose to extract at 50-250 g/L).

1.3.3 Determine the phase ratio of extract and distribution coefficient of phenolic compounds, gallic acid and ellagic acid in the extract at suitable glucose concentration.

1.3.4 Measure and compare the antioxidant activity of the extract before and after sugaring out concentration in water-EtOH with water-PG systems.

## 1.4 Expected benefits

1.4.1 Provide an efficient alternative method for extraction of plant-derived phytochemical bioactive compounds by using water-ethanol and water-propylene glycol system.

1.4.2 Provide the information about sugaring out effect for concentration process of biomolecules.

1.4.3 The results of this research can be used as reference for herbal or natural products for pharmacological studies.

# CHAPTER II

# BACKGROUND AND LITERATURE REVIEWS

## Background

### 2.1 Introduction of *T. chebula*

*Terminalia chebula* Retzius is a perennial plant that grows to about 25-30 meters tall. It belongs to one genus of the *Terminalia* family and is widely distributed in tropical areas of the world. The fruit of *T.chebula* is commonly known as black Myroblans (Saleem et al., 2002). The color of the unripe fruit is green and the ripe fruit is brown. The dried ripe fruit has a bitter and sour taste. Traditionally, the dried ripe fruit of *T.chebula* has been used in the treatment of asthma, sore throat, vomiting, hiccough, diarrhea, bleeding piles, gout, heart and bladder diseases (Malekzadeh et al., 2001). In Thailand, the fruit is commonly known as Samor Thai. Both fresh and dried fruits of Samor thai have been reported to have high content of phenolic compounds which have strong antioxidant capacity such as ellagic acid (EA) and gallic acid (GA). The compounds also have anticancer, antimicrobial, and anti-inflammatory activities (Rangsriwong et al., 2009).

**Figure 2.1** Fresh fruits of *Terminalia chebula* Retz

**Figure 2.2** Dried fruits of *Terminalia chebula* Retz.

## 2.2 Phenolic compounds

The phenolic compound is defined as that containing the phenol functional group compounds. The simplest compound containing the phenolic group is $C_6H_4OH$, with one benzene ring and one hydroxyl group (Bookrags staff, 2005). Phenolic compounds are found in fruits and vegetables and are considered to deliver health benefits by several mechanisms, including: (1) free radical scavenging; (2) protection and regeneration of other dietary antioxidants i.e. vitamin E; and (3) chelating of pro-oxidant metal ions. Phenolic acids constitute about one-third of the dietary phenols; that is present in plants in free and bound forms. The species and levels of phenolic compounds vary dramatically among fruits and vegetables. The different functional properties of phenolic compounds are dependent on different structures or levels. Recently, phenolic compounds derived from fruits have received more attention because of their bioactive functions (Sahelian, 2005; Fang et al., 2009). In *T. chebula* fruits, the phenolic compounds are mostly present as the main components containing the major compounds identified as ellagic acid and gallic acid (Rangsriwong et al., 2009). These phenolic compounds potentially show antioxidant, antimutagen, antitumor, anti-inflammatory, and anticarcinogenic properties (Budrat and Shotipruk, 2009).

Ellagic acid (EA) is a dimeric derivative of gallic acid; a bicyclic derivative, that mainly exists in higher plants, including fruits and nuts. Plants produce EA to protect themselves from microbial infection and pests. The barks of trees are rich in polyphenol components that help to protect the trees against predators and pathogens (Seerama et al., 2005; Vekiari et al., 2008; Zambuchini et al., 2008). Elllagic acid is a

very stable compound with low solubility in water. EA illustrates both antibacterial and antioxidant activity by preventing or slowing down the deterioration of cells caused by microorganisms. In the 1960s, EA was mainly studied for its effects on blood clotting, haemostatic activity and whitening of the skin. But reports about effects of EA on carcinogenesis were published in the following decades. During the past few years, interest in EA has increased due to its possible antimutagenic, antiviral and anticarcinogenic effects. For instance, the abilities for induction of cell-cycle arrest and apoptosis and also for inhibition of tumor formation and growth in animals of EA have been proven by several studies. Also, EA has applied in the food industry as a food additive.

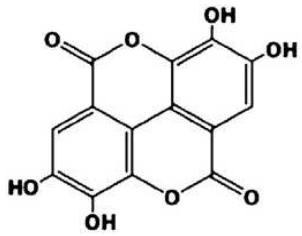



**Figure 2.3** Ellagic acid

Gallic acid (GA) is one of the hydroxybenzoic acids that occurs naturally in plants and has been found to be pharmacologically active as antioxidant, antimutagenic and anticarcinogenic agent. GA is a compound that is able to inhibit the growth of cells from several types of tumor. In addition, GA is used as a material for inks, paints, and color developers. Thus, as a common dietary constituent, GA may affect the digestive and absorptive functions of the gut (Dmitrienko et al., 2002; Gupta et al., 2007; Da Silva et al., 2009).

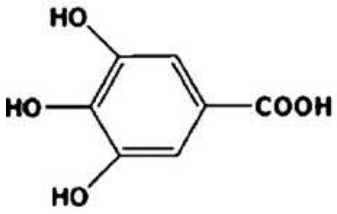



**Figure 2.4** Gallic acid

## 2.3 Solvent Extraction

Solvent extraction is a technique that not only meets the strict environmental regulations but also produces high purity value added products (Kumar et al., 2009). Solvent extraction is usually used to recover a component from either a solid or liquid. The sample is contacted with a solvent that will dissolve the solutes of interest. Solvent extraction is of major commercial importance for chemical and biochemical industries, as it is often the most efficient method of separation of valuable products from complex feed stocks or reaction products.

For engineering purposes, the solvents can be divided into two main categories:

1. Physical Extraction: the existing compounds are transferred from one phase to the other, by means of simple distribution or solvation.

2. Chemical Extraction: the extraction is a chemical process, causing the creation of new molecules, either by ion pairing or complexation (Marinsky and Marcus, 1985).

Some extraction techniques involve partition between two immiscible liquids; others involve either batch extractions or continuous extractions. The solvent can be liquid, vapor or supercritical fluid. The sample can be solid, liquid or gas. Nowadays, wide range of techniques is used for solvent extraction.

## 2.4 Propylene Glycol

Propylene glycol (PG) is a colorless, odorless and miscible with water. It is also known by the systematic name, propane-1,2-diol. Propylene glycol is generally recognized as safe (GRAS) for use as a direct and indirect food additive under prescribed conditions by the U.S. Food and Drug Administration (FDA). Propylene glycol is nontoxic and essentially non-irritating to the skin and mildly irritating to the eyes. Numerous studies support that PG is not a skin sensitizer or a carcinogen. It is not expected to bio-accumulate and it is not acutely toxic to water organisms except at very high concentrations. Propylene glycol has a wide range of practical applications such as antifreeze, coolants, cosmetic, excipient (inert solvent or carrier) in pharmaceuticals and solvent in personal care products.

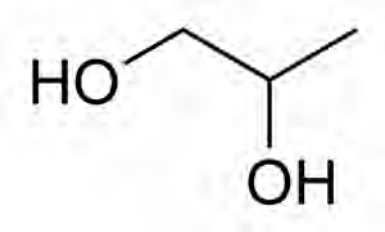



**Figure 2.5** Propylene glycol

## 2.5 Sugaring out concentration

Sugaring-out concentration is a novel phase partition method in which a monomeric carbohydrate or a disaccharide is used to trigger phase separation. The addition of sugar (monomers or disaccharides) above a critical concentration in the mixture induces two-phase formation (Dhamole, 2009). Sugars, uncharged but polar biomolecules, readily dissolve in water because of the stabilizing effect of hydrogen bonds between the hydroxyl groups or the carbonyl oxygen of the sugars and the polar water molecules. The study about sugaring out effect showed the formation of an immiscible acetronitrile (ACN) by the addition of a monomeric sugar or disaccharide as mass-separating agent (MSA) into an ACN–water solution. Some original hydrogen bonds in the mixture are replaced by hydrogen bonds formed between sugar and water molecules, which may force ACN molecules to separate from water molecules and form a new phase (Wang et al., 2008).

## 2.6 Experimental design

The experimental design techniques normally used for process analysis and modeling are the full factorial, partial factorial and central composite design (CCD). A full factorial requires many experiments if the large number of factors and levels are considered. A partial factorial design requires fewer experiments than a full factorial. The central composite design gives almost as much information as factorial but requires fewer tests than the full factorial design. Central composite design allows modeling and determination of optimal conditions. It is used to optimize experimental conditions by drawing response surface (Brachet et al., 2000; Zhang et al., 2007; Aslan, 2008). The central composite design can be rotatable by the choice of $\alpha$. The choice of $\alpha$ in the CCD is dictated primarily by the region of interest. For a spherical

region of interest, the best choice of $\alpha = \sqrt{k}$ it is called a spherical central composite design. The spherical CCD puts all the factorial and axial design points on the surface of a sphere of radius $\sqrt{k}$ (Montgomery, 2004). When this region is a sphere, the design must include center runs to provide an independent estimate of the experimental error. Generally, three to five center runs are recommended.

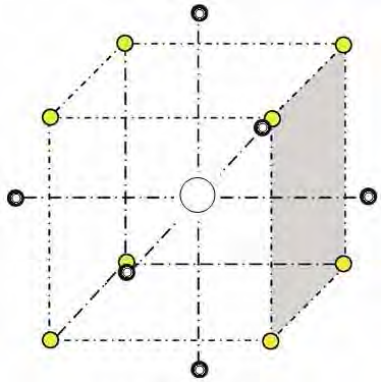


**Figure 2.6** Central composite design

## 2.7 Literature reviews

In the last few decades, there has been a considerable growth in the field of herbal medicine. It is becoming popular in developing and developed countries due to its natural origin and lesser side effects (Naik et al., 2004). Such interest in the phytochemical content of fruits, vegetables and grains has increased because of consumer awareness of its various health and nutraceutical benefits. An intense interest in plant polyphenols in particular is evidenced by numerous papers devoted to various aspects of these compounds (Hayouni et al., 2004). Polyphenols, are widely distributed in the plant kingdom and are important components of common foods, including tea, red wine, fruits, beverages and various medicinal plants. The importance of polyphenols arises from their effects on sensory properties, including astringency and color, and possible health effects (Vekiari et al., 2008). They are antioxidants with redox properties, allowing them to act as reducing agents, hydrogen donators, and singlet and triplet oxygen quenchers. They also have metal chelation properties (Hayouni et al., 2004).

In Indian traditional system of medicine, the *Terminalia chebula* Retzius is a popular folk medicine which contains high contents of phenolic compounds. In Thailand, the fruit of *T.chebula* known as Samor thai, has been reported to have

strong antioxidant capacity and contains phenolic compounds such as gallic acid (GA) and ellagic acid (EA). These compounds have been shown to have anticancer, antimicrobial, and anti-inflammatory properties. To extract the phenolic compounds from *T. chebula* fruits, solvent extraction is used because it is efficient, adaptable, reproducible, and can be easily used for performing continuous and mass production (Rydberg, 2004; Guo et al., 2008). The different solvent systems have been used for the extraction of polyphenols from plant materials. The extraction yield is depended on the solvent and the method of extraction. Water, aqueous mixtures of ethanol, methanol, and acetone, are commonly used in plant extraction (Hayouni et al., 2004). Due to the different polarities of the active constituents, and the acceptability of this solvent system for human consumption, the water-EtOH mixtures are possibly the most suitable solvent systems. Since water has a strong polar solvent while ethanol is a low-polar solvent, and they are capable of mixing in any ratio. With the addition of water to ethanol, the polarity of complex solvent will increase continuously (Zhang et al., 2007). The extraction of *T. chebula* using ethanol as solvent has been used extensively. (Rangsriwong et al., 2009; Gao et al., 2007; Bhattacharya et al., 2009). Kaur et al (1998) claimed that successful extraction of *T. chebula* was obtained by using 95% ethyl alcohol and ethyl acetate extraction for 48 hrs. The extracts of *Emblica officinalis*, *Terminalia bellerica* and *Terminalia chebula* in 95% ethanol for one day showed that all the extracts could effectively prevent lipid peroxidation by many measuring assays (Bhattacharya et al., 2009). Durling et al. (2007) extracted phenolics and essential oil from dried sage (*Salvia officinalis*) using ethanol–water mixtures. Their result showed that water-EtOH solvent ratio had the most pronounced effect on the recoveries of sage bioactive compounds. At high ethanol contents there was a high recovery of the more lipophilic compounds but a low recovery more hydrophilic compounds. Cho et al. (2009) investigated the optimization of ethanol extraction of isoflavones from soybean sprout cotyledon. The maximum amount of isoflavone in aqueous ethanol extracts was obtained when isoflavones in cotyledons were extracted with 80–90% (v/v) aqueous ethanol. The study on optimization of ethanol–water extraction of lignans from flaxseed revealed that the acquired ratio of lignans (organic matter) increased with increasing of the ethanol proportion in the extraction medium up to 70% and then began to decline with the further increase of ethanol proportion in the extraction medium. The solvent with 70% ethanol content was chosen for the determination of optimal extraction temperature and time (Zhang

et al., 2007). Moreover, the study on ethanol–water solvent system for extraction of podophyllotoxin showed that at high concentration of ethanol in the solvent significantly reduced the yield and extraction rate of podophyllotoxin. At 30% ethanol and 53°C, the maximum yield and extraction rate were obtained. Also the effective diffusivity and relative equilibrium concentration of podophyllotoxin obtained their minimum and maximum values at 30°C, 100% ethanol and 53°C, 30% ethanol, respectively (Izadifar and Baik, 2008). Cacace and Mazza (2003) reported that the extraction rate of phenolic compounds was enhanced with ethanol concentration from 39 to 67% and then was reduced with further increase in the concentration from 67 to 95% ethanol. In addition to water-ethanol mixtures, water-polyvalent alcohol mixtures have been used in pharmaceuticals in order to increase the solubility of drugs that are poorly soluble in water during the design of homogeneous pharmaceutical dosage forms such as syrups and elixirs. Among the polyvalent alcohols, propylene glycol is one of the most important cosolvent and cosurfactant because of its remarkable nontoxicity and its ability to increase the solubility of drug. Propylene glycol can alter the polarity in water-PG system if its concentration increases. Propylene glycol is used to solubilize lipophilic drugs in aqueous vehicles (Kale and Allen 1989; Bendas et al., 1995; Zhou et al., 2009). Valdecoxib is a newly introduced nonsteroidal anti-inflammatory drug which is poor solubility in water. The solubility of valdecoxib can be enhanced by the addition of propylene glycol in water (Liu et al. 2005). Water-Propylene glycol mixture can increase the solubility of paracetamol compared with the solubility of paracetamol in water. High amount of cosolvent enhanced the solubility of drug until the maximum solubility was reached (Jouyban et al. 2006). Besides, propylene glycol is used as an emulsification agent in Angostura and orange bitters because of its properties of an emulsifier. Also propylene glycol can use as preservative for example it has been successfully used in the preservation of erythrocytes and mammalian embryos from a variety of species (Villalba et al., 1996).

As the next step of natural compound separation, concentration of the extract is the important process. One of the most interesting concentration processes is aqueous two phase extraction (ATPE) which has been widely used for concentration and separation of biomacromolecules such as proteins and nucleic acids. Nowadays, aqueous two phase concentration using short chain alcohol/salt system called salting-

out effect is often used. The aqueous two-phase system is formed after adding alcohol/salt system. The top phase is rich with alcohol and the bottom phase is rich in inorganic salt. This technique is applied to purify natural compounds because of its advantages; low cost, easy recovery of alcohol by evaporation and simple scale-up. However, salting out occurs at high concentration of salt which may cause some unwanted chemical reactions and may not be suitable to concentrate and separate biological components.

Bin Wang et al (2008) studied the new process for separation and extraction called sugaring out effect. Monomeric sugar or diasaccharide were added into an acitronitrile (ACN)-water solution and kept at low temperature to performed phase separation. The sugaring out effect caused the two phases form and ACN was separated from water into top phase. Increasing the sugar concentration also increased the observed phase separation ratio in water-ACN solutions.

As mentioned above exhibited that water-ethanol system and water-propylene glycol system are ones safe method which can increase hydrophobicity of solvent and enhance interesting product from extraction process. Most of extraction process from previous study intended to extracted phenolic compounds from *T.chebula* for pharmacological study only. From the above mentioned reasons, this study focused on the extraction of phenolic compounds from *T. chebula* fruits using water-ethanol and water-propylene glycol solvent systems. Water-ethanol and water-propylene glycol systems would increase the phenolic compounds in extract and help to extract less polar compounds from *T.chebula* fruits such as ellagic acid. The experimental design was used to find the optimal extraction condition of both solvent systems. The considering variables in extraction were the temperature of extraction, the concentration of ethanol in water and concentration of propylene glycol in water, and the extraction time.

After extraction, the extracts in both solvent systems which contained the highest phenolic compounds were concentrated by sugaring out effects. Monomeric sugar, glucose, was used in the experiment. The amount of adding glucose in particular was studied. Finally, the extracts were measured and compared antioxidant activity of concentrated extract with original extract.

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Materials and chemicals

The dried fruits of *Terminalia chebula* were obtained from Chulabhorn Research Institute. All the samples were finely powdered through a mesh of size 500 μM. The reference standard chemicals (gallic acid and ellagic acid) and Folin-Ciocalteau reagent were purchased from Sigma-Aldrich Chemicals (Missouri, USA). Water used in the experiments was distilled and de-ionized water. Ethanol, formic acid and HPLC grade methanol, were purchased from Merck (Darmstadt, FR, Germany). Propylene glycol was purchased from Namsian (Bangkok, Thailand). Glucose was purchased from Ajax Finechem (NSW, Australia)

## 3.2 Extraction

Dried ripe fruit of *T.chebula* was powdered using blender. The powdered *T. chebula* was sieved through a 500μM mesh. One gram of powdered *T. chebula* fruits was suspended in a 2 neck round bottom flask, connected to a reflux column and a thermometer. The sample was extracted with 150 ml of extraction solvent: water-EtOH or water-PG mixture. The effect of temperature, concentration of organic solvent and time were studied on the amount of phenolic content in the extracts.

### 3.2.1 Experimental design for extraction

In this study, the optimization of phenolic compounds from *T.chebula* extracts in water-ethanol (EtOH) and water propylene glycol (PG) systems was studied using an experimental design. The spherical CCD was used in which the region of interest was $\alpha = \sqrt{k}$, where k is the number of variables. Three independent variables were chosen; temperature ($X_1$), concentration($X_2$) and time($X_3$). This design puts all the factorial and axial design points on the surface of a sphere of radius $\sqrt{k}$ as shown in Figure 3.1.

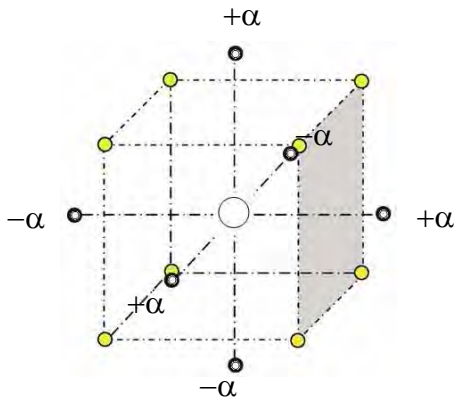



**Figure 3.1** Central composite orthogonal rotatable design

The low, middle and high levels of each variable were designated -1.73, 0 and +1.73, respectively. The corresponding actual values for each variable are listed in Table 3.1. Seventeen experiments were performed; eight factorial points for three factors each at five levels, six axial points and three center runs for the measurement of the error. The experimental matrix for three-variable spherical central composite design is shown in Table 3.2.

**Table 3.1:** Coded variables for extraction

| Variables | -1.73 | -1 | 0 | 1 | 1.73 |
|---|---|---|---|---|---|
| $X_1$ = Temp (°c) | 29 | 40 | 55 | 70 | 81 |
| $X_2$ = EtOH (%v/v) | 0 | 17 | 40 | 63 | 80 |
| $X_2$ = PG (%v/v) | 13 | 20 | 30 | 40 | 47 |
| $X_3$ = Time (min) | 19 | 30 | 45 | 60 | 71 |

**Table 3.2:** The Spherical CCD for three independent variables.

| Run | X1 | X2 | X3 |
|---|---|---|---|
| 1 | -1 | -1 | -1 |
| 2 | 1 | -1 | -1 |
| 3 | -1 | 1 | -1 |

| Run | X1 | X2 | X3 |
|---|---|---|---|
| 4 | 1 | 1 | -1 |
| 5 | -1 | -1 | 1 |
| 6 | 1 | -1 | 1 |
| 7 | -1 | 1 | 1 |
| 8 | 1 | 1 | 1 |
| 9 | -1.73 | 0 | 0 |
| 10 | 1.73 | 0 | 0 |
| 11 | 0 | -1.73 | 0 |
| 12 | 0 | 1.73 | 0 |
| 13 | 0 | 0 | -1.73 |
| 14 | 0 | 0 | 1.73 |
| 16 | 0 | 0 | 0 |
| 17 | 0 | 0 | 0 |
| 17 | 0 | 0 | 0 |

The correlation between the independent variables and the response was calculated by the following second-order polynomial equation (3.1).

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^{\,2} + \sum_{i=1}^{3} \sum_{j=1}^{3} \beta_{ij} X_i X_j \; ; \; i \neq j \qquad (3.1)$$

Where Y = response, $X_i$ = coded variables of input variables, $\beta_0$ = interception, $\beta_{ii}$ = regression coefficient and $\beta_{ij}$ is cross-product coefficient. Design and analysis of the central composite experiment were carried out using Statistical Package for the Social Sciences (SPSS).

### 3.3 Sugaring Out Concentration

To concentrate the extract by sugaring out method, 10 ml of extracts in water-EtOH and water-PG solutions obtained from the previous experiments giving the highest total phenolic compounds were placed into a test tube. Glucose was then added into the extracts, after which, the extracts were heated until all added glucose dissolved completely, at which point the phase separation was induced. The effects of

the glucose concentrations (50, 100,150, 200, and 250 g/L) were determined on the efficiency of the phase separation, which was quantified in terms of the phase ratio (R) in (3.2).

$$R= V_{top}/V_{bottom}, \quad (3.2)$$

where $V_{top}$ and $V_{bottom}$ are the volumes of the top phase and the bottom phase, respectively. At a selected glucose concentration giving the highest phase ratio, the concentrations of the total phenolic compound and specific compounds (GA and EA) in each phase were analyzed using HPLC.

The distribution coefficients (D) of were determined for total phenolic compound, gallic acid and ellagic acid in water-EtOH and water-PG from the following equation.

$$D= C_{top}/C_{bottom} \quad (3.3)$$

where $C_{top}$ and $C_{bottom}$ are the corresponding compound concentrations of the top phase and the bottom phase , respectively.

## 3.4 Analysis

### 3.4.1 Determination of total phenolic content

The determination of the total phenolic content was carried out using Folin-Ciocalteau method modified from that described in previous study (Rodríguez-Meizoso et al., 2006). Initially, 0.1 ml of the extracts from water-ethanol and water-propylene glycol extractions were dissolved in 2.8 ml distilled water. 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 vortex. The mixture was left at room temperature for 30 min, after which the absorbance was measured at 750 nm using distilled water as a reference. The total phenolic content was calculated on the basis of calibration curve of gallic acid.

### 3.4.2 HPLC analysis of *T. chebula* extracts

The analysis of ellagic acid and gallic acid was performed using high performance liquid chromatography (HPLC). 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.

### 3.4.3 Antioxidant activity

Antioxidant activity of the water-EtOH and water-PG extracts which contained the highest phenolic compounds was measured before and after concentration by using ABTS method, which was modified from that described by Re et al., 1999. For the purpose of comparing the antioxidant activity in various extracts, 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 first diluted in series of distilled water, then each diluted extract was then 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) at 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 measure at 734 nm using distilled water as a reference.

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

$$PI(\%) = [(1-A_t/A_r)]\times 100 \qquad (3.4)$$

$A_t$ 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 $IC_{50}$

Beside the $IC_{50}$ value above, the antioxidant activity of the extracts before and after concentration were also compared in terms for percent inhibition, which was determined using various volume ratios of extract:ABTS solution, summarized in the following table.

**Table 3.3:** The volume ratio of extract:ABTS solution

| | |
|---|---|
| Volume ratio (ml) of Extract : ABTS solution | 0.025:2, 0.025:5, 0.025:10, 0.025:15 |

# CHAPTER IV

# RESULTS AND DISCUSSION

This study can be divided into two parts. The first deals with *T.chebula* extraction, carried out using solvent systems of water-ethanol (EtOH) and water-propylene glycol (PG). The spherical central composite design (spherical CCD) and data analysis were employed to investigate the main effects and the interactions between each factor. The results from extraction were then fitted with a response surface equation to find the optimal extraction condition. In the second part, the extracts containing the highest amount of phenolic compounds obtained for both water-EtOH and water-PG solvent systems in Part I were concentrated by using a new "sugaring out" technique, in which glucose was added to the extracts to induce phase separation. The phase ratio and the distribution coefficient of total phenolic, and specific phenolic compounds such as gallic acid (GA) and ellagic acid (EA) were measured. Finally, the antioxidant activities of the extracts obtained by water-EtOH and water-PG extraction, and those of the sugaring out concentrated extracts were measured and compared.

## 4.1 Statistical analysis and main effects of extraction conditions on phenolic content (mg/gDW).

In this study, the spherical CCD experimental design was used to evaluate the main and the interaction effects on the extraction of phenolic compounds (response Y) from *T.chebula*. The considered variables were temperature ($X_1$), concentration of organic solvent ($X_2$) and time ($X_3$). The factors and levels of spherical central composite design are shown in Table 4.1. The results of total phenolic content are illustrated in Table 4.2.

**Table 4.1:** Factors and levels in experimental design

| Variables | Levels | | | | |
|---|---|---|---|---|---|
| | -1.73 | -1 | 0 | 1 | 1.73 |
| $X_1$ = Temp (°c) | 29 | 40 | 55 | 70 | 81 |
| $X_2$ = EtOH (%v/v) | 0 | 17 | 40 | 63 | 80 |
| $X_2$ = PG (%v/v) | 13 | 20 | 30 | 40 | 47 |
| $X_3$ = Time (min) | 19 | 30 | 45 | 60 | 71 |

**Table 4.2:** Extraction results from all experiments.

| Run | $X_1$ | $X_2$ | $X_3$ | $Y_{water\text{-}EtOH}$ | $Y_{water\text{-}PG}$ |
|---|---|---|---|---|---|
| 1 | -1 | -1 | -1 | 179.41 | 165.96 |
| 2 | 1 | -1 | -1 | 177.34 | 177.62 |
| 3 | -1 | 1 | -1 | 177.01 | 176.97 |
| 4 | 1 | 1 | -1 | 184.89 | 171.49 |
| 5 | -1 | -1 | 1 | 158.86 | 157.00 |
| 6 | 1 | -1 | 1 | 162.97 | 168.53 |
| 7 | -1 | 1 | 1 | 174.59 | 177.58 |
| 8 | 1 | 1 | 1 | 218.40 | 205.64 |
| 9 | -1.73 | 0 | 0 | 162.36 | 142.38 |
| 10 | 1.73 | 0 | 0 | 173.08 | 167.62 |
| 11 | 0 | -1.73 | 0 | 128.67 | 175.47 |
| 12 | 0 | 1.73 | 0 | 187.26 | 188.39 |
| 13 | 0 | 0 | -1.73 | 197.37 | 186.80 |
| 14 | 0 | 0 | 1.73 | 176.53 | 165.30 |
| 15 | 0 | 0 | 0 | 205.74 | 174.65 |
| 16 | 0 | 0 | 0 | 200.09 | 176.13 |
| 17 | 0 | 0 | 0 | 201.32 | 175.97 |

From Table 4.2, the analysis of variance (ANOVA) was conducted using SPSS 16.0 program to determine the factors that have important effects on the amount of phenolic content. The analysis indicated the model significance of 95% confidence intervals, and the results are summarized in Table 4.3-4.4 for water-EtOH and water-PG extractions, respectively.

**Table 4.3:** ANOVA table for phenolic content from water-EtOH extraction

Dependent Variable:Y

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 7142.080[a] | 14 | 510.149 | 57.879 | .017 |
| Intercept | 303022.492 | 1 | 303022.492 | 34379.272 | .000 |
| $X_1$ | 1860.177 | 3 | 620.059 | 70.349 | .014 |
| $X_2$ | 4811.742 | 3 | 1603.914 | 181.971 | .005 |
| $X_3$ | 504.776 | 3 | 168.259 | 19.090 | .050 |
| $X_1 * X_2$ | 308.099 | 1 | 308.099 | 34.955 | .027 |
| $X_1 * X_3$ | 221.648 | 1 | 221.648 | 25.147 | .038 |
| $X_2 * X_3$ | 544.692 | 1 | 544.692 | 61.798 | .016 |
| $X_1 * X_2 * X_3$ | 110.789 | 1 | 110.789 | 12.570 | .071 |
| Error | 17.628 | 2 | 8.814 | | |
| Total | 560077.059 | 17 | | | |
| Corrected Total | 7159.708 | 16 | | | |

a. R Squared = .998 (Adjusted R Squared = .980)

From the ANOVA analysis of water-EtOH system, the results showed that the main effect of all factors, temperature ($X_1$), concentration ($X_2$) and time ($X_3$), and all pairwise interactions were significant to the phenolic contents (Y). On the other hand, for extraction with water-PG, the ANOVA analysis in Table 4.4 showed that the main effects of all three factors and the interactions between temperature and time as well as that of temperature and concentration were significant to phenolic content (Y) from *T.chebula* fruits.

**Table 4.4:** ANOVA table for phenolic content from water-PG extraction

Dependent Variable:Y

| Source | Type III Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|
| Corrected Model | 2921.504[a] | 14 | 208.679 | 318.297 | .003 |
| Intercept | 306178.104 | 1 | 306178.104 | 467012.623 | .000 |
| $X_1$ | 1088.977 | 3 | 362.992 | 553.671 | .002 |
| $X_2$ | 621.000 | 3 | 207.000 | 315.737 | .003 |
| $X_3$ | 266.168 | 3 | 88.723 | 135.328 | .007 |
| $X_1 * X_2$ | .047 | 1 | .047 | .071 | .814 |
| $X_1 * X_3$ | 139.595 | 1 | 139.595 | 212.924 | .005 |
| $X_2 * X_3$ | 348.746 | 1 | 348.746 | 531.941 | .002 |
| $X_1 * X_2 * X_3$ | 141.800 | 1 | 141.800 | 216.288 | .005 |
| Error | 1.311 | 2 | .656 | | |
| Total | 516053.467 | 17 | | | |
| Corrected Total | 2922.816 | 16 | | | |

a. R Squared = 1.000 (Adjusted R Squared = .996)

## 4.2 The optimal condition for water-EtOH and water-PG extraction of phenolic compounds from *T.chebula* fruits.

The relations between each factor and the phenolic compounds were modeled with a response surface using 2nd order polynomial model. By the use of statistical program SPSS 16.0, the analysis obtained was summarized in Table 4.5-4.7 and 4.8-4.10, for water-EtOH and water-PG systems, respectively.

**Table 4.5:** Model summary for $Y_{water\text{-}EtOH}$ (phenolic content in water-EtOH system)

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
|---|---|---|---|---|
| 1 | .949[a] | .901 | .775 | 10.042 |

a. Predictors: (Constant), $X_3X_3$, $X_2X_3$, $X_1X_3$, $X_1X_2$, $X_3$, $X_2$, $X_1$, $X_2X_2$, $X_1X_1$

**Table 4.6:** ANOVA Table for optimal condition of phenolic content of $Y_{water\text{-}EtOH}$ system

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1 | Regression | 6453.797 | 9 | 717.089 | 7.111 | .008[a] |
| | Residual | 705.911 | 7 | 100.844 | | |
| | Total | 7159.708 | 16 | | | |

a. Predictors: (Constant), $X_3X_3$, $X_2X_3$, $X_1X_3$, $X_1X_2$, $X_3$, $X_2$, $X_1$, $X_2X_2$, $X_1X_1$

b. Dependent Variable: Y

**Table 4.7:** Coefficients[a] for response surface equation for $Y_{water\text{-}EtOH}$

| Model | | Unstandardized Coefficients | | Standardized Coefficients | t | Sig. |
|---|---|---|---|---|---|---|
| | | B | Std. Error | Beta | | |
| 1 | (Constant) | 202.360 | 5.798 | | 34.903 | .000 |
| | $X_1$ | 5.167 | 2.685 | .228 | 1.924 | .096 |
| | $X_2$ | 12.703 | 2.685 | .561 | 4.731 | .002 |
| | $X_3$ | -2.851 | 2.685 | -.126 | -1.062 | .324 |
| | $X_1X_2$ | 6.206 | 3.550 | .207 | 1.748 | .124 |
| | $X_1X_3$ | 5.264 | 3.550 | .176 | 1.483 | .182 |
| | $X_2X_3$ | 8.251 | 3.550 | .276 | 2.324 | .053 |
| | $X_1X_1$ | -9.975 | 2.880 | -.447 | -3.463 | .011 |
| | $X_2X_2$ | -13.234 | 2.880 | -.594 | -4.594 | .003 |
| | $X_3X_3$ | -3.550 | 2.880 | -.159 | -1.232 | .258 |

a. Dependent Variable: Y

**Table 4.8:** Model summary for $Y_{water\text{-}PG}$ (phenolic content in water-PG system)

| Model | R | R Square | Adjusted R Square | Std. Error of the Estimate |
|---|---|---|---|---|
| 1 | .910[a] | .828 | .607 | 8.473 |

a. Predictors: (Constant), $X_3X_3$, $X_2X_3$, $X_1X_3$, $X_1X_2$, $X_3$, $X_2$, $X_1$, $X_2X_2$, $X_1X_1$

**Table 4.9:** ANOVA Table for optimal condition of phenolic content of $Y_{water\text{-}PG}$ system

| Model | | Sum of Squares | df | Mean Square | F | Sig. |
|---|---|---|---|---|---|---|
| 1 | Regression | 2420.200 | 9 | 268.911 | 3.745 | .048[a] |
| | Residual | 502.587 | 7 | 71.798 | | |
| | Total | 2922.787 | 16 | | | |

a. Predictors: (Constant), $X_3X_3$, $X_2X_3$, $X_1X_3$, $X_1X_2$, $X_3$, $X_2$, $X_1$, $X_2X_2$, $X_1X_1$

b. Dependent Variable: Y

**Table 4.10** Coefficients[a] for response surface equation for $Y_{water\text{-}PG}$

| Model | | Unstandardized Coefficients | | Standardized Coefficients | t | Sig. |
|---|---|---|---|---|---|---|
| | | B | Std. Error | Beta | | |
| 1 | (Constant) | 175.574 | 4.892 | | 35.889 | .000 |
| | $X_1$ | 6.395 | 2.266 | .442 | 2.822 | .026 |
| | $X_2$ | 6.071 | 2.266 | .420 | 2.679 | .032 |
| | $X_3$ | -1.465 | 2.266 | -.101 | -.647 | .538 |
| | $X_1X_2$ | -.077 | 2.996 | -.004 | -.026 | .980 |
| | $X_1X_3$ | 4.177 | 2.996 | .219 | 1.394 | .206 |
| | $X_2X_3$ | 6.602 | 2.996 | .345 | 2.204 | .063 |
| | $X_1X_1$ | -6.089 | 2.431 | -.428 | -2.505 | .041 |
| | $X_2X_2$ | 2.909 | 2.431 | .204 | 1.197 | .270 |
| | $X_3X_3$ | .946 | 2.431 | .066 | .389 | .709 |

a. Dependent Variable: Y

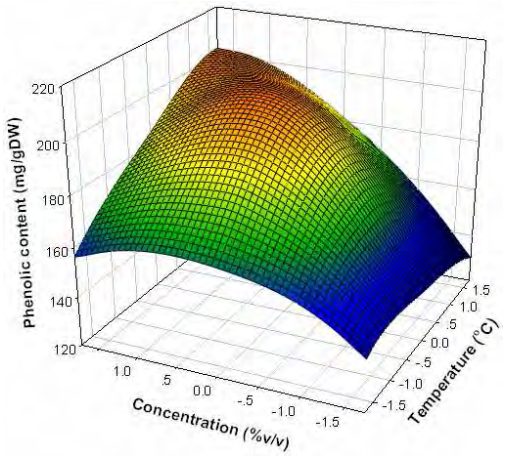
The response surfaces for water-EtOH and water-PG systems obtained from the analysis are given by the following equations.

$$Y_{water\text{-}EtOH=} 202.36+5.167X_1+12.703X_2-2.851X_3+6.206X_1X_2+5.264X_1X_3+ 8.251X_2X_3-9.975(X_1)^2-13.234(X_2)^2-3.55\ (X_3)^2 \quad (4.1)$$

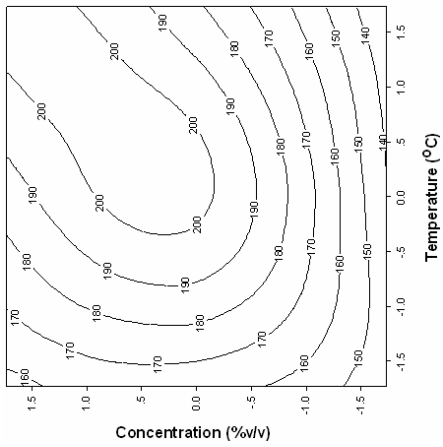
$$Y_{water\text{-}PG} = 175.574+6.395X_1+6.071X_2-1.465X_3-0.077X_1X_2+4.177X_1X_3+6.602X_2X_3-6.089\ (X_1)^2+2.909\ (X_2)^2+0.946(X_3)^2 \quad (4.2)$$

where Y is the amount of phenolic content (mg/gDW), $X_1$, $X_2$ and $X_3$ are coded variables for temperature, concentration of organic solvent and time. The significance of each coefficient was determined from the P-value, if the value of P less than 0.05, the model coefficients were significant. For water-EtOH system, the concentration ($X_2$) of EtOH, the quadratic terms of temperature ($X_1^2$) and concentration ($X_2^2$) were significant. For water-PG system, the temperature ($X_1$) and concentration ($X_2$) and the quadratic term of temperature ($X_1^2$) were significant. The response surfaces for the amount of total phenolic compounds in the extracts are displayed in three-dimensional (3D) plots as shown in Figure 4.1(a)-(c) and 4.2(a)-(c) for water-EtOH and water-PG systems respectively. These 3D plots present the response as a function of the combination of two test variables with the other maintained at its respective zero level. The corresponding contour plots (2D) are shown in Figure 4.1(d)-(f) and 4.2(d)-(f) respectively.

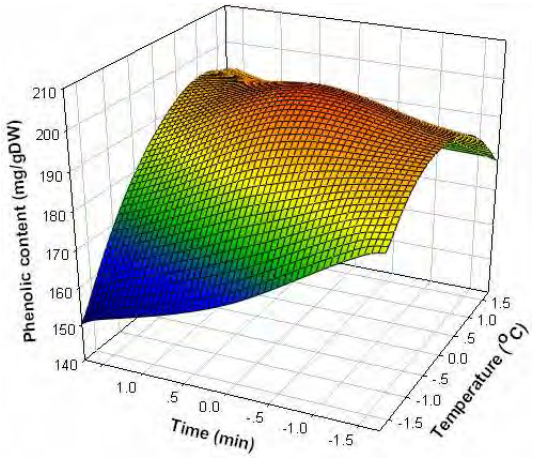
It is noted that these models (4.1 and 4.2) were appropriate for the extraction temperature between 29-81°C, concentration of EtOH from 0-80 %v/v and concentration of PG from 13-47 %v/v.



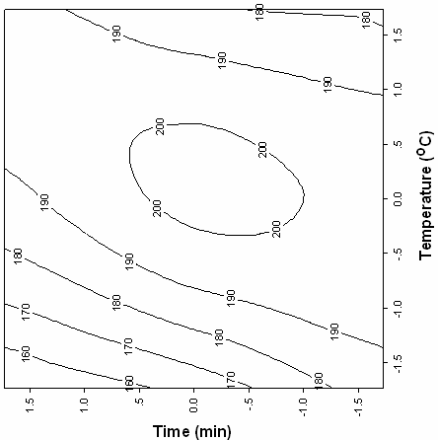

(a)



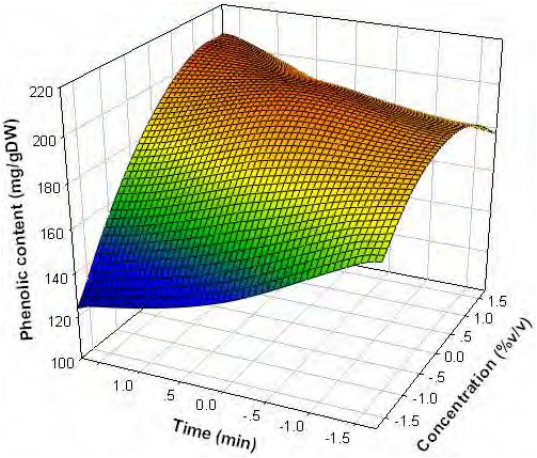

(d)



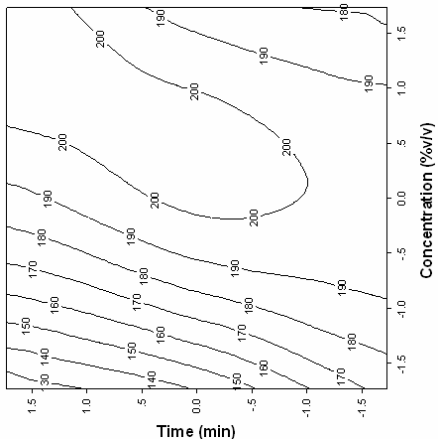

(b)




(e)

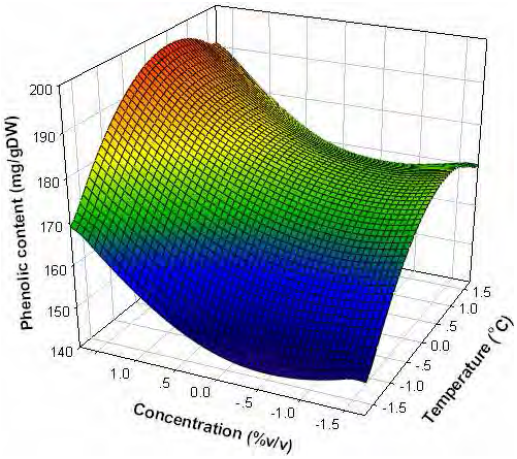



(c)

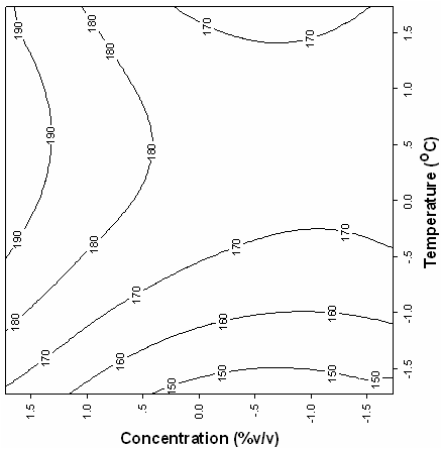



(f)

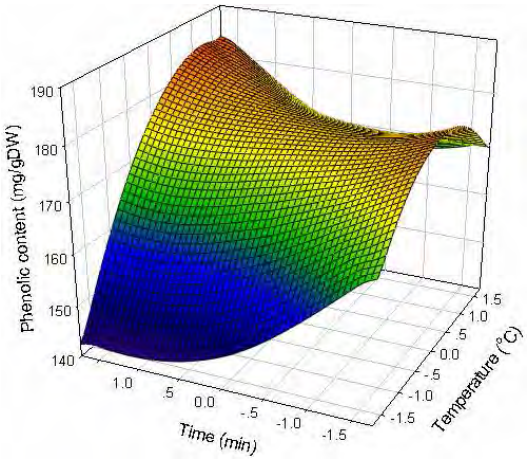
**Figure 4.1** Response surface plot and contour plot on total phenolic contents in water-EtOH system as a function of (a),(d) temperature and concentration at center level of time (b),(e) temperature and time at center level of concentration (c),(f) concentration and time at center level of temperature.


Phenolic content (mg/gDW)
200
190
180
170
160
150
140
1.0
.5
0.0
-.5
-1.0
-1.5
Concentration (%v/v)
Temperature (°C)

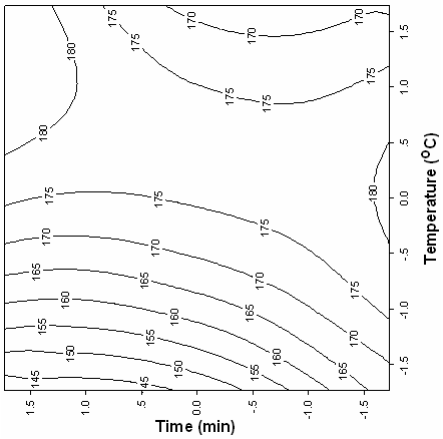
(a)


Concentration (%v/v)
Temperature (°C)

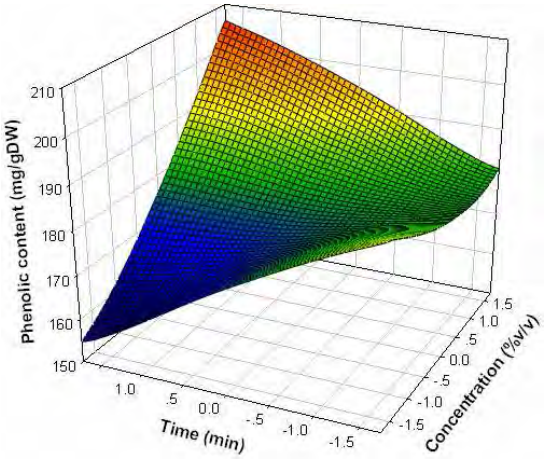
(d)


Phenolic content (mg/gDW)
190
180
170
160
150
140
Time (min)
Temperature (°C)

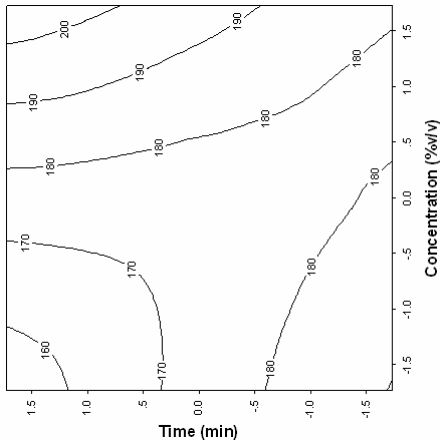
(b)


Time (min)
Temperature (°C)

(e)


Phenolic content (mg/gDW)
210
200
190
180
170
160
150
Time (min)
Concentration (%v/v)

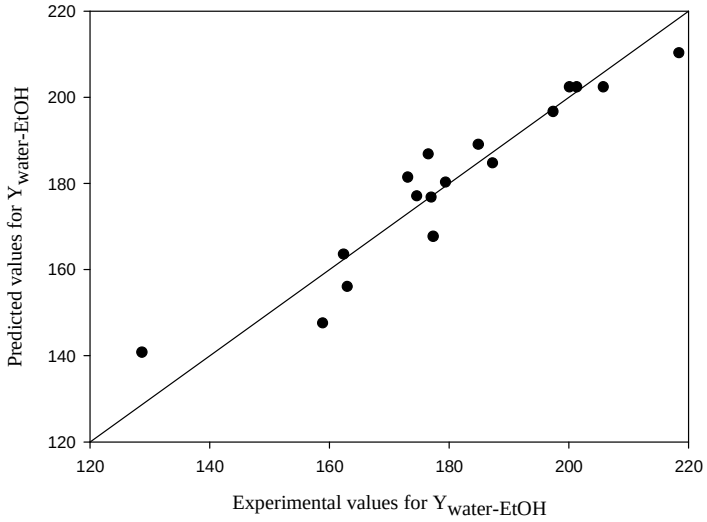
(c)


Time (min)
Concentration (%v/v)

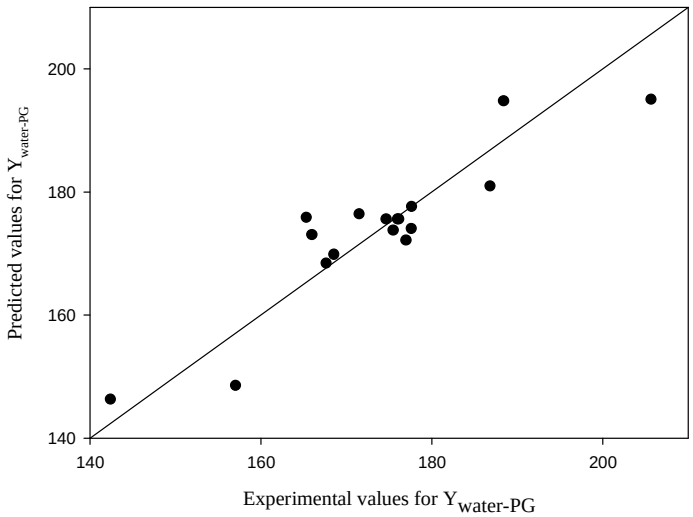
(f)

**Figure 4.2** Response surface and contour plot on total phenolic contents in water-PG system as a function of (a), (d) temperature and concentration at center level of time (b), (e) temperature and time at center level of concentration (c), (f) concentration and time at center level of temperature.

The experimental and the predicted values were plotted in Figure 4.3 (a) and (b) for water-EtOH and water-PG systems respectively. The results indicated a reasonable prediction of experimental results by the models.




(a)




(b)

**Figure 4.3** (a) Relation between experimental values and predicted values for water-EtOH system (b) Relation between experimental values and predicted values for water-PG system.

### 4.3 Response surface estimation for maximum phenolic content

Response surface methodology (RSM) was used to determine the optimal operational extraction conditions. Here, RSM was used to determine the optimal response for *T.chebula* extraction using water-EtOH and water-PG systems.

The optimal conditions for extraction can be determined by taking the partial derivative for the equation of (4.1) and (4.2) with respect to each of the factors, and then by setting the partial derivative equal to 0, the equations (4.3 -4.6) can be solved and the optimal conditions be determined.

$$\left[\frac{\partial Y_{(4.1)}}{\partial X_1}\right]_{x_2 x_3} = 0 \tag{4.3}$$

$$\left[\frac{\partial Y_{(4.1)}}{\partial X_2}\right]_{x_1 x_3} = 0 \tag{4.4}$$

$$\left[\frac{\partial Y_{(4.1)}}{\partial X_3}\right]_{x_1 x_2} = 0 \tag{4.5}$$

$$\left[\frac{\partial Y_{(4.2)}}{\partial X_1}\right]_{x_2 x_3} = 0 \tag{4.6}$$

$$\left[\frac{\partial Y_{(4.2)}}{\partial X_2}\right]_{x_1 x_3} = 0 \tag{4.7}$$

$$\left[\frac{\partial Y_{(4.2)}}{\partial X_3}\right]_{x_1 x_2} = 0 \tag{4.8}$$

The response $Y_{water\text{-}EtOH}$ presents the maximum phenolic content of 212.51 mg/gDW, at the optimal condition of $X_1$ =1.41 and $X_2$ =0.158 and$X_3$ = 2.48. In other words, the model predicted the highest phenolic content of 212.51 mg/gDW, which was obtained at the temperature of 76°C and concentration of EtOH 76.4 %v/v and 82 min. On the other hand, the response $Y_{water\text{-}PG}$ shows the phenolic content 178.64 mg/gDW at the optimal condition of $X_1$ =0.013, $X_2$ =0.63 and $X_3$ = -1.48. This optimum condition was equivalent to 57°C, 36%v/v of PG and extraction time of 23 min.

In addition to total phenolic compounds, the amounts of gallic acid (GA) and ellagic acid (EA) in the *T. chebula* extracts were also measured using HPLC. The chromatogram in Figure 4.4 shows that the retention times of gallic acid and ellagic acid were 12.0 and 24.8 minute respectively. The amount (mg) of GA and EA of the extracts obtained from water-EtOH and water-PG system are compared with those obtained with subcritical water and soxhlet extraction with water and alcohol from previous study (Rangsriwong et al., 2009), and the results are summarized in Table 4.11.

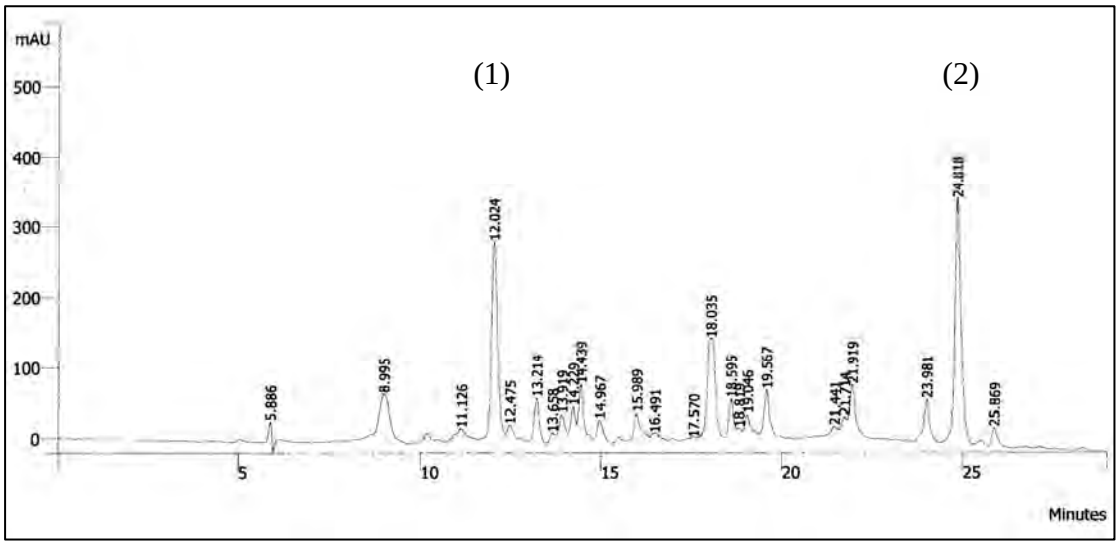



**Figure 4.4:** HPLC chromatogram of *T. chebula* extract (1) gallic acid and (2) ellagic acid.

**Table 4.11:** The component content of gallic acid and ellagic acid after extraction from *T.chebula* fruits

| Extraction methods | Temperature (°C) | Extraction time (min) | Total phenolic content (mg/gDW) | Component content (mg) | |
|---|---|---|---|---|---|
| | | | | GA | EA |
| Water-EtOH | 70 | 60 | 218.40 | 11.00±0.04 | 9.11±0.06 |
| Water-PG | 70 | 60 | 205.64 | 9.83±0.055 | 5.93±0.07 |
| Subcritical water extraction* | 120 | 37.5 | 172.74 | 7.4±0.51 | 2.36±0.11 |
| | 180 | 37.5 | 116.16 | 14.72±0.22 | 5.38±0.15 |

| Extraction methods | Temperature (°C) | Extraction time (min) | Total phenolic content (mg/gDW) | Component content (mg) | |
|---|---|---|---|---|---|
| | | | | GA | EA |
| Soxhlet water extraction* | 100 | 120 | 173.4 | 6.37±0.21 | 2.27±0.15 |
| | 100 | 240 | 151.627 | 7.59±0.16 | 2.14±0.21 |
| Soxhlet ethanol extraction* | 78.3 | 120 | 84.45 | 4.92±0.20 | 3.01±0.22 |
| | 78.3 | 240 | 76.093 | 3.33±0.11 | 2.35±0.40 |
| Hot water extraction in stirred vessel* | 100 | 120 | 157.73 | 7.04±0.23 | 1.48±0.14 |

*(Rangsriwong et al., 2009)

As seen from these results, the amounts of GA obtained from water-EtOH and water-PG extraction were higher than those obtained by other methods except for subcritical water extraction at 180°C. Ellagic acid from water-EtOH and water-PG extracts were higher than those obtained with the other. Compared with subcritical water extraction, these results suggested that extraction with water-EtOH and water-PG system requires considerably lower temperature (70°C vs 180°C) since the compound degradation could be significantly reduced.

### 4.4 Sugaring out Concentration

The extracts containing the highest phenolic compounds were used for further study on sugaring out concentration. The concentration process was carried out by adding glucose to the extract to induce the separation of the extract into phase solvent rich phase (EtOH or PG) and water rich phase. Here, the phase ratios (R) or the ratio of the volume of solvent rich phase (top) and water rich phase (bottom) were first determined at various glucose concentrations. The glucose concentration giving the lowest phase ratio giving the most concentrated extract was selected for the further determination of the distribution coefficients (D) of total phenolic compounds, GA and EA between two phases for both solvent systems. In addition, the antioxidant activities of the concentrated extracts was also measured and compared with those of the extracts prior to concentration. Here, the antioxidant activity was presented in terms of percent inhibition (%PI) and $IC_{50}$.

### 4.4.1 Phase ratio

In sugaring out concentration, the phase separation (Figure 4.5) takes place. Glucose addition to the extracts, followed by heating the system until glucose was completely dissolved. The organic rich phase is at the top and the aqueous phase is at the bottom. In the original extracts, EtOH or PG was relatively soluble in water, as a result of the OH groups, which form hydrogen bonding with water. Nevertheless sugar molecules have a larger number of OH groups, which may cause the phase separation possibly by forming the hydrogen bonding with water molecules at a greater extent, thus having the tendency to pull the water molecules further away from EtOH (or PG), and thus causing the glucose-water rich phase to be separated from the original solutions. Since EtOH and PG are relatively soluble, a small amount of heating was needed to help induce the phase separation. The phase ratio (R) of the extract was measured as the ratio of the volume of the top phase to that of the bottom phase (equation 3.2). The results are shown for various glucose concentrations in Figure 4.6 which indicated that as the glucose concentration increased, more water was separated into the bottom phase. From this figure, at 200 g/L of glucose, the phase ratio for water-EtOH system and water-PG system are, 1.94 and 1.85 respectively. However, adding more glucose than 200 g/L, the phase ratios were nearly constant. This was observed for both the water-EtOH and water-PG systems, and thus, this concentration was therefore appropriate and was then selected for subsequent sugaring out concentration experiments.

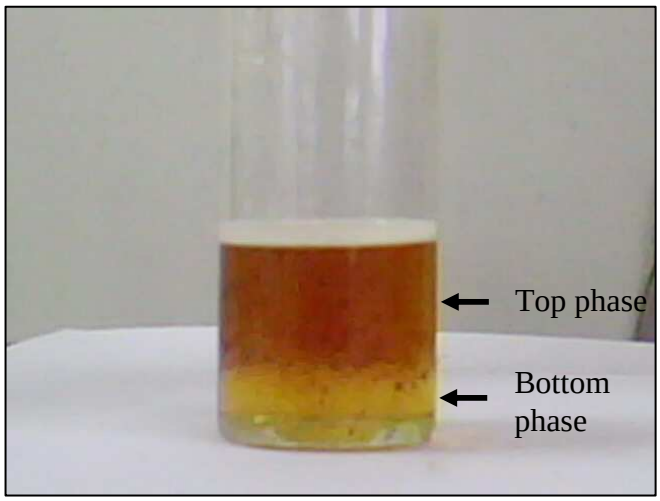



**Figure 4.5** Top and bottom phase after concentration

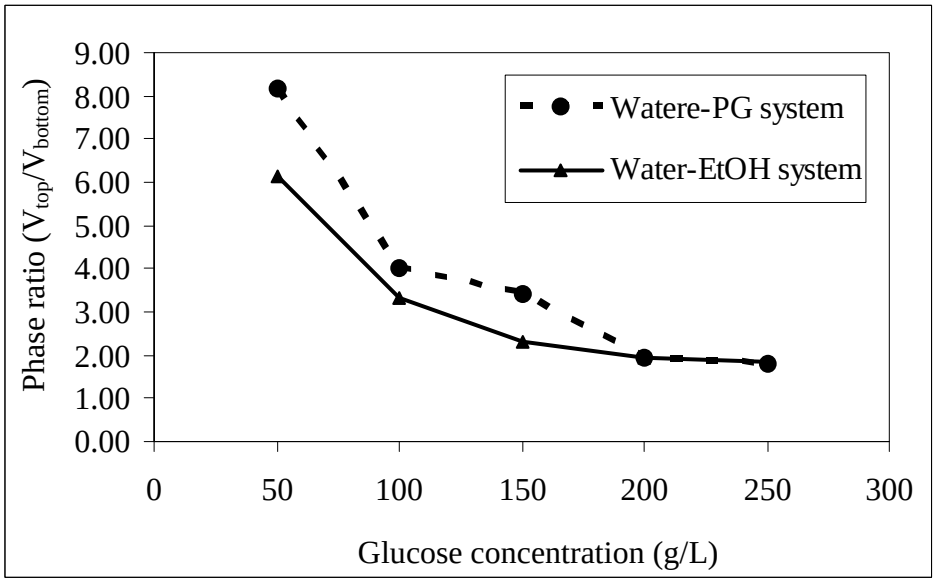



**Figure 4.6** Phase ratios of extracts at different glucose concentration

At glucose concentrations lower than 200 g/L, the phase ratio of the water-PG system was higher than that of the water-EtOH system. This was possibly due to the higher miscibility of propylene glycol in water, so water is more difficult to be separated from the orginal water-PG mixture.

After the sugaring out process, the phenolic extract was concentrated in to the top phase as can be observed by the darker top phase color, compared with the bottom and the original extract. The change in the color is related to the change in concentration of the phenolic compounds. The quantitative examination of the total phenolic content of the extract before and after sugaring out concentration was carried out using spectrophotometer on the basis of a standard curve with gallic acid, which is the major component in *T. chebula* fruit. The concentrations of phenolic compounds of the top and bottom phase in comparison with that of phenolic in the original samples for both water-EtOH and water-PG systems are shown in Figure 4.7.

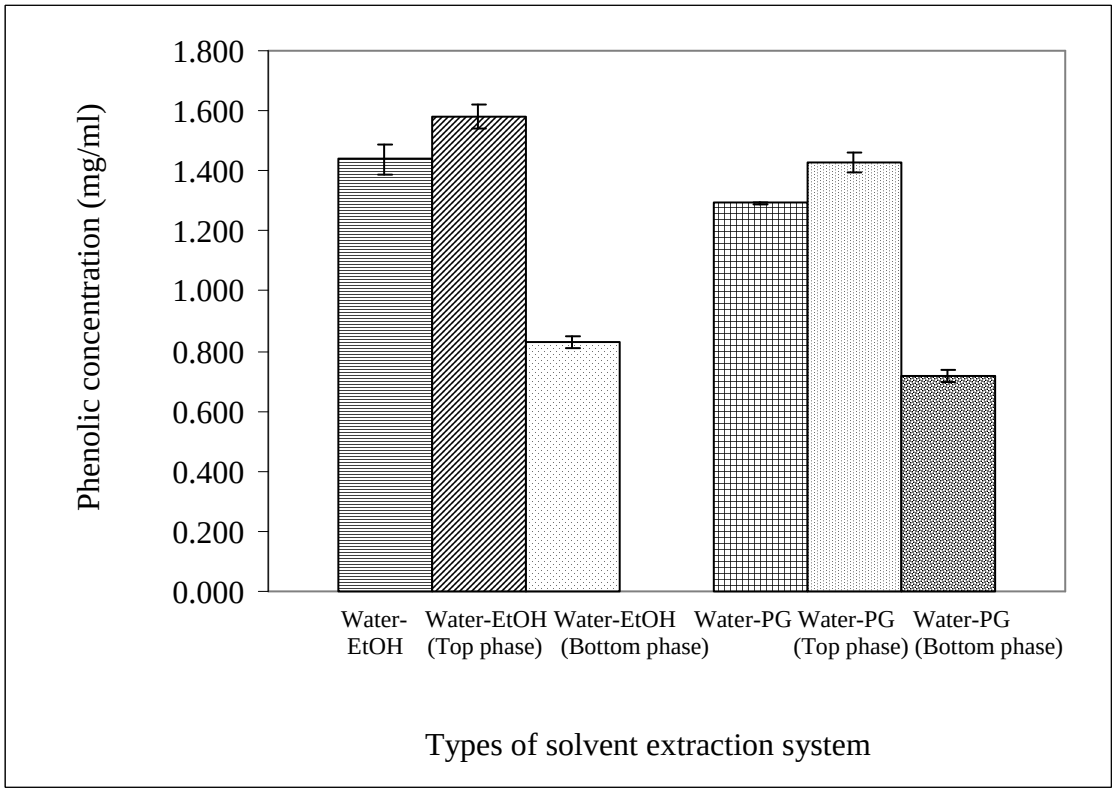



**Figure 4.7** Concentration of phenolic compounds before and after sugaring out concentration.

**Table 4.12:** Concentration of phenolic compounds and distribution coefficient in water-EtOH and water-PG solvent systems.

| Extraction method | Phenolic concentration (mg/ml) | Distribution coefficient |
|---|---|---|
| Water-EtOH | 1.44 | 1.98 |
| Water-EtOH (top phase) | 1.58 | |
| Water-EtoH (bottom phase) | 0.80 | |
| Water-PG | 1.29 | 1.98 |
| Water-PG (top phase) | 1.43 | |
| Water-PG (bottom phase) | 0.72 | |

Figure 4.7 shows the concentration of total phenolic compounds after sugaring out effect. The phenolic concentration increased 10% in water-EtOH system; from 1.44 to 1.58 mg/ml. In water-PG system, the phenolic concentration increased 11% from 1.29 to 1.43 mg/ml.

### 4.4.2 Distribution coefficients of phenolic compounds

In a typical concentration process, the distribution coefficient (D) is a key parameter determining the efficiency of concentration process. D is defined as the ratio of the solute concentration of the concentrated phase (top phase) to that of the diluted phase (bottom phase). From the total phenolic measurements, for sugaring out concentration was carried out using 200 g/L of glucose, the distribution coefficients of total phenolic compounds for water-EtOH and water-PG systems were both 1.98 (Table 4.12). In addition, the distribution coefficients were determined for the specific phenolic compounds: gallic acid (GA) and ellagic acid (EA) between the two phases. Table 4.13 summarizes the experimental results for extract volumes and concentrations of the GA and EA in each phase, which are used to determined the distribution coefficients.

The distribution coefficient of water-EtOH and water-PG ($D_{GA}$) are 4.89 and 4.34 respectively and that of water-EtOH and water-PG ($D_{EA}$) are 3.52 and 3.22, respectively.

**Table 4.13:** Concentrations of GA and EA and distribution coefficients

| System of Extraction | Volume (ml) | Concentration of compounds (mg/ml) | | Amount of GA and EA (mg) | | Distribution coefficient | | Phase ratio (From Fig. 4.4) |
|---|---|---|---|---|---|---|---|---|
| | | GA | EA | GA | EA | GA | EA | |
| Water-EtOH (Original) | 10 | 0.073± 0.0028 | 0.061± 0.0043 | 0.73 | 0.61 | 4.89 | 3.52 | 1.94 |
| Water-EtOH (Top) | 7 | 0.072± 0.0058 | 0.061± 0.0026 | 0.51 | 0.43 | | | |
| Water-EtOH (Bot) | 3.6 | 0.015± 0.0006 | 0.017± 0.0014 | 0.05 | 0.06 | | | |
| Water-PG (Original) | 10 | 0.066± 0.0037 | 0.040± 0.0048 | 0.66 | 0.40 | 4.34 | 3.22 | 1.85 |
| Water-PG (Top) | 6.95 | 0.062± 0.0027 | 0.045± 0.0021 | 0.43 | 0.31 | | | |
| Water-PG (Bot) | 3.75 | 0.014± 0.0044 | 0.014± 0.0056 | 0.05 | 0.05 | | | |

It is noted that the total volume after concentration process was increased as the dissolved sugar took up some volume of water. The concentrations (mg/ml) of GA and EA in top phase of water-EtOH system are almost the same compared with the

original sample. The concentrations of GA are 0.073 and 0.072 mg/ml for original sample and top phase respectively. The concentrations of EA are 0.061 mg/ml in both original sample and top phase. For water-PG system, the concentration of GA slightly decreased from 0.066 to 0.062 mg/ml in top phase, while the concentration of EA increased around 12.5%; from 0.040 to 0.045 mg/ml in top phase. Although the total phenolic compound was concentrated into the top phase, the concentrations of GA in the top phase slightly decreased in water-EtOH and water-PG system and that the concentration of EA stayed relatively the same in water-EtOH system, this was possible caused by the degradation of GA and EA during the heating process of the sugaring out concentration. This was confirmed by the calculation of the amount (mg) of GA and EA after sugaring out effects. Table 4.13 shows the total amount of GA and EA after concentration process in both solvent systems decreased. For water-EtOH system, the amount of GA decreased from 0.73 to 0.56 mg. The amount of EA decreased from 0.61 to 0.49 mg. For water-PG system, the total amount of GA decreased from 0.66 to 0.48 mg. The amount of EA decreased from 0.40 to 0.36 mg.

Despite the decrease, from the distribution coefficients of GA and EA are high. It can be concluded from these results that sugaring out effect is potentially an effective way to concentrate and partition the compounds, considering that the compound degradation should minimized.

### 4.5 Antioxidant activity

From the section of concentration of total phenolic compounds, we can see that the total phenolic concentration of the extracts after sugaring out effect increased (Figure 4.6). Here, the antioxidant activity of the concentrated fractions was further examined and compared with the original extracts. The antioxidant activities of the extracts were measured by the ability of the extract to inhibit the $ABTS^{\bullet+}$ free radical and was shown in terms of percent inhibition (%PI) and $IC_{50}$ values.

#### 4.5.1 Percent Inhibition

The percent inhibition (%PI) shows the ability of phenolic extracts from *T.chebula* fruits to perform as a free radical inhibitor. The antioxidant activity of each extract was compared using the criterion of percent inhibition of $ABTS^{\bullet+}$ radicals.

The percent inhibition was calculated using the equation (3.4). The extracts for this study obtained from the optimal extraction conditions of water-EtOH, water-PG and water as well as the same extracts which were processed by sugaring out concentration at glucose 200 g/L.

Figure 4.8 shows the %PI of extract using water, water-EtOH and concentrated extract of water-EtOH solvent systems. The volume ratio of extract:ABTS was varied at 0.025:2, 0.025:5, 0.025:10 and 0.25:15. The graph illustrated that for all the volume ratios of extract:ABTS, the concentrated extract in water-EtOH showed the highest %PI compared with the extracts obtained from water, water-EtOH and water extraction alone without concentration. The same result was also observed for water-PG solvent system. Figure 4.9 showed the %PI of extract in water-PG and concentrated extract of water-PG after sugaring out concentration compared with water extraction. At all the volume ratio of extract:ABTS the concentrated extract showed the highest percent inhibition of the free radical.

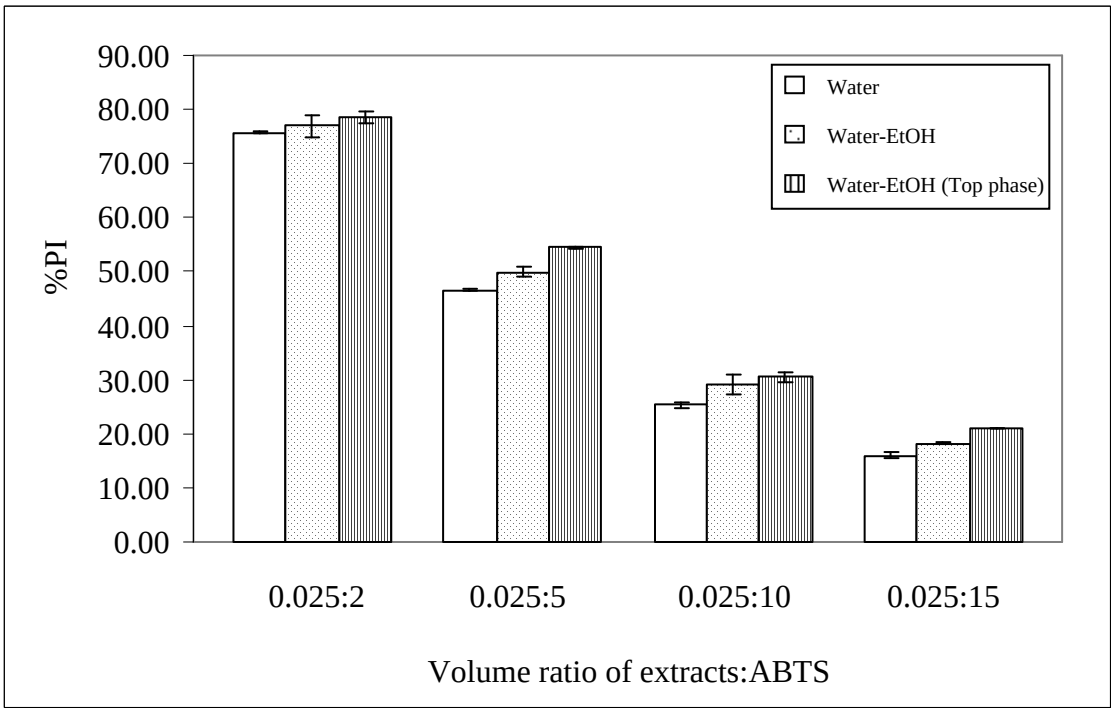



**Figure 4.8:** %PI of various volume ratio of extracts:ABTS in water-EtOH solvent systems before and after sugaring out concentration compared with water extraction

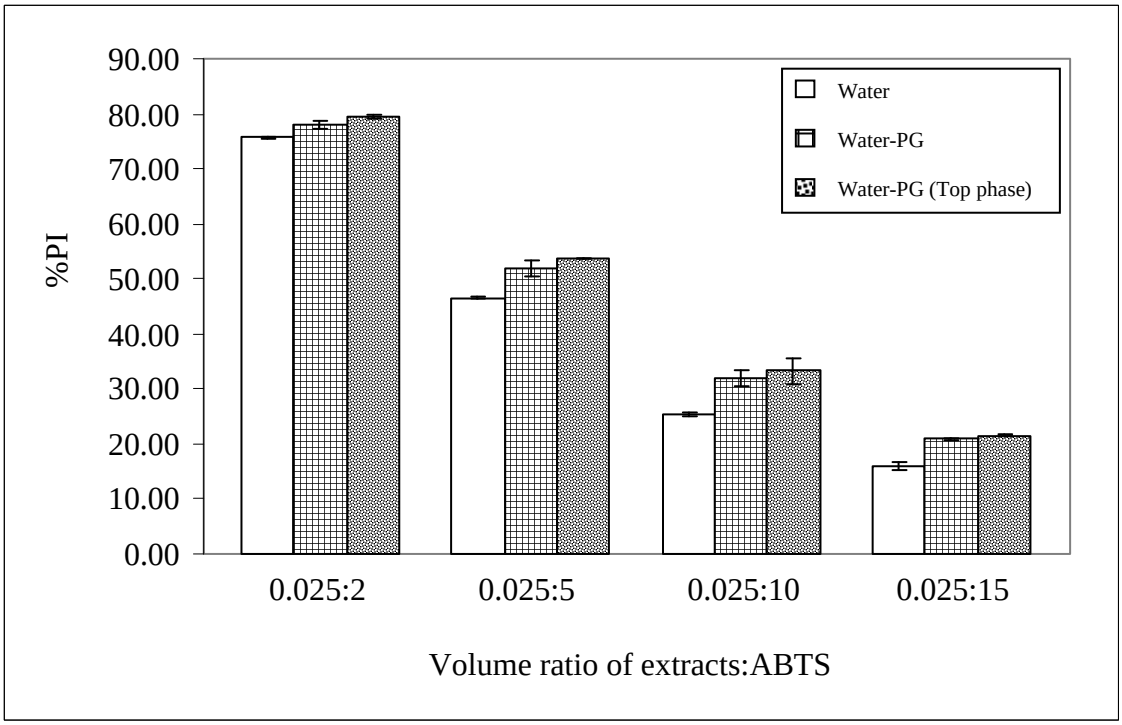



**Figure 4.9:** %PI of various volume ratio of extracts:ABTS in water-PG solvent systems before and after sugaring out concentration compared with water extraction

Although the concentration of GA and EA in the top phase was not significantly increased, the percent inhibition above correlated well with the total phenolic concentration in the top phase which increased approximately 10% after extract concentration (Figure 4.7). The increase total phenolic content caused the concentrated extract to be more efficiently scavenge the free radicals.

### 4.5.2 $IC_{50}$

Other than the inhibition of free radicals, the antioxidant activity of the extract was also presented using $IC_{50}$ index, which was the concentration of the sample solution reducing a 50 % of the radical absorbance, thus the higher $IC_{50}$, the lower antioxidant activity.

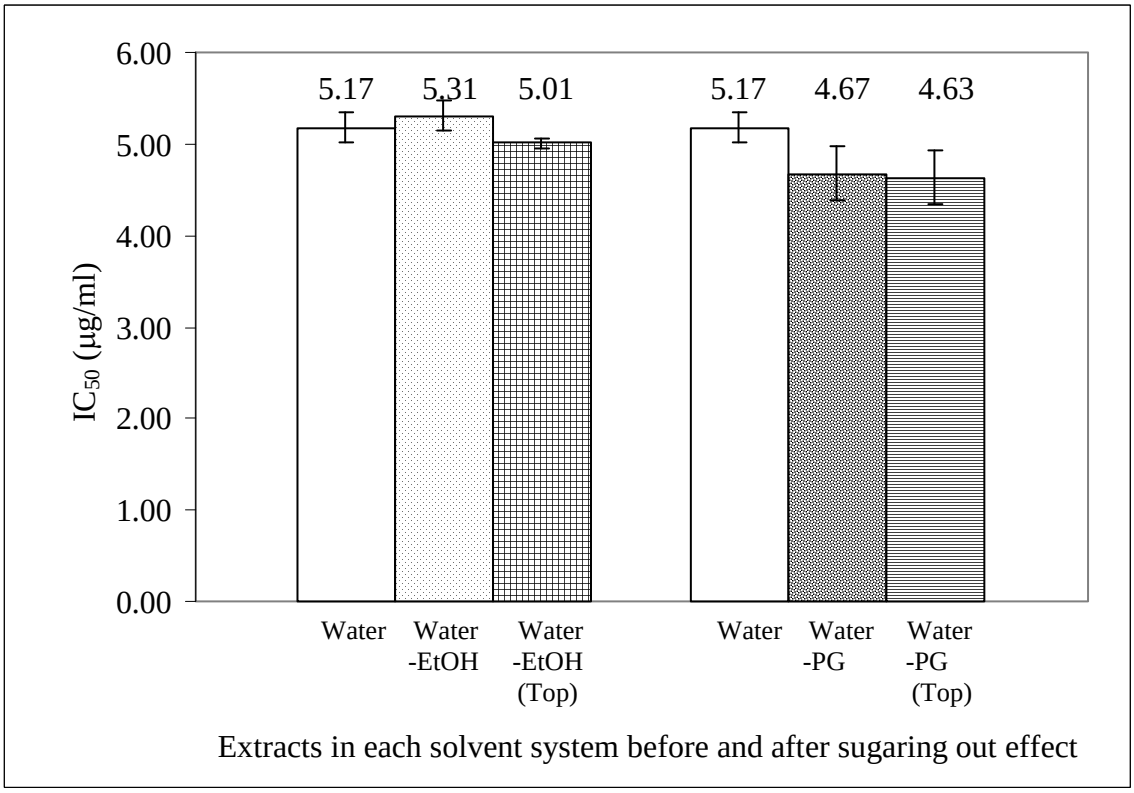



**Figure 4.10** Antioxidant activity ($IC_{50}$) of the *T. chebula* extracts in water-EtOH and water-PG systems before and after concentration process.

Figure 4.10 shows $IC_{50}$ values of the water, water-EtOH and water-EtOH (Top phase) extracts, which were 5.17, 5.31 and 5.01 and μg/ml, respectively, and those for water-PG and water-PG (Top phase) systems were 4.67and 4.63 μg/ml, respectively. It can be seen from these results that the water-EtOH extract has comparable $IC_{50}$ to that of water extract, while that of water PG-extract has lower $IC_{50}$ value than that of water. Compared with water-EtOH original extract, the concentrated water-EtOH has lower $IC_{50}$ (higher antioxidant activity), while the concentrated water-PG extract has comparable $IC_{50}$ to the original water-PG extract. Water-PG extracts was lower than the water extract. Considering the extraction system alone based on the $IC_{50}$ values, water-PG was an interesting solvent system for extraction of phenolic compounds, giving the extract with the lowest $IC_{50}$ value. Moreover, the increase in the antioxidant activity of the extract obtained by this system was possibly due to the preservative property of PG, which might help inhibit the free radicals to some extent. The concentrated extract of both systems have relatively the same $IC_{50}$ with the original extract, which indicated that the antioxidant property of the extract was not

affected by the sugaring out concentration process. Thus, from these results, sugaring out concentration has been shown to potentially concentrate the phenolic compounds from *T. chebula* extract.

# CHAPTER V

# CONCLUSIONS AND RECOMMENDATIONS

## 5.1 Conclusions

1. Phenolic compounds from *T. chebula* can be effectively extracted using water-ethanol (EtOH) and water-propylene glycol (PG) solvent systems. Higher yield could be achieved with considerably lower extraction temperature, compared with subcritical water extraction.

2. The analysis of variance (ANOVA) of the experimental results based on CCD design determined by SPSS 16.0 indicated that at 95% confidence interval ($p < 0.05$), the significant factors affecting the yield of water-EtOH extraction are the main effects of temperature, concentration of EtOH and time, and the interactions between temperature and concentration, temperature and time, and concentration and time. For water-PG systems, the significant factors affecting the yield were the main effects of temperature, concentration and time, and the interactions between temperature and time, and concentration and time. The relations between each factor and the phenolic compounds were modeled with $2^{nd}$ order polynomial models and the equations for the response surfaces for extraction with water-EtOH and water-PG are:

$$Y_{water\text{-}EtOH=} 202.36+5.167X_1+12.703X_2-2.851X_3+6.206X_1X_2+5.264X_1X_3+ 8.251X_2X_3-9.975(X_1)^2-13.234(X_2)^2-3.55\ (X_3)^2 \quad (5.1)$$

$$Y_{water\text{-}PG} = 175.574+6.395X_1+6.071X_2-1.465X_3-0.077X_1X_2+4.177X_1X_3+6.602X_2X_3-6.089\ (X_1)^2+2.909\ (X_2)^2+0.946(X_3)^2 \quad (5.2)$$

From the response surface equation for $Y_{water\text{-}EtOH}$, the optimal condition was found to be $X_1$ =1.41 and $X_2$ =0.1.58 and$X_3$ = 2.48, which give the yield of total phenolic compounds of 212.51 mg/gDW. In other words, the model predicted the highest phenolic compounds yield of 212.51 mg/gDW could be was obtained at the temperature of 76°C and concentration of EtOH 76.4 %v/v and 82 min. For water-PG extraction, the response surface equation for $Y_{water\text{-}PG}$ shows the phenolic content 178.64 mg/gDW at the optimal condition at $X_1$ =0.013, $X_2$ =0.63 and $X_3$ = -1.48. This

optimum condition corresponded to the condition at 57°C, 36%v/v of PG and at extraction time of 23 min. The model predictions at optimal parameters of water-EtOH and water-PG system were experimentally validated.

3. Sugaring out concentration of the phenolic compounds in both solvent systems was achieved by addition of glucose at the concentration of 200 g/L. This concentration resulted in the highest phase separation. At this concentration, the phase ratio of volume of the top phase to that of the bottom phase was found the smallest at 1.94 and 1.85 respectively for water-EtOH and water-PG.

4. After sugaring out concentration, the total phenolic concentrations in the top (concentrated phase) increased by 10% and 11% respectively for water-EtOH and water-PG system. The distribution coefficients of the total phenolic in both solvent systems were 1.98.

5. The distribution ratio of gallic acid after sugaring out process was 4.89 and 4.34 for water-EtOH and water-PG systems respectively. The distribution ratio of ellagic acid was 3.52 and 3.22 for water-EtOH and water-PG systems respectively. The distribution ratio showed that the sugaring out concentration has the ability to concentrate gallic acid and ellagic acid into desired phase. However, heating process during the sugaring out concentration could cause the degradation of gallic acid and ellagic acid.

7. The antioxidant activities of the extract were measured in terms of percent inhibition (%PI) and $IC_{50}$. The concentrated extract from water-PG system showed the highest antioxidant activity among the other extracts.

8. The water-PG solvent system is an effective and interesting solvent for extraction of *T.chebula* fruits yielding high amount of phenolic compounds. After sugaring out concentration, the concentrated extract in water-PG also presented the highest antioxidant activity (lowest $IC_{50}$) than other extracts obtained from water and water-EtOH extraction.

9. The sugaring out concentration is an alternative and effective method for concentration and separation of water-organic solvent system, and can potentially be considered as an alternative concentration process for natural compounds.

## 5.2 Recommendations

In the extraction systems in this study, the organic solvents (EtOH and PG) have OH (Hydroxyl group) functional groups which form the hydrogen bonding with water, which is the same form O—H bonding water molecules form with glucose. The phase separation could be induced more easily if the forms of interactions are different between water-solvent and water-glucose. Other solvent systems could be further study, and in which case heating might not be necessary for the induction of phase separation, and as a result degradation of phenolic compounds could be minimized. Solvents that form N—H hydrogen bonding with water, for example, could be considered. In addition to solvent type for concentration, optimization of the process could be carried out in the future study to determine the effects of different factors such as sugar type, sugar concentration, and concentration equilibrium temperature. Nevertheless, the results in this study suggested that the sugaring out concentration process can potentially be applied for the concentration of various natural extracts.

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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

| Peak area (UV dectector at 270 nm) | Concentration of gallic acid (mg/ml) |
|---|---|
| 16093717 | 0.03 |
| 27063400 | 0.06 |
| 40939208 | 0.09 |
| 57462588 | 0.12 |

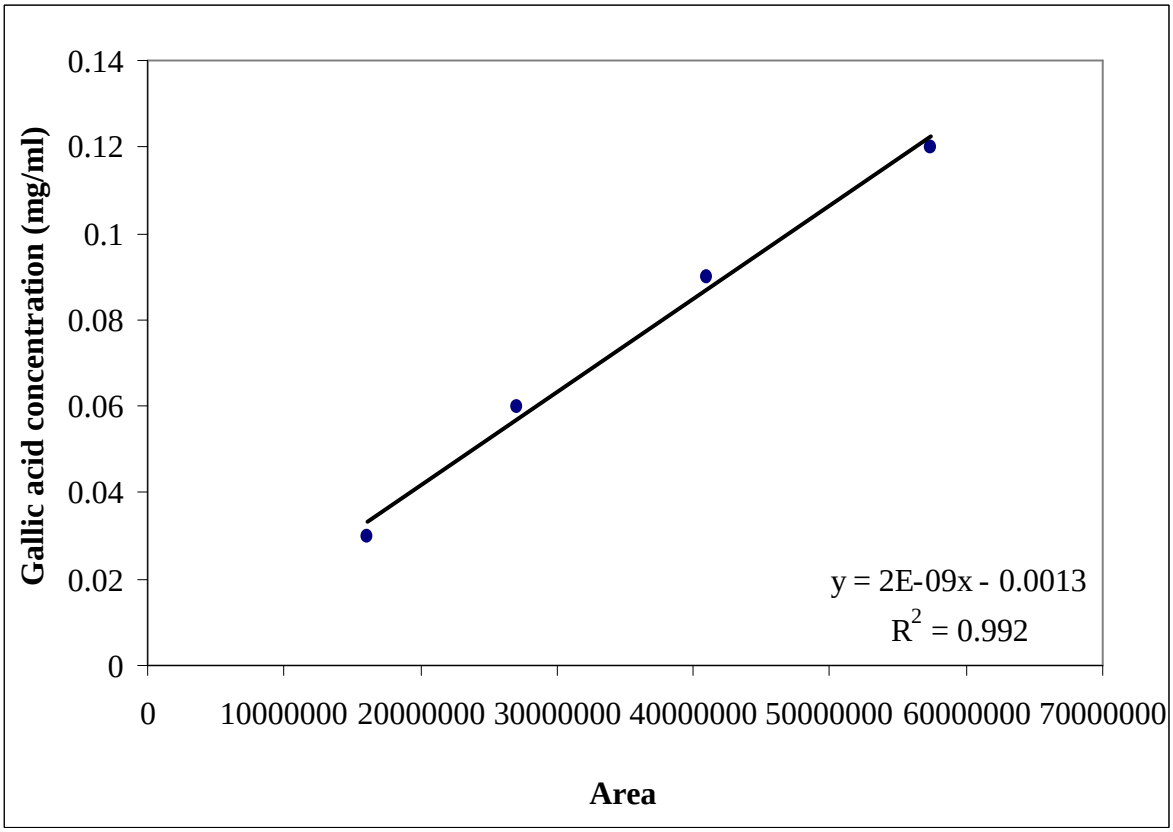


**Figure A-1.1** Standard calibration curve of gallic acid.

**Table A-1.2:** Standard calibration curve data of ellagic acid

| Peak area (UV dectector at 270 nm) | Concentration of gallic acid (mg/ml) |
|---|---|
| 10113888 | 0.02 |
| 23271514 | 0.04 |
| 39052308 | 0.06 |
| 54528124 | 0.08 |

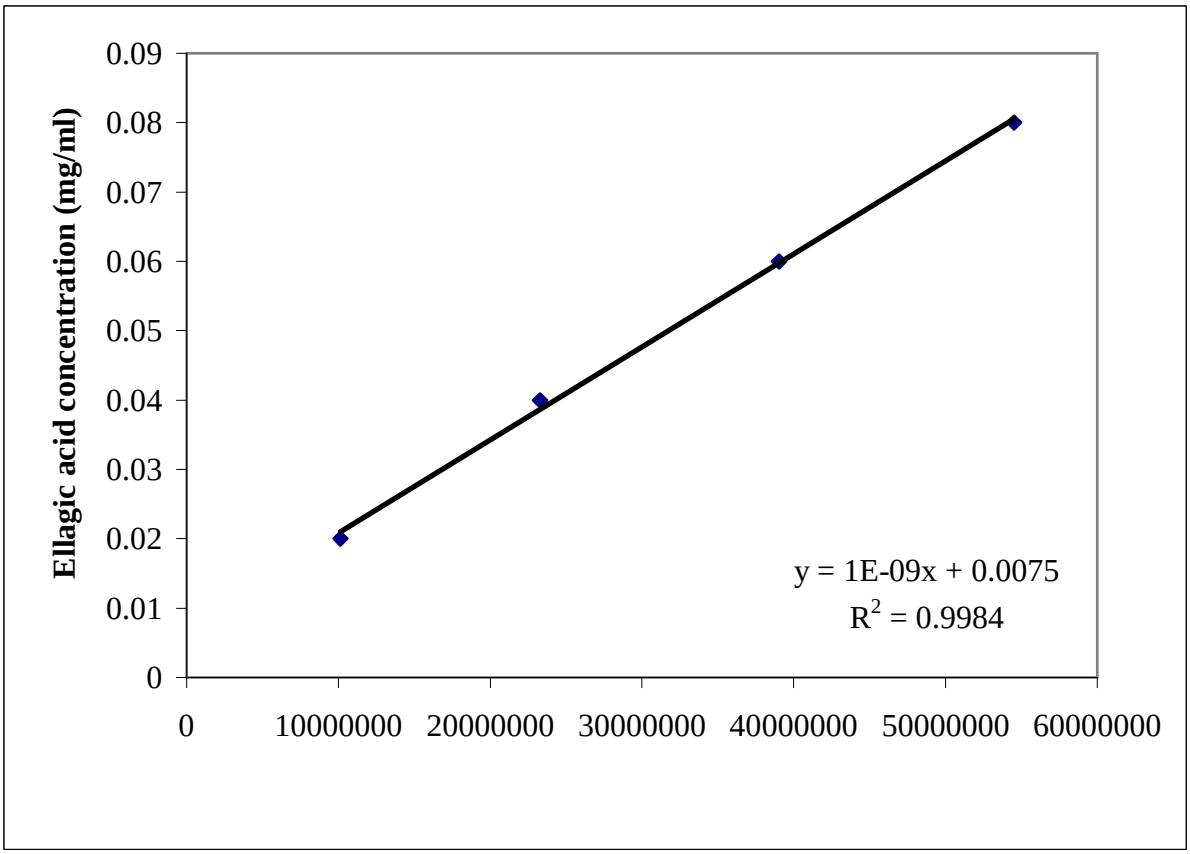


**Figure A-1.2** Standard calibration curve of ellagic acid.

## 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 |

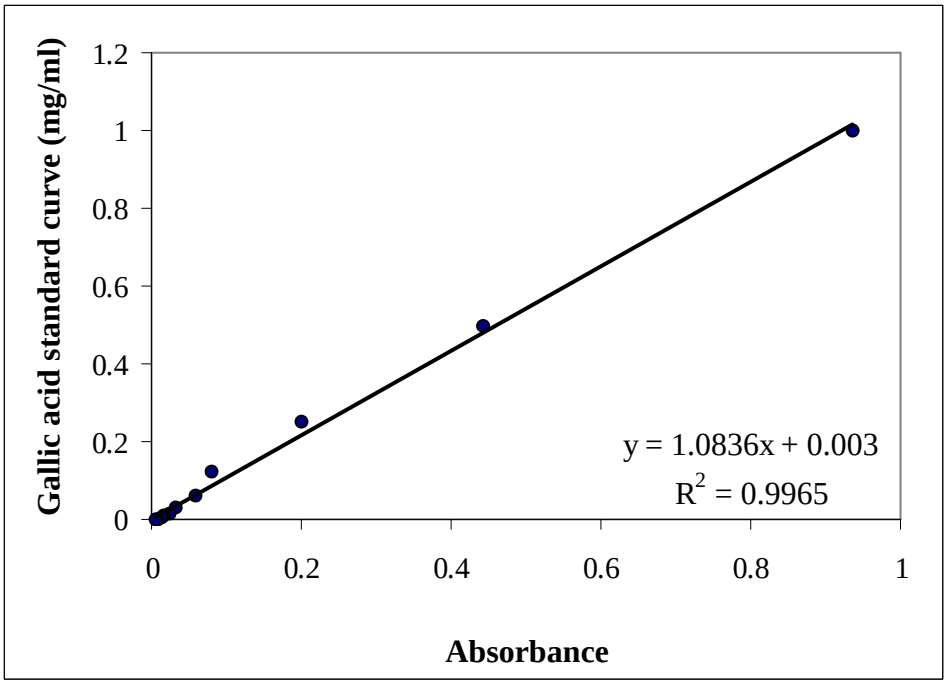


**Figure A-2.1** Standard calibration curve of gallic acid (average).

**A-3** Phase ratio after sugaring out concentration

**Table 3.1:** The phase ratio after sugaring out concentration

| Extraction methods | Glucose concentration (g/L) | Average of volume of top phase (ml) | Average of volume of bottom phase (ml) | Phase ratio (R) | Total volume (ml) |
|---|---|---|---|---|---|
| Water-PG | 50 | 8.9 | 1.1 | 8.09 | 10 |
| Water-PG | 100 | 8.0 | 2.0 | 4.00 | 10 |
| Water-PG | 150 | 7.9 | 2.3 | 3.43 | 10.2 |
| Water-PG | 200 | 7.0 | 3.6 | 1.94 | 10.6 |
| Water-PG | 250 | 6.9 | 3.8 | 1.82 | 10.7 |
| Water-EtOH | 50 | 8.6 | 1.4 | 6.14 | 10 |
| Water-EtOH | 100 | 8.0 | 2.4 | 3.33 | 10.4 |
| Water-EtOH | 150 | 7.6 | 3.3 | 2.30 | 10.9 |
| Water-EtOH | 200 | 6.95 | 3.75 | 1.85 | 10.7 |
| Water-EtOH | 250 | 7.0 | 3.8 | 1.84 | 10.8 |

**A-4** Concentration of phenolic compounds before and after sugaring out concentration.

**Table 4-1:** Concentration of phenolic compounds before and after sugaring out concentration.

| Extraction methods | Average concentration of original extract (mg/ml) | STDV |
|---|---|---|
| Water-EtOH (Original) | 1.44 | 0.050 |
| Water-EtOH (Top phase) | 1.58 | 0.040 |
| Water-EtOH (Bottom phase) | 0.80 | 0.021 |
| Water-PG (Original) | 1.29 | 0.002 |
| Water-PG (Top phase) | 1.43 | 0.034 |
| Water-PG (Bottom phase) | 0.72 | 0.020 |

**A-5** %PI of various volume ratio of extract:ABTS in water, water-EtOH and water-PG solvent systems before and after sugaring out concentration.

**Table A-5.1:** %PI of various volume ratio of extracts:ABTS in water

| Volume ratio of Extract:ABTS | %PI of water extraction | STDV |
|---|---|---|
| 0.025:2 | 75.71 | 0.20 |
| 0.025:5 | 46.50 | 0.20 |
| 0.025:10 | 25.32 | 0.45 |
| 0.025:15 | 16.04 | 0.66 |

**Table A-5.2:** %PI of various volume ratio of extracts:ABTS in water-EtOH

| Volume ratio of Extract:ABTS | %PI of water-EtOH extraction | STDV |
|---|---|---|
| 0.025:2 | 76.93 | 2.12 |
| 0.025:5 | 49.89 | 0.96 |
| 0.025:10 | 29.18 | 1.77 |
| 0.025:15 | 18.21 | 0.10 |

**Table A-5.3:** %PI of various volume ratio of extracts:ABTS in water-EtOH (top phase)

| Volume ratio of Extract:ABTS | %PI of water-EtOH (Top phase) | STDV |
|---|---|---|
| 0.025:2 | 75.71 | 1.21 |
| 0.025:5 | 46.50 | 0.10 |
| 0.025:10 | 25.32 | 0.91 |
| 0.025:15 | 16.04 | 0.15 |

**Table A-5.4:** %PI of various volume ratio of extracts:ABTS in water-PG

| Volume ratio of Extract:ABTS | %PI of water-PG extraction | STDV |
|---|---|---|
| 0.025:2 | 78.00 | 0.61 |
| 0.025:5 | 51.96 | 1.46 |
| 0.025:10 | 31.89 | 1.57 |
| 0.025:15 | 20.96 | 0.15 |

**Table A-5.5:** %PI of various volume ratio of extracts:ABTS in water-PG (top phase)

| Volume ratio of Extract:ABTS | %PI of water-PG (Top phase) | STDV |
|---|---|---|
| 0.025:2 | 79.57 | 0.40 |
| 0.025:5 | 53.82 | 0.05 |
| 0.025:10 | 33.25 | 2.37 |
| 0.025:15 | 21.50 | 0.10 |

**A-6** $IC_{50}$ of extracts in water, water-EtOH and water-PG solvent systems before and after sugaring out concentration.

**Table A-6.1:** $IC_{50}$ of extracts in water, water-EtOH and water-PG solvent systems before and after sugaring out concentration.

| Extraction methods | $IC_{50}$ | STDV |
|---|---|---|
| Water | 5.17 | 0.164 |
| Water-EtOH | 5.31 | 0.165 |
| water-EtOH (Top phase) | 5.01 | 0.055 |
| water-PG | 4.67 | 0.296 |
| water-PG (Top phase) | 4.63 | 0.293 |

# APPENDIX B

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## Hot Water Extraction of Polyphenolic Compounds from *Terminalia Chebula* Fruits

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**Abstract**

Fresh and dried fruits of *Terminalia chebula* Retz. are commonly used as herbal medicine as they contain various phytochemicals. These polyphenolic compounds exhibit therapeutic properties such as antioxidant, anticarcinogenic, and antimicrobial activities. This study investigated the extraction of polyphenolic compounds from *T. chebula* fruits by subcritical water extraction (SWE) as well as the use of polymeric surfactant, propylene glycol, to reduce the extraction temperature. The experiment was carried out in a semi-continuous flow system at the water flow rate of 4 ml/min. The effect of extraction temperature (120-220ºC) was examined on the amounts of total phenolic compounds extracted at a pressure of 4 MPa. In addition, the yields of the extracts were analyzed and compared to those obtained by water extraction and soxhlet extraction. The result showed that, the yield decreased with increasing temperature. In this study, the addition of propylene glycol (PG) was found to help reducing extraction temperature to 70ºC. At such condition, the total phenolic content was higher than that obtained by hot water extraction at the same temperature without surfactant and higher than that obtained by SWE.

### 1. Introduction

*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. Samor thai fruit, either fresh or dried, which have been reported to have the strongest antioxidant capacity and the highest phenolic contents over 133 Indian medicinal plants [1]. The fruits of *T. chebula* contain several polyphenolic compounds including gallic acid (GA), ellagic acid (EA), and corilagin (CG) [2]. These compounds are associated with a lower risk of various chronic diseases. Phenolic compounds were reported to be effectively extracted by organic solvents such as ethanol, ethyl acetate [3], ether [4], and 70% methanol [5]. 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) [6], 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. Particularly, when the water temperature is increased to above its normal boiling point (100°C) with applied pressure, its ability to extract various organic compound dramatically increases. At this condition, the water is called pressurized hot water (PHW) or subcritical water and the range of temperature in which SWE is used for extraction of natural compunds is between the boiling temperature (100°C) and the critical temperature (374°C). 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. Despite these several advantages, high temperature operation might cause thermal degradation of the compounds. For example, the degradation of damnacanthal from roots of *Morinda citrifolia* was observed at the operating temperature above 200°C [7]. In this study, we proposed to investigate the effects of temperature for subcritical water extraction of total phenolic contents from Samor thai fruits. Furthermore, we attempted to reduce the extraction temperature by adding propylene glycol (PG) into the water. The results were then compared with those

obtained with SWE and other conventional methods.

## 2. Materials and Methods

### 2.1 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 and Folin-Ciocalteau reagent were purchased from Sigma-Aldrich Chemicals (Missouri, USA). Water used in the experiments was distilled and deionized water.

### 2.2 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.

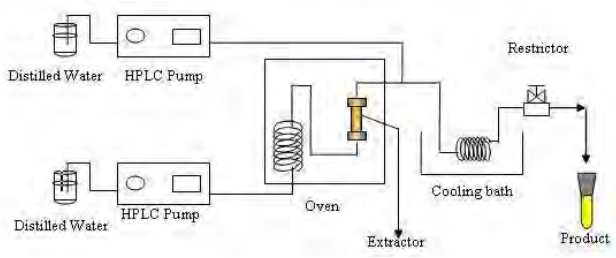

**Fig 1.** Diagram of experimental setup subcritical water extraction.

**2.3 Water extraction**

1 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. Moreover, the extraction by adding of 40% v/v PG aqueous solution at 70°C was studied. Theses extracted with hot water and PG aqueous solution were carried out for 2 h. After that, the extracted was analyzed for total phenolic content.

**2.4 Soxhlet extraction**

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

**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 [8]. 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. Results and Discussion**

**3.1 Effect of temperature of subcritical water extraction**

In this study, the effect of subcritical water extraction was carried out at flow rate of 4 ml/min and pressure of 4 MPa for 2 hours. Fig. 2 shows that the increase in temperature caused the total phenolic content to decrease. At 120°C, total phenolic compounds extracted were the highest. On the other hand, at 220°C, thermal degradation of the product has occurred.

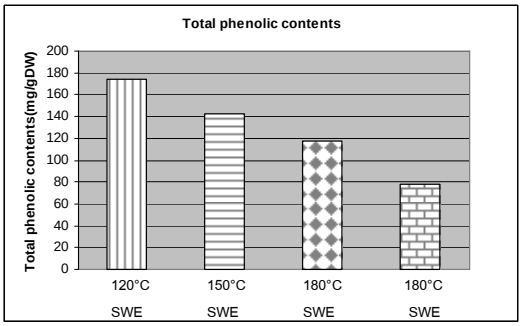


**Fig.2** Effect of temperature on the amount of total phenolic contents.

### 3.2 Effect of PG addition

To determine the effect of PG addition on the possibility of reducing the extraction temperature, extraction was carried out in a 40% v/v PG aqueous solution for 2 hours at 70°C. The results in Fig 3 illustrate that the total phenolic compounds obtained by extraction with PG solution was the highest. With the addition of PG, the total phenolic compounds could be extracted at low extraction temperature, and therefore higher amount could be achieved.

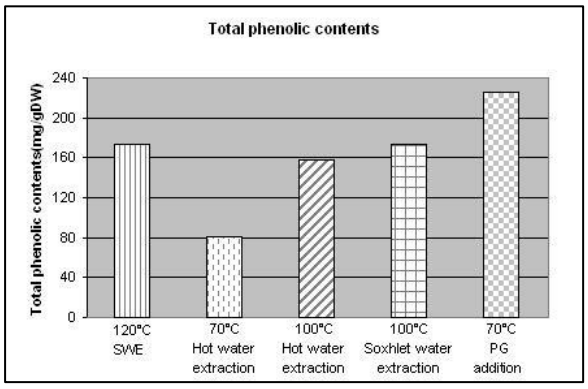


**Fig 3.** Comparison of total phenolic comtents obtained with various methods.

### 4. Conclusions

The results in this study demonstrated that the higher the SWE temperature, the lower the amount of phenolic compounds extracted, especially at the temperature above 120°C as the degradation of phenolic contents took place. To reduce the degradation, we investigated the use of PG aqueous solution (40% v/v) which was found to achieve the highest recovery at lower extraction temperature (70°C), compared with SWE and other conventional methods. Thus, the use of PG is an environmentally friendly means to reduce the energy consumption, and the product degradation could be avoided.

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# Extraction and concentration of Phenolic compounds from *Terminalia Chebula* fruits

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## Abstract

The fruits of *Terminalia chebula* Retz. are traditionally used as herbal medicine in India. Both fresh and dried fruits have been reported to have high content of phenolic compounds, which are known to deliver health benefits. This study aims to investigate the extraction of polyphenolic compounds from *T. chebula* fruits by water-ethanol (EtOH) and water-propylene glycol (PG) systems. The results showed that, for water-EtOH system, the highest phenolic content was obtained after 60 min of extraction at 70°C using 63%v/v EtOH in water. For water-PG system, the suitable condition was extraction with 40%v/v PG in water at 70°C for 60 min. After extraction, the concentration of the extract by means of sugaring out showed that the phase separation can be suitably triggered at 200 g/L of glucose in both water-EtOH and water-PG systems. The phase ratio of water-EtOH system was 2.0 and water-PG system was 2.1. The distribution ratios of gallic acid and ellagic acid in water-EtOH system were 4.5 and 3.1, respectively, which were higher than water-PG system.

## 1. Introduction

Fresh and dried fruits *Terminalia chebula* Retzius, known as Samor thai have 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 corilagin (CG), ellagic acid (EA), and gallic acid (GA) (Worasuttayangkurn et al., 2001).

Although phenolic compounds of *T. chebula* are partially soluble in water, the solubility of phenolic compounds can be improved employing appropriate water-cosolvent systems. An example is water-ethanol (water-EtOH) mixture, which has been successfully used for extraction of phytochemical compounds from many plants (Durling et al., 2007, Cho et al., 2009). Another interesting water-cosolvent system for extraction of natural compounds is water-propylene glycol (water-PG) system, which is used commonly to enhance the solubility of lipophilic drug such as Valdecoxib (Liu et al., 2005) and paracetamol (Jouyban et al., 2006). However, to the best of our knowledge, no previous studies have been conducted using propylene glycol for extraction of natural phytochemicals.

In this study, water-EtOH system and water-PG system will be employed for extraction of phenolic compounds from *T. Chebula.* The effects of extraction conditions on the total phenolic yields were examined using the central composite design (CCD). Moreover, the possibility the extract concentration by means of sugaring out concentration, a new alternative for separation and concentration using monosaccharide (Wang et al., 2008), was investigated.

## 2. Materials and Methods

### 2.1 Extraction of total phenolic compounds

#### 2.2.1 Water-ethanol and water-PG extraction

One gram of powdered *T. chebula* fruits were suspended in round bottom flask and refluxed in 150 ml of extraction mixtures. The effect of different variables on extraction efficiency such as temperatures ($X_1$) cosolvent (EtOH or PG) concentrations ($X_2$), extraction times ($X_3$) was studied using spherical CCD.

### 2.2 Sugaring out concentration of the extract

Initially, 10 ml of extracts in EtOH and PG solutions was placed in test tube. Glucose was added into the extracts, after which, the extracts were heated until all added glucose dissolved completely, at which point the phase separation was induced. The effects of the glucose concentrations (50-250 g/L) were determined on the efficiency of the phase separation, which was quantified in terms of the phase ratio (R), which is the ratio of the volumes of the top and the bottom phase, respectively. At a selected glucose concentration giving the highest phase ratio, the concentrations

of the phenolic compounds (GA and EA) in each phase were analyzed (using HPLC) and the distribution coefficients (D) or the ratio concentrations of the solute in the top and the bottom phases were determined.

**2.3 Analysis of total phenolic compounds, GA and EA**

The determination of the total phenolic content in the extract was carried out using Folin-Ciocalteau method modified from that described in previous study using gallic acid as a reference compound (Rodríguez-Meizoso et al., 2006). The analysis of GA and EA is performed using high performance liquid chromatography (HPLC).

**3. Results and Discussion**

**3.1 Extraction**

The extraction results showed that the highest amounts of total phenolic contents were 242 mg/g DW and 216mg/gDW for water-EtOH and water-PG system, respectively. These were obtained after 60 min of extraction with 63%v/v of EtOH in water at 70°C for water-EtOH system, and after 60 min of extraction with 40%v/v of PG in water at 70°C for water-PG system, respectively. The extraction without using cosolvent system showed gave the extracts containing the lowest phenolic contents. Based on the analysis of variance (ANOVA) for the CCD experiments, temperature and time are significant factors for extraction with water-EtOH system, where as temperature and PG concentrations are significant factors for extraction with water-PG system ($p<0.05$). The model equations for the total phenolic yields are described by Eq 1 and 2, respectively. The coefficients of variation (R-squared) for YEtOH was 0.893 and for YPG was 0.786.

$$Y_{EtOH} = 202.367 + 10.066X_2 - 20.849X_2^2 - 13.061X_3^2 \quad (1)$$

$$Y_{PG} = 175.575 + 7.472X_1 + 5.699X_2 \quad (2)$$

**3.2 Concentration**

The extracts containing the highest total phenolic compound concentrations (obtained with by 63%v/v ethanol in water and 40%v/v propylene glycol in water) were used for the investigation of sugaring out concentration. The addition of glucose into the extracts, followed by heating until the sugar dissolved, resulted in the separation into the organic rich phase (top phase) and the aqueous phase (bottom). Fig 1 shows the phase ratios at different glucose concentrations. From this figure, the highest phase ratios (R= 2.0 for water-

EtOH system and R= 2.1 for water-PG system) were obtained with 200 g/L of glucose added into the mixtures. At this concentration, the concentrations of GA and EA in the two phases were determined (Table 2), from which the distribution coefficients of GA and EA were then calculated. The results are summarized in Table 2, where the DGA,EtOH and DGA,PG were 4.860 and 3.212 for water-EtOH system and water-PG system, respectively, while DEA,EtOH and DEA,PG were 3.427 and 2.583 for water-EtOH system and water-PG system, respectively.

**Table 2.** Concentration using glucose 200g/L in 10 ml extracts

(a) water-ethanol system (b) water-propylene glycol system.

(a)

| Water-EtOH | Concentration (mg/ml) | |
|---|---|---|
| | Gallic acid | Ellagic acid |
| Top phase | 0.076 | 0.063 |
| Bottom phase | 0.016 | 0.018 |
| Distribution coefficient | 4.860 | 3.427 |

(b)

| Water-PG | Concentration (mg/ml) | |
|---|---|---|
| | Gallic acid | Ellagic acid |
| Top phase | 0.066 | 0.046 |
| Bottom phase | 0.021 | 0.018 |
| Distribution coefficient | 3.212 | 2.583 |

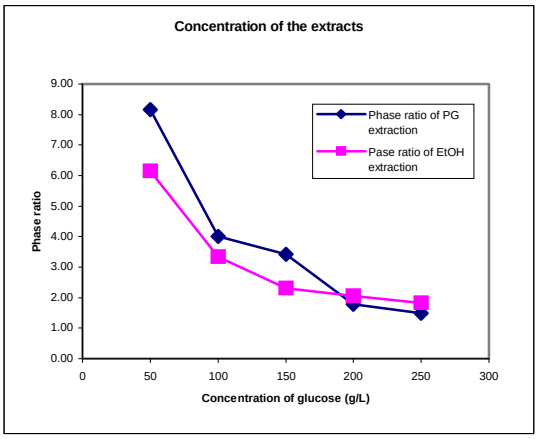


**Fig 1.** Phase ratio of extracts

## 4. Conclusions

The results in this study demonstrated that water-EtOH and water-PG system can improve the amount of phenolic compounds from *T.chebula* fruits extracts. Moreover, the extraction can be carried out at low temperature. The phase separation can be formed by using glucose as separating media.

## 5. References

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# VITA

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