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SUBCRITICAL WATER EXTRACTION OF RESVERATROL FROM BARKS OF *SHOREA ROXBURGHII G. DON*.

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Field of Study	Chemical Engineering
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สุมาลี ไชยณุกูล : การสกัดสารเรสเวอราทรอลจากเปลือกของลำต้นพะยอมด้วยน้ำกึ่งวิกฤต (SUBCRITICAL WATER EXTRACTION OF RESVERATROL FROM BARKS OF *SHOREA ROXBURGHII G. DON*.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร อาทิวรรณ โชติพฤกษ์, 44 หน้า.

พะยอมเป็นพืชสมุนไพรไทยที่มีสรรพคุ ณทางด้านยา เช่น ต้านมะเร็ง ต้านการอักเสบ ป้องกันโรคหัวใจและโรคอัลไซเมอร์ สารสำคัญที่พบในส่วนของเปลือกของลำต้นพะยอมคือสารพอลิฟีนอลิก ประกอบด้วย สารทรานส์เรสเวอราทรอลและทรานส์พีซิด โดยในธรรมชาติมักพบสารทรานส์พีซิดมากกว่าสารทรานส์เรสเวอราทรอล อย่างไรก็ตาม ควา มสามารถในการออกฤทธิ์ของสารทรานส์พีซิด น้อยกว่าสารทรานส์เรสเวอราทรอล ดังนั้น วัตถุประสงค์ของ งานวิจัยนี้เพื่ อศึกษาการสกัดสารทรานส์เรสเวอราทรอลและทราส์พีซีดจากเปลือกของลำต้นพะยอมด้วยน้ำกึ่งวิกฤต และศึกษาการเปลี่ยนรูปของสารทราน ส์พีซิดเป็นสารทราน ส์เรสเวอราทรอล ด้วยการไฮโดรไลซิสด้วยเอนไซม์บีต้ากลูโคซิเดส ในส่วนแรก ได้ทำการศึกษาสภาวะที่เหมาะสมของการสกัดด้วยน้ำกึ่งวิกฤต ได้แก่ อุณหภูมิ (100 – 190 องศาเซลเซียส) และอัตราการไหลของน้ำ (2 – 4 มิลลิลิตรต่อนาที) โดยสกัดนาน 6 ชั่วโมง ที่ความดันคงที่ คือ 10 เมกกะปาสคาล และการเปรียบเทียบผลกับปริมาณสารที่สกัดได้จากวิธีการสกัดแบบดั้งเดิม จากผลการทดลองพบว่าปริมาณสารทรานส์เรสเวอราทรอลที่สกัดได้มีปริมาณน้อย อยู่ในช่วง 0.68 – 13.01 ไมโครกรัมต่อกรัมน้ำหนักแห้ง ในขณะที่ได้ปริมาณสารสกัดทรานส์พีซิด เท่ากับ 301.70 ไมโครกรัมต่อกรัมน้ำหนั กแห้ง ในสภาวะที่เหมาะสมที่สุดคือที่ อุณหภูมิ 190 องศาเซลเซียส อัตรา การไหลของน้ำ 3 มิลลิลิตรต่อนาที เมื่อเปรียบเทียบปริมาณสารสกัดที่ได้จากวิธีน้ำกึ่งวิกฤตกับวิธีอื่นๆ พบว่าปริมาณสารสกัดทรานส์พีซิดที่ได้จากการสกัดด้วยน้ำกึ่งวิกฤตจะให้ปริมาณสารสกัดทราน ส์พีซิดสูงกว่าการสกัดด้วยวิธีใช้ตัวทำละลายและวิธีซอคเลท คือได้สารสกัดเท่ากับ 130.88 68.37 และ 74.87 ไมโครกรัมต่อกรัมน้ำหนักแห้ง ตามลำดับ งานวิจัยในส่วนที่สองเกี่ยวข้องกับ การศึกษาการเปลี่ยนรูปของสารทรานส์พีซิดเป็นสารทรานส์เรสเวอราทรอล โดยอาศัยการไฮโดรไลซิสด้วยเอนไซม์บีตากลูโคซิเดสที่สภาวะการบ่มคงที่ที่อุณหภูมิ 30 องศาเซลเซียส นาน 17 ชั่วโมง และเปรียบเทียบผลการไฮโดรไลซิสของสารสกัดที่ได้จากวิธีน้ำกึ่งวิกฤตกับการไฮโดรไลซิสของสารสกัดที่ได้จากวิธีดั้งเดิม พบว่า ปริมาณสารสกัดทรานพีซิดหลังจากการไฮโดรไลซิสสารสกัดที่ได้จา กวิธีซอคเลทและวิธีใช้ตัวทำละลายด้วยน้ำลดลง ในขณะที่ปริมาณสารทรานส์เรสเวอราทรอลเพิ่มขึ้น ในส่วนการไฮโดรไลซิสสารสกัดที่ได้จากวิธีน้ำกึ่งวิกฤตที่อุณหภูมิ 190 องศาเซลเซียส ให้ผลการทดลองคล้ายคลึงกับสารสกัดที่ได้จากการสกัดด้วยเอทานอล กล่าวคือปริมาณสารทรานส์พีซิดไม่สามารถเปลี่ยนเป็นสารทรานส์เรสเวอราทรอลได้ ดังนั้นการศึกษาเพื่อลดอุณหภูมิของการสกัดและการหาสภาวะที่เหมาะสมของการไฮโดรไลซิสด้วยเอนไซม์บีตากลูโคซิเดสจึงต้องทำการศึกษาเพิ่มเติมต่อไป

ภาควิชา……วิศวกรรมเคมี...……ลายมือชื่อนิสิต.....

สาขาวิชา…....วิศวกรรมเคมี……ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก……

ปีการศึกษา ……2554……

5270557321 : MAJOR CHEMICAL ENGINEERING
KEYWORDS: SUBCRITICAL WATER EXTRACTION / *SHOREA ROXBURGHII G. DON*. / PHA-YOM / PHENOLIC COMPOUND / *TRANS*-RESVERATROL / *TRANS*-PICEID

SUMALEE CHAINUKOOL: SUBCRITICAL WATER EXTRACTION OF RESVERATROL FROM BARKS OF *SHOREA ROXBURGHII G. DON*. ADVISOR : ASSOC. PROF. ARTIWAN SHOTIPRUK, Ph.D., 44 pp.

Phayom *(Shorea roxburghii G. Don.)* is a Thai plant traditionally known for its medicinal properties such as anti-cancer, anti-inflammation, cardiovascular disease and alzheimer disease protection. Phayom contains important polyphenolic compounds consisting of *trans*-resveratrol and glycosylated forms (known also as piceid), found especially in the barks. In general, the form existed in the plant is *trans*-piceid rather than free *trans*-resveratrol. Nevertheless, bioavailability of the *trans*-piceid is much lower, compared with that of the free *trans*-resveratrol. Therefore, this study aimed to extract *trans*-resveratrol and *trans*-piceid from Phayom barks obtained by subcritical water extraction (SCWE) and to obtain free form of *trans*-resveratrol via β-glucosidase hydrolysis of the extract. Firstly, the investigation was carried out to find suitable conditions for extraction of resveratrol from bark of Phayom with subcritical water. The effects of temperature (100-190 °C) and water flow rate (2-4 ml/min) were considered for extraction carried out for 6 hours, at a constant pressure of 10 MPa. The results were then compared with those obtained with conventional methods. The amount of *trans*-resveratrol in the extracts was found to be small (0.68 – 13.01 μg/g DW) compared with the amount of *trans*-piceid, whose maximum amount of 301.70 μg/g DW was obtained at 190 °C and at the flow rate of 3 ml/min. The amounts of *trans*-piceid obtained by the SCWE, solvent extraction and soxhlet extraction were 130.88, 68.37, 74.87 μg/g dry weight (DW), respectively. Overall, the extract obtained by SCWE has higher amount of *trans*-piceid than those of solvent extraction and soxhlet extraction. Secondly, the transformation of *trans*-piceid into free *trans*-resveratrol by using the enzymatic hydrolysis with β-glucosidase was studied. The hydrolysis was carried out at the incubation temperature of 30 °C and the incubation time of 17 hours. The results were compared for hydrolysis of various extracts obtained from SWE and other conventional methods. The amount of *trans*-piceid in the extracts obtained with soxhlet and reflux extraction with water was found to decrease as a result of β-glucosidase hydrolysis, while to the amount of *trans*-resveratrol increased. This result demonstrated that the conversion took place as a result of the enzymatic treatment of these water extracts. The hydrolysis of the subcritical water extract obtained at 190 °C gave similar results as that of the ethanol extract, that is, *trans*-piceid could not be converted to *trans*-resveratrol. Conversion of *trans*-piceid to *trans*-resveratrol may further be improved further by decreasing the extraction temperature and optimizing the hydrolysis condition.

Department :..…Chemical Engineering… Student's Signature.....

Field of Study :...Chemical Engineering… Advisor's Signature…….

Academic Year : ……2011…….....

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CHAPTER I

1.1 Motivation

Shorea roxburghii G. Don. (S. roxburghii) is a plant in the Dipterocarpaceae family. It is found to be distributed in the mixed deciduous forests or in evergreen forests of Asia, Africa as well as South America [Sasaki et al., 2008]. The color of the wood is light olive yellow to light yellowish or reddish-brown [Pande et al., 2007]. The inner bark is generally brownish [Jayanthi et al.]. Traditionally, the wood of *S. roxburghii* has been used to make plywood and furniture [Nakamura et al., 2006]. Moreover, the flowers of *S. roxburghii* have been used to treat diarrhea, dysentery, bloody stool and astringent. In Thailand, the plant is commonly known as Pha-Yom. The barks of Pha-Yom have been reported to have high content of phenolic compounds which are known that have antioxidant properties and specific biological action in preventing and treating various diseases. Bioactive compounds contains in barks of Pha-Yom including tannin, flavonoid, coumarins [Temsiririrkkul et al., 2004] and resveratrol oligomers [Ito et al., 2003; Ito et al., 2005]. These compounds have strong antioxidant capacity. Of the most interest is resveratrol due to its high biological activity such as anticancer, anti-inflammatory, blood sugar-lowering properties, protecting human health by diverse mechanisms. However, the compound exists in a very low content in plant materials. When it does exist, it occurs as glycosylated forms rather than the more active free form. To increase quantities of biologically active *trans*-resveratrol in barks of *S. roxburghii* by, the glucoside form of resveratrol in the extract must be converted into the free form. This can be done via a hydrolysis process, a method currently used to transform glucoside form into aglycons. Previous studies demonstrated that acid or base can transform glycosylated and esterified phenolics into their aglycons [Hong and Wrolstad., 1986; Markham, 1982], however, the process is environmentally unfriendly. To reduce the environmental impact, enzymatic hydrolysis is the new alternative for the transformation of glucoside form into aglycons. β-Glucosidase is a glucosidase enzyme shown to increase aglycone in wine [Todaro et al., 2008; Torre et al., 2004], grapevine [Jeandet et al., 1997] and onion waste [Turner et al., 2006] by hydrolyzing its glucoside forms. In this study, after

extraction, the hydrolysis of glycosylated resveratrol in Pha-yom extract will be carried out using glucosidase enzyme.

As for extraction of resveratrol, different organic solvents have been investigated, for instance, 100% acetone [Ana et al., 2001], 80% ethanol [Maite et al., 2000; Jocelyn et al., 2009; Irena et al., 2004], 100% ethyl acetate [Bravo et al., 2008; Fan et al., 2008] and 90% methanol [Mahmood et al., 2008] have been employed for resveratrol extraction from various plants. Supercritical carbon dioxide extraction (SC-CO_2) has also been applied to extract resveratrol from dried grape, including grape pomace, seed, stem and skin [Casas et al., 2010]. The result showed that, SC-CO_2 is not suitable for the extraction of polar analytes without use of organic solvent modifier.

Alternatively, subcritical water extraction (SCWE), extraction with water under sufficient pressure to maintain it in a liquid state at temperatures well between boiling and critical point, has become an interesting technique for extraction of herbal plants. The technique has shown promise for replacing conventional extraction methods for the extraction of polar and slightly non-polar compounds from raw materials by modifying the polarity of water through changes in temperature and pressure. The important advantage of SCWE is the reduction in the usage of organic solvent. Moreover, water is available, non-flammable, non-toxic solvents and can be recycled or disposed with minimal environmental problems. Furthermore, this technique is a fast, clean, low cost and highly efficient method of extraction. Extraction with subcritical water has been shown to be effective for several compounds such as lignans, proteins and carbohydrates from flaxseed [Ho et al., 2007], lactones from kava roots [Kubatova et al., 2001], anti-cancer damnacanthal from roots of Yor [Anekpankul et al., 2007], phenolic compounds from fruits of Bitter melon [Budrat et al., 2008] and from fruits of Samor Thai [Rangsriwong et al., 2009].

The objective of this study is to extract phenolic compounds from the barks of *S. roxburghii* with SCWE. The effect of solvent flow rate and extraction temperature on total phenolic content will be investigated, and the results will be compared with reference soxhlet extraction method. Furthermore, the extract obtained from the best suitable extraction conditions were further hydrolyzed by β-glucosidase enzyme. The hydrolysis

results will be compared with those of the extracts obtained by conventional methods such as soxhlet and stirred vessel water and ethanol extraction.

1.2 Objectives

1.2.1 To investigate the suitable conditions for extraction of phenolic compounds from *Shorea roxburghii G. Don*. with subcritical water.

1.2.2 To obtain free form of *trans*-resveratrol via β-glucosidase hydrolysis of the extract.

1.3 Working scopes

1.3.1 Determination of the suitable condition for subcritical water extraction of phenolic compounds from *Shorea roxburghii G. Don.* considering the effects of two factors: temperature (100-190 °C), and flow rate (2-4 ml/min), at a fixed pressure of 10 MPa, on the amount of the compounds extraction and the extraction rate. The extraction time is approximately 6 hr.

1.3.2 Determination and identification of *trans*-resveratrol and glucoside of resveratrol (*trans*-piceid) using HPLC on the basis of reference standard solution.

1.3.3 Determination of the content of free *trans*-resveratrol obtained by converting the glucoside form using β-glucosidase hydrolysis. The hydrolysis conditions were taken from literature and the results were compared for hydrolysis of various extracts obtained from SWE and other conventional methods.

1.4 Expected benefit

The results of this research can be used as reference for SCWE study of herbal or natural products.

CHAPTER II

BACKGROUND AND LITERATURE REVIEWS

Background

2.1 Introduction of *S. roxburghii*

Shorea roxburghii G. Don. is a genus of plant in the Dipterocarpaceae family. It is found to be distributed in the mixed deciduous forests or in evergreen forests of Asia, Africa, and South America [Sasaki et al., 2008]; in which it grows to over 40 meters tall and 1 meter in diameter. The larger fruit is a capsule with winged calyx lobes and is up to 9 centimeter × 1.2 centimeter. The bark is 2-2.5 centimeter thick, gray, and rather deeply fissured; the inner bark has brownish concentric bands [Jayanthi et al.]. In general, *S. roxburghii* is used in plywood and furniture [Nakamura et al., 2006]. The variety commonly found in Thailand is called Pha-Yom, which is used as food and medicine. *S. roxburghii* has been used for the treatment of diarrhea, dysentery, bloody stool and astringent. Although parts are used including barks, flowers, leaves, roots and seeds, the barks are the most widely used medicinally [Ito et al., 2003; Ito et al., 2005].

Figure 2.1 Barks of *Shorea roxburghii G. Don.*

2.2 Resveratrol compound

Resveratrol (3,4,5-trihydroxystilbene) is stilbene phenolic compound consisting of two aromatic rings joined by a methylene bridge [Das et al., 2010]. Resveratrol synthesis is activated in response to a variety of biotic such as microbial invasion, and abiotic stresses such as wounding, UV, ultrasound (US), ozone and chemical treatments [Jocelyn et al., 2009]. The molecular formula of resveratrol is ($C_{14}H_{12}O_3$) and its molecular weight is 228.25. Resveratrol is found as a free form or glycosylated forms, but naturally, it is mostly found in glycosylated forms. Both free and bound resveratrol exists in *cis*- and *trans*- isomeric forms, and the glycosylated resveratrol is known as a piceid. The amount of resveratrol is more or less depending on location, season, and temperature in which the plants are grown. The isomerization of *trans*-resveratrol to *cis*-resveratrol can occur by exposure to sunlight. Resveratrol is widely used in medicine, food, as well as cosmetics because of its pharmacological properties. The interest in resveratrol has increased due to its possible cardioprotective action, including the inhibition of low-density lipoprotein oxidation, inhibition of smooth muscle cell proliferation, and platelet aggregation [Cecil et al., 1995; Kirk et al., 2000], which are well-known to contribute to heart attack or strokes. Resveratrol is also highly active in inhibiting the formation of preneoplastic lesions in a mouse mammary organ culture model [Bhat et al., 2001; Bhat et al., 2002]. Resveratrol has also been shown to enhance the production of nitric oxide which is a chemical that helps keep arteries relaxed, allowing for improved blood flow [Wallerath et al., 2002]. However, resveratrol is generally found in a low content in plants and the compound is quite sensitive to light, air and temperature.

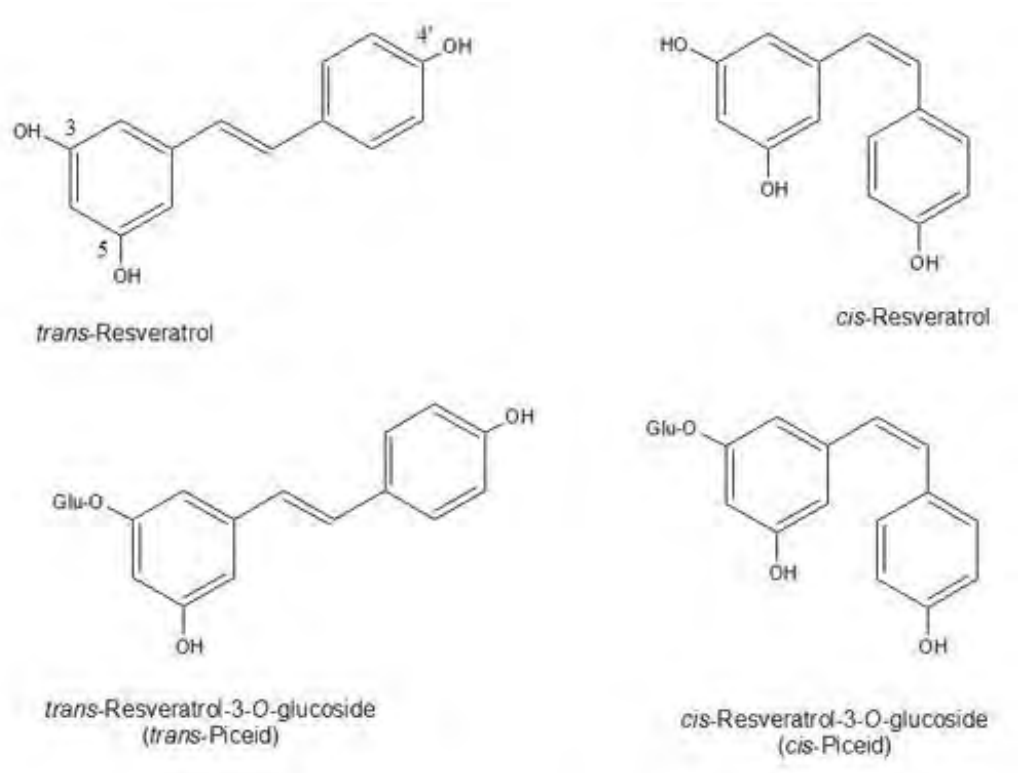


Figure 2.2 Molecular structure of *trans*-, *cis*-resveratrol and glucoside of resveratrol.

2.3 Subcritical water extraction technology

Subcritical water (SCW) is defined as the region of condensed phase of water at temperature between the boiling point temperature (100 °C) and the critical point temperature (374 °C) and at a pressure high enough to maintain the liquid state as shown in Figure 2.3. It is also known by other names such as hot water extraction, pressurized hot water extraction (PHWE), pressurized low polarity water extraction (PLPWE), high-temperature water extraction, superheated water extraction or hot liquid water extraction. Subcritical water extraction (SCWE) is a promising “green” technique based on the use of water as the extraction solvent. At ambient pressure and temperature, water is a highly polar solvent with a high dielectric constant (ε) at room temperature of approximately 80 due to the presence of extensive hydrogen-bonded structure. Hence, water is not a

suitable extraction fluid for non-polar or organic compounds at this condition. When the temperature of water is raised to 250 °C while the liquid state is maintained by keeping the pressure high enough , its ε is reduced to approximately 27, which falls between those of methanol (ε = 33) and ethanol (ε = 24) at 25 °C [Hawthorne et al., 1994]. Thus, under these conditions, water behaves like certain organic solvents which can dissolve a wide range of organic solutes from different matrixes. Therefore this technique is an alternative for benign extraction of solid sample. The major advantage of SCWE is the reduction in the usage of organic solvent. Moreover, water is available, non-flammable, non-toxic solvents and can be recycled or disposed with minimal environmental problems. SCWE is therefore a fast, clean, low cost and highly efficient method of extraction for less-polar organic components from environmental soil, sediments and plant materials.

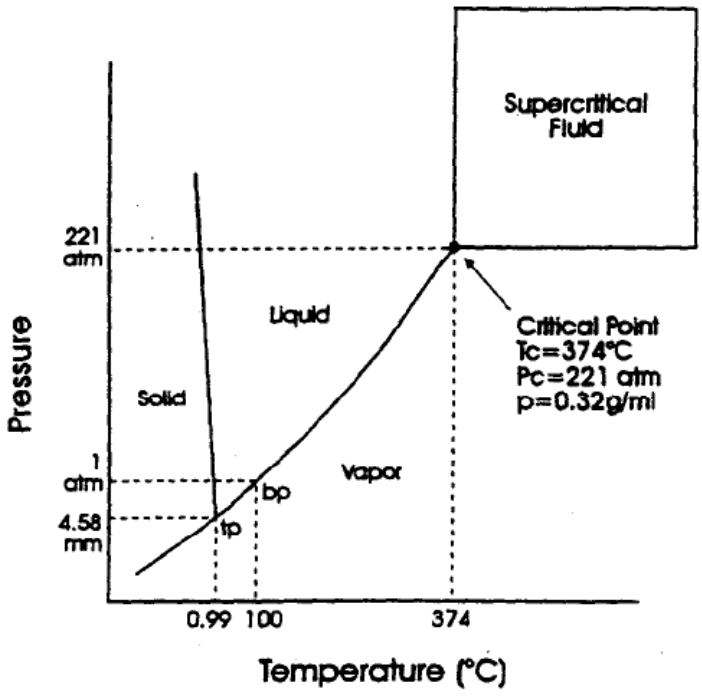


Figure 2.3 Phase diagram for water as a function of temperature and pressure.

2.4 Literature reviews

2.4.1 Various forms of resveratrol and conversion of *trans*-piceid to *trans*-resveratrol

In recent years, there has been an interest in the study of important compounds in herbal medicine. Among various groups plant compounds, phenolics are of the most important components broadly distributed in the plant kingdom and, therefore are found in many common foods including wine, tea, coffee, beer, fruits, beverages and various medicinal plants [Ana et al., 2007]. One of the most important phenolic compounds is resveratrol which is generally found in wine, grape, mulberry, bilberry, cranberry, blueberry, Ko-J-Kon in japan, knotweed, peanut [Ito et al., 2005; Ito et al., 2006]. In Thailand, barks of *S. roxburghii* (a plant in Dipterocarpaceae family, known as Pha-Yom) are well known to be the rich source of various resveratrol oligomers. Although all parts of Pha-Yom (barks, flowers, leaves, roots and seeds) are used medicinally as they contain several phenolic compounds particularly tannin, flavonoid and coumarins [Temsiririrkkul et al., 2004], the barks are found to contain the highest content of reveratrol. The compound has been shown to have significant effects on the growth inhibition of human lung carcinoma A549 cells (non-small-cell lung cancer) by blocking cell cycle progression [Chen et al., 2010], the action on multiple direct targets of carcinogenesis [Sengottuvelan et al., 2009], the inhibition of the generation of reactive oxygen species in blood platelets [Olas et al., 2001]. In addition, it has also been reported that the antioxidant activity of resveratrol is comparable with that of vitamin E. [Agrawal et al., 2007].

In plants, resveratrol generally occurs in two isomeric forms, *trans* and *cis*, and is found as a free or glycosylated forms. For instance, in *Polygonum cuspidatum*, a plant originated in China, the average content of piceid is six times higher than that of free resveratrol [Zhou et al., 2002]. Also, Patcharamun et al., (2009) demonstrated that resveratrol compounds exist mostly as glycosylated forms (known also as piceid) in the roots of *S. roxburghii*. Nevertheless, bioavailability of the glycosylated forms is much lower, compared with that of the free resveratrol [Meng et al., 2004]. To increase

contents of the free resveratrol, the glucoside functional group must be removed. Traditionally, aglycone is acquired by hydrolyzation of its glucoside, and acid or alkaline is used as the hydrolytic reagent. However, the hydrolytic reaction is always carried out under violent conditions and causes pollution [Rommel and Wrolstad, 1993]. In contrast, biotransformation only requires mild conditions and results in less pollution [Wendy, 2000]. Wang et al., (2007) compared different hydrolysis of raw herb of *Polygonum cuspidatum* such as H_2SO_4 hydrolysis, enzymatic hydrolysis and fermentation by *Aspergillus oryzae.* The results showed that the enzymatic hydrolysis of raw herb, *trans*-piceid was converted to *trans*-resveratrol with the highest yield was equal to that from fermentation of raw herb was performed by *Aspergillus oryzae.*, but enzymatic hydrolysis used shorter time than fermentation.

In recently years, there has been an interest in the study of β-glucosidase enzymatic hydrolysis in raw material. Todaro et al., (2008) demonstrated that β-glucosidase can be produced *trans*-resveratrol increases of up to 75%. Torre et al., (2004) studied the effect of different temperature, 30 - 70 °C in enzymatic hydrolysis and revealed that the product increased with the increase of temperature, from 30 to 60 °C. However, the product started to decrease when the temperature reached 70 °C due to the fast deactivation of the enzyme. In order to enhance the performance of enzymes, the incubate temperature should be decreased. Jeandet et al., (1997); Goldberg et al., (1995) demonstrated that the incubation time of 17 hrs, and the incubation temperature 30 °C, was able to convert all the resveratrol glucosides into their aglycones. The optimum pH of β-glucosidase is 5 [Zhang et al., 2007].

2.4.2 Methods for Resveratrol Extraction

Extraction is an important step for removal of bioactive compounds from plant sources, and the efficiency of the process is much affected by choice of extraction solvents. Ana et al., (2001) compared different solvents including 100% ethanol, 80% ethanol, 50% acetone/methanol, 75% acetone, and 100% acetone for extraction of

resveratrol related compounds. They found that 80% ethanol gave the highest extraction efficiency for *trans*-picied, *cis*-picied and *trans*-resveratrol. However, the disadvantage of conventional solvent extraction is long extraction time, labor intensive procedures and consumption of large quantities of organic solvents. To increase the speed of the extraction process, using less solvent, Liazid et al., (2007) proposed a method of extraction of resveratrol from grapes with microwave-assisted extraction using methanol as the extraction solvent. They found that high microwave assisted extraction temperature minimizes the duration of the process compare with solvent extraction and reduces the solvent requirement. Supercritical carbon dioxide (SC-CO_2) extraction is an alternative technique, in which no organic solvents are utilized. However, this technique is limited to compounds of low or medium polarity. The extraction of resveratrol by SC-CO_2 is only feasible in the presence of organic solvent modifiers [Casas et al., 2001; Pascual-Marti et al., 2001] to give comparable the extraction efficiencies with reference methods, such as soxhlet extraction [Beňova et al., 2010]. Alternatively, SCWE has shown promise for replacing conventional extraction methods for the extraction of polar and slightly non-polar compounds. The main advantages of SCWE are its simplicity, shorter extraction time, low cost of the extracting agent and being environmentally friendly technique. Previous reports demonstrated that SCWE was an effective method for lactone extraction from kava root [Kubatova et al., 2001] and for anti-cancer damnacanthal from roots of *Morinda citrifolia* [Anekpankul et al., 2007] and for antraquinones from roots of *Morinda citrifolia* and for polyphenolic compounds from fruits of *Terminalia chebula* Retz. [Rangsriwong et al., 2009].

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and chemicals

The barks of *S. roxburghii* were obtained from Kasetsart University. Gallic acid, *trans*-resveratrol, *trans*-piceid and β-glucosidase were obtained from Sigma Chemical Co. (St Louis, Mo, USA.). Water used in the experiments was distilled and deionized water. AR grade acetic acid, HPLC grade ethanol and acetonitrile, were purchased from Merck (Darmstadt, FR Germany).

3.2 Preparation of sample and aqueous *cis*-resveratrol solution

3.2.1 Sample preparation

The barks of *S. roxburghii* were dried in an oven at 50 °C about 1 day until it reached a constant weight. The dried sample was crushed into fine powder using moulinex blender. All the finely powdered samples were sieved through a mesh of size 500 μM. This powder was stored at 4 °C in a domestic refrigerator until use.

3.2.2 Preparation of aqueous solutions of *cis*-resveratrol

Cis-resveratrol is not commercially available because of its instability in solid form. It was therefore prepared by exposing 2.5 ppm aqueous solution of *trans*-resveratrol to sunlight at 40 °C, whose intensity is approximately 36,500 lux, for 10 min.

3.3 Subcritical water extraction (SCWE)

Subcritical water extraction was carried out in a laboratory-built apparatus shown in Figure 3.1. The extraction system consisted of two HPLC pumps (PU 980, JASCO,

Japan) used to deliver the water and solvent though the system at constant flow rates, a degassing instrument (ERC 3215, CE, Japan), an oven (D63450, HARAEUS, Germany), where the extraction cell (10 ml, Thar Design, USA) was mounted, a pressure gauge, and a back pressure regulator value (AKICO, Japan), a heating cable (2 metre, 220 Volt, Suan Luang Engineering) and a temperature control . All connections were made with stainless steel capillaries (1/16 inch inside diameter).

Water was passed through a degassing equipment to remove dissolved oxygen. The degassed water was pumped through the tubing which was wrapped with a heating cable used to preheating the water before it entered the oven. The preheated water was then passed through the preheating coil, made from 3 m length stainless steel tubing, installed inside the oven before it entered the extraction vessel, which was preloaded with 0.5 g of sample. The back pressure regulator value placed at the outlet of the extraction system was used to maintain the system pressure to ensure that the water was in liquid state at all temperature tested. Before starting the extraction, all connections were checked for possible leakage. The second pump was then turned on to deliver degassed water at constant flow rate of 1 ml/min to wash off any residual product in the outlet line behind the extraction to prevent clogged up the line. The extract was cooled in a coil immersed in a water bath to prevent possible product degradation, and the extract was collected in fractions in sample collecting vials every 30 minutes in a first second hours and after which it was collected every 60 minutes. The extracts were then evaporated under vacuum to remove the water until volume of the extracts were remained about 10 ml and all the extract and standard were thoroughly filtered using 0.45 μm Nylon Filter Media with Polypropylene Housing disk held in 13-mm diameter syringe filter and stored at 4 °C until they were analyzed.

The experimental variables to be studies and their range were listed in Table 3.1

Table 3.1: Condition for experiment

Variables	Condition
Temperature	100-190 °C
Flow rate	2-4 ml/min
Pressure	10 MPa

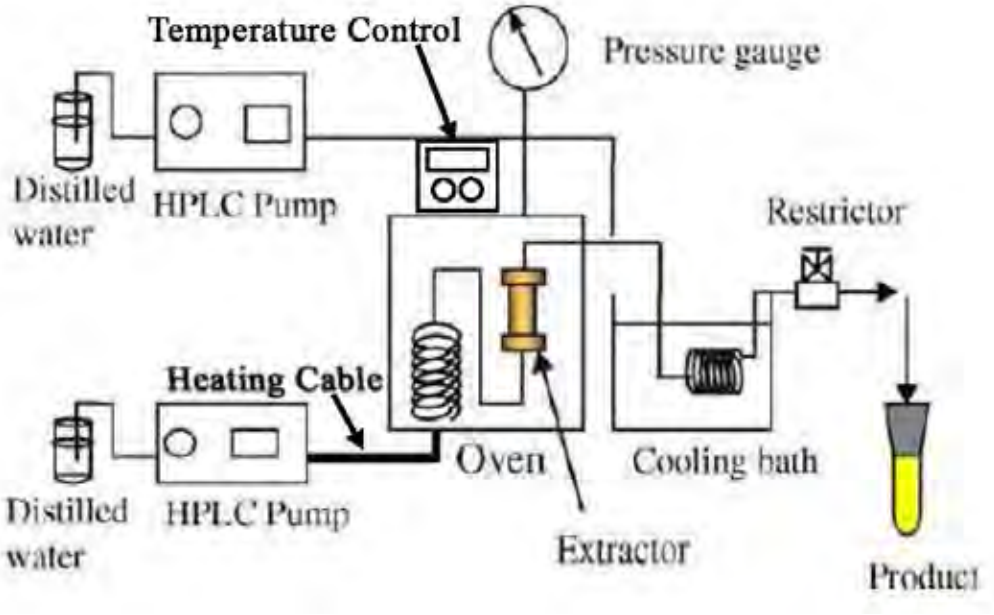


Figure 3.1 Diagram of experimental setup of subcritical water extraction.

3.4 Soxhlet extraction

To determine the total amount of phenolic compounds, particularly *trans*-resveratrol and *trans*-piceid in the plant sample, extraction with Soxhlet apparatus (Figure 3.2) was carried out. To do this, 0.5 gram of the powder sample was put into the cellulose thimble and was extracted with 200 ml of 80% (v/v) ethanol and 100% (v/v) water for a

long time 3 hours. The extract was evaporated under vacuum to remove the volume was about 10 ml. The concentration extract was stored at 4 °C until analysis.

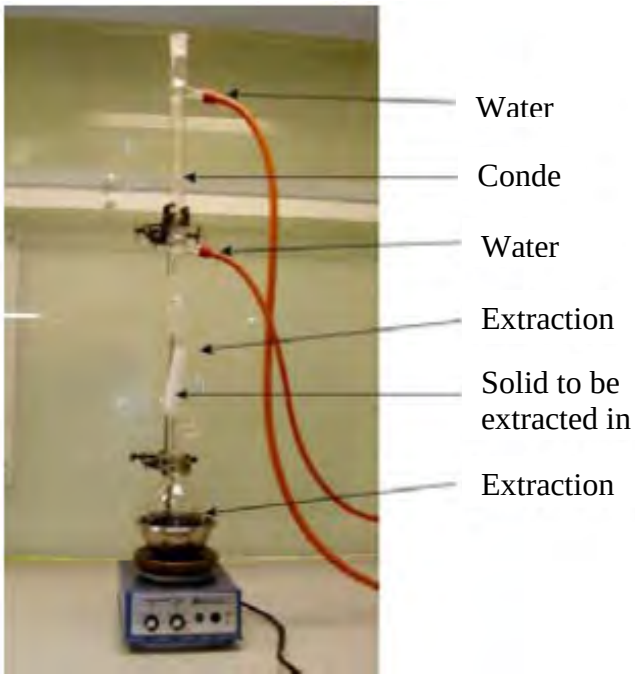


Figure 3.2 Diagram of soxhlet apparatus.

3.5 Enzymatic hydrolysis of resveratrol glucosides

The enzymatic hydrolysis of the extracts obtained by Soxhlet extraction and SWE was performed to convert glucosides form into *trans*-resveratrol free form using β-glucosidase, following the method modified from previously reported literature [Torre et al., 2004]. Initially, 10 ml of the extracts were adjusted to pH 5.0 with 0.1 M NaOH. Then 1 ml aqueous β-glucosidase solution (1 mg ml^{-1}) was added into the extracts. Hydrolysis was carried out by incubation the mixtures at fixed temperatures and time 30 °C for 17 hrs. The mixture was taken immediately placed in an ice bath to stop the reaction. The samples were filtered before HPLC analysis.

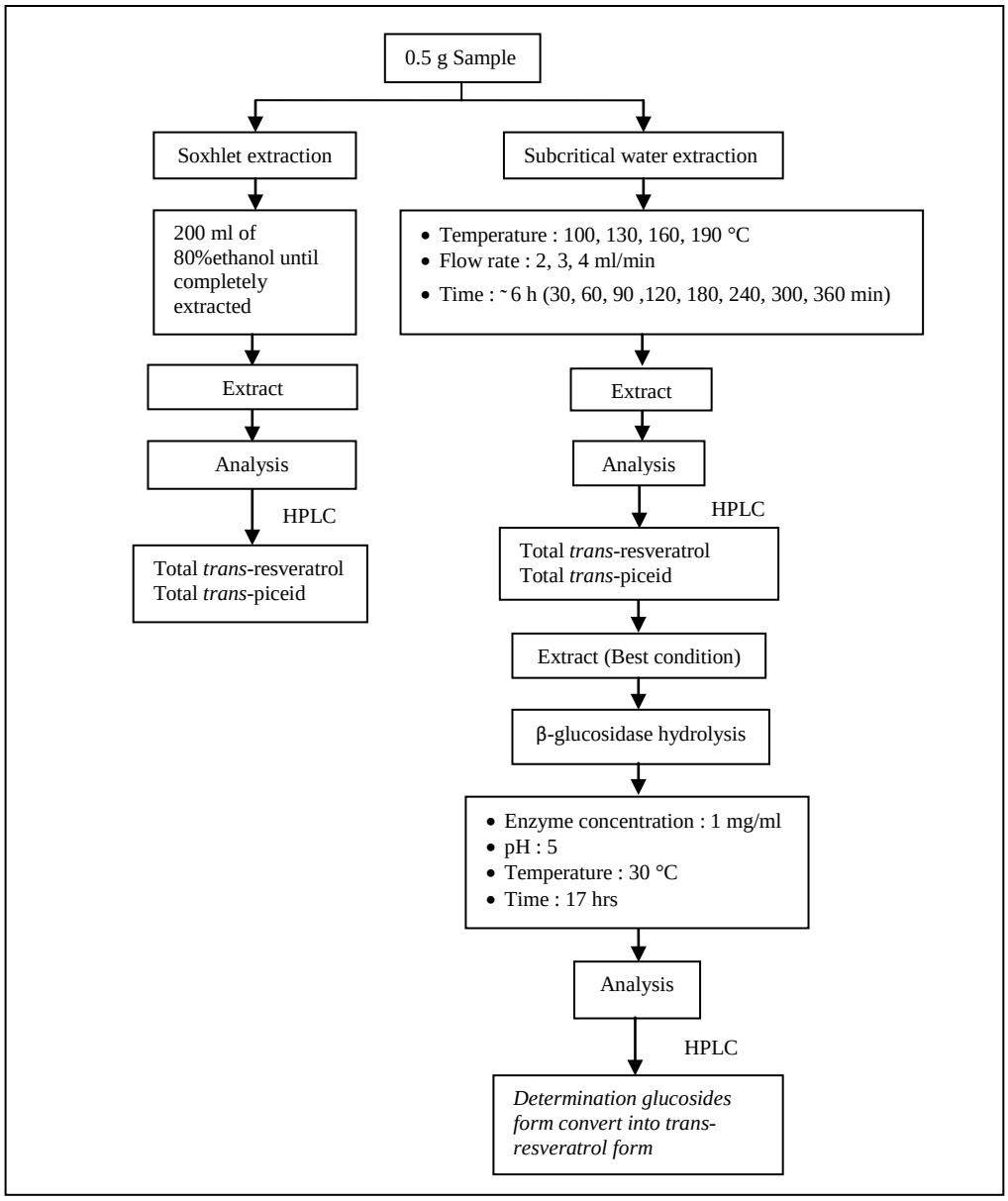


Figure 3.3 Summarizes all experimental studies carried out in this research.

3.6 Analysis

3.6.1 Total phenolic analysis

The determination of the total phenolic content in the extract of *S. roxburghii* 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 was 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 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.

3.6.2 HPLC analysis of *S. roxburghii* extract

The analysis of *trans*-resveratrol, *trans*-piceid and *cis*-resveratrol were determined using High Performance Liquid Chromatography (HPLC) modified from previously reported of [Rudolf et al., 2005]. HPLC were performed with a C_{18} Inertsil ODS-3 column (250 x 4.6 mm ID, 5 µm particle size) and equipped with photodiode array (PDA) detector. The mobile phase consists of solvent A (0.1% acetic acid in water) and solvent B (100% acetonitrile, HPLC grade). All the sample extract injection volume was 20 µL. The flow rate was set at 1.5 ml/min and column temperature was maintained at 25 °C throughout the test.

For the analysis of *trans*-resveratrol and *trans*-piceid were monitored with PDA absorbance at 307 nm. The gradient system started with 5% solvent B at 0 min and was change to 37% solvent B at 23 min followed by an increase of solvent B to 72% over 5 min, with the total run time of 30 min.

For the analysis of *cis*-resveratrol was monitored with PDA absorbance at 285 nm. The gradient system started with 5% solvent B at 0 min and then 42% solvent B in 23 min followed by an increase of solvent B 71% over 5 min. The total run time was 30 min. The concentration of *trans*-resveratrol, *trans*-piceid and *cis*-resveratrol in the sample was calculated from each standard curve, a plot of peak area versus concentration for a series of standard solutions

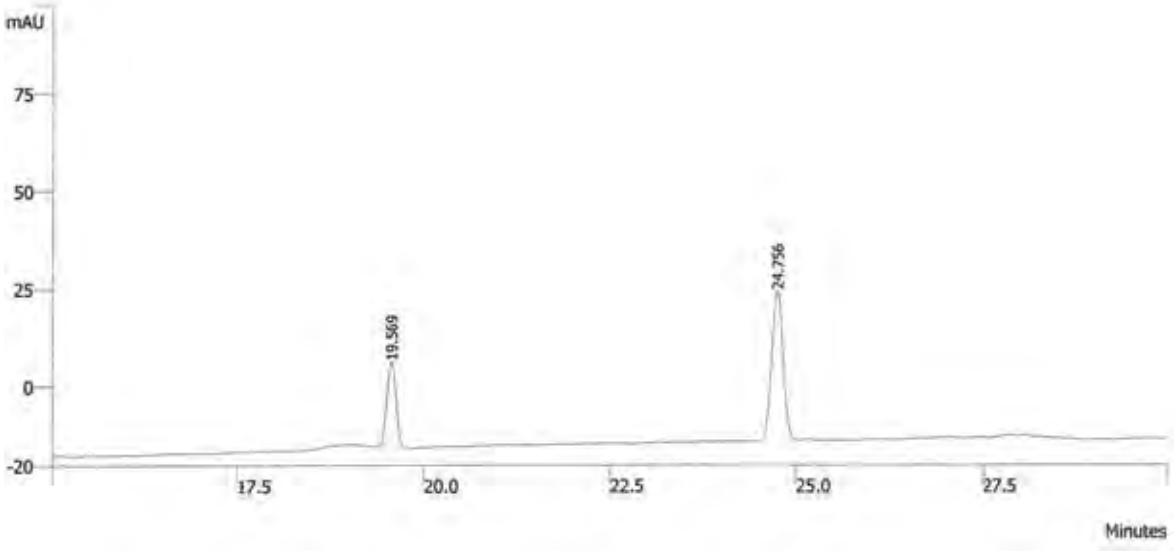
CHAPTER IV

RESULTS AND DISCUSSION

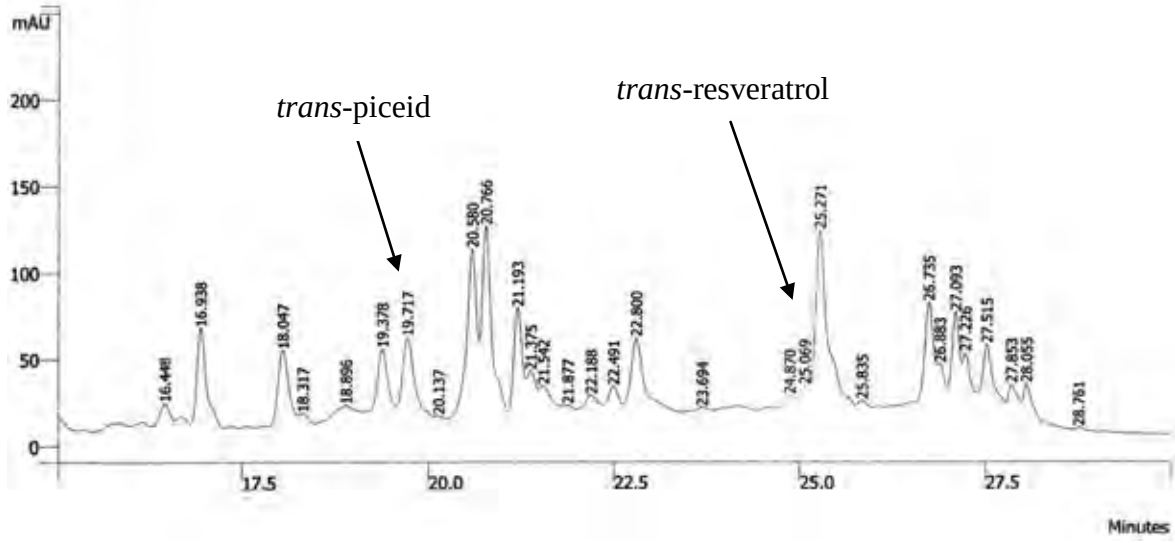
This chapter presents the experimental results of subcritical water extraction of the stilbenes: *trans*-resveratrol and *trans*-piceid from *Shorea roxburghii G. Don*. The effects of temperature and the water flow rate on the extraction performance of the stilbene constituents were presented and the suitable extraction conditions were determined and discussed. The results were compared with those of the conventional water and ethanol extractions using a stirred vessel and soxhlet apparatus. In addition, the hydrolysis of the extracts obtained by SCWE and the conventional extraction methods with β-glucosidase in attempting to convert *trans*-piceid to *trans*-reveratrol was examined.

4.1 Stilbene constituents in *S. roxburghii* bark extract

Stilbene constituents from barks of *S. roxburghii* in subcritical water extract were quantified by HPLC, three of which are of particular interest including *trans*-piceid, *trans*-resveratrol and *cis*-resveratrol. The chromatograms the standard solution of *trans*-piceid and *trans*-resveratrol and the subcritical water extracts of *S. roxburghii* barks are shown in Figure 4.1. From the chromatograms, *trans*-piceid and *trans*-resveratrol peaks have the retention time of about 19.6 and 24.8 min, respectively. Other than in the trans-form, reveratrol also exists in the *cis*-form in *S. roxburghii* bark. The chromatogram of the standard *cis*-resveratrol, obtained by exposing the *trans*-resveratrol standard to sunlight and the *roxburghii* bark extracts are shown in Figure 4.2.

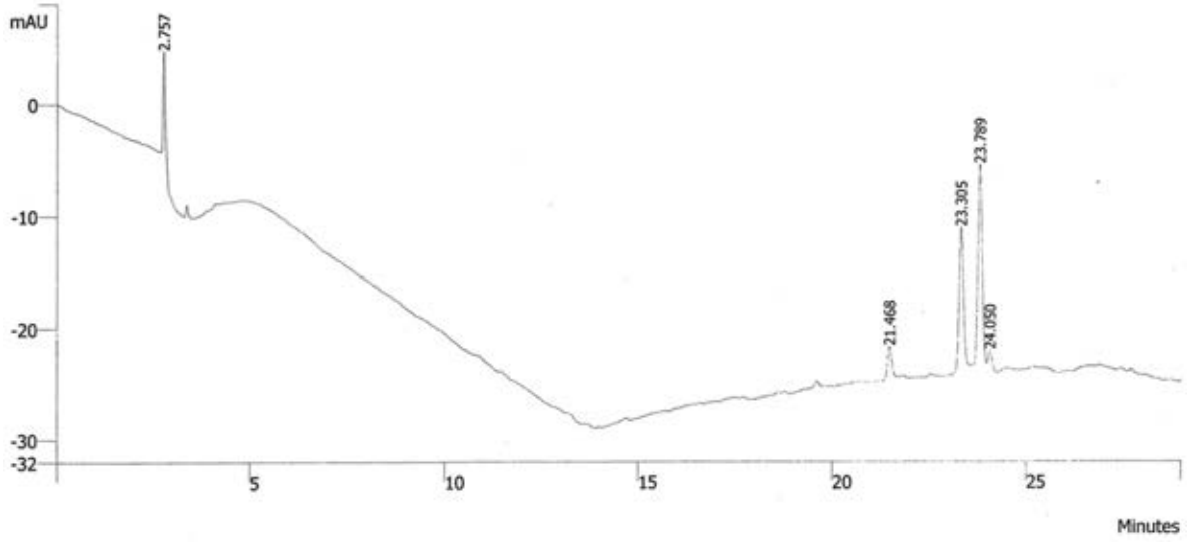


A)

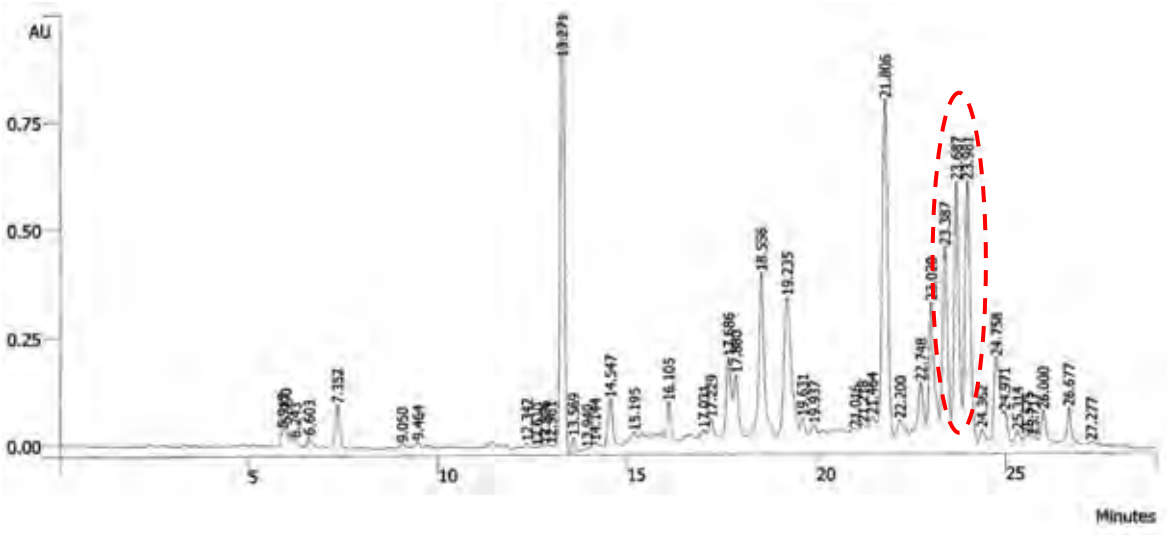


B)

Figure 4.1 Chromatograms of A) *trans*-resveratrol and *trans*-piceid standards B) subcritical water extract from *S. roxburghii* barks.



A)



B)

Figure 4.2 Chromatograms of A) *cis*-resveratrol standard B) subcritical water extract from *S. roxburghii* barks.

4.2 Subcritical water extraction of *trans*-resveratrol and *trans*-piceid contents

4.2.1 Effect of temperature on subcritical water extraction

In this study, subcritical water extraction was carried out to determine the effect of water temperature in the range of 100 to 190 °C on the *trans*-resveratrol and *trans*-piceid contents. A constant water flow rate of 2 ml/min was used and the extraction was carried out at a constant pressure of 10 MPa for up to 6 hours. The cumulative amount of *trans*-resveratrol and *trans*-piceid contents obtained during the extraction is shown in Figure 4.3, which indicates that *trans*-reveratrol is present largely in the *trans*-piceid form in *S. roxburghi* barks, while the amount of *trans*-resveratrol in the extracts was very small (0.68 – 13.01 μg/g DW). Thus the effect of temperature on the amount of *trans*-resveratrol extracted was difficult to quantify. Rather, the recovery of *trans*-reveratrol from this plant requires another hydrolysis step that converts *trans*-piceid to *trans*-resveratrol. The effect of the subcritical water temperature on the amount of extracted *trans*-piceid, on the other hand, was more pronounced, in which it was found that the amount of *trans*-piceid increased with increasing extraction temperature up to 190 °C. In all cases, the initial rate of extraction was high and starts to slow down after 30 min. The high initial rate of extraction was due to the high concentration gradient of the extracted solutes across the surface of the *S. roxburghii* sample at the start of the extraction process. As the extraction proceeded to 360 min, the maximum amount of *trans*-piceid of 301.70 μg/g DW was obtained at the extraction temperature of 190 °C. The increase in extraction yield with temperature is due to the fact that the polarity of water decreases with temperature, resulting in increased solubility of *trans*-piceid in water. It is noted that dielectric constant (ε) of subcritical water at 190 °C is relatively close to that of 80% ethanol at 25 °C [Lide et al., 2009], the concentration of ethanol which was reported to give the highest yield for *trans*-piceid extraction [Cho et al., (2006); Romero-Perez et al., (2001)]. In addition, despite that the high temperature could cause compound degradation, extraction by subcritical water, in many cases (especially for phenolic compounds extraction), was reported to have higher yield, due to the increase in the

ionization constant of water at high temperature condition [Budrat and Shotipruk, 2008]. Goto et al., (2008) demonstrated that hydrolysis reaction promotes the decomposition of lignin into smaller aromatic molecular compound including phenolic compounds. Based on the above results, the extraction temperature of 190 °C, giving highest yield, was chosen as the suitable temperature and used for the subsequent studies.

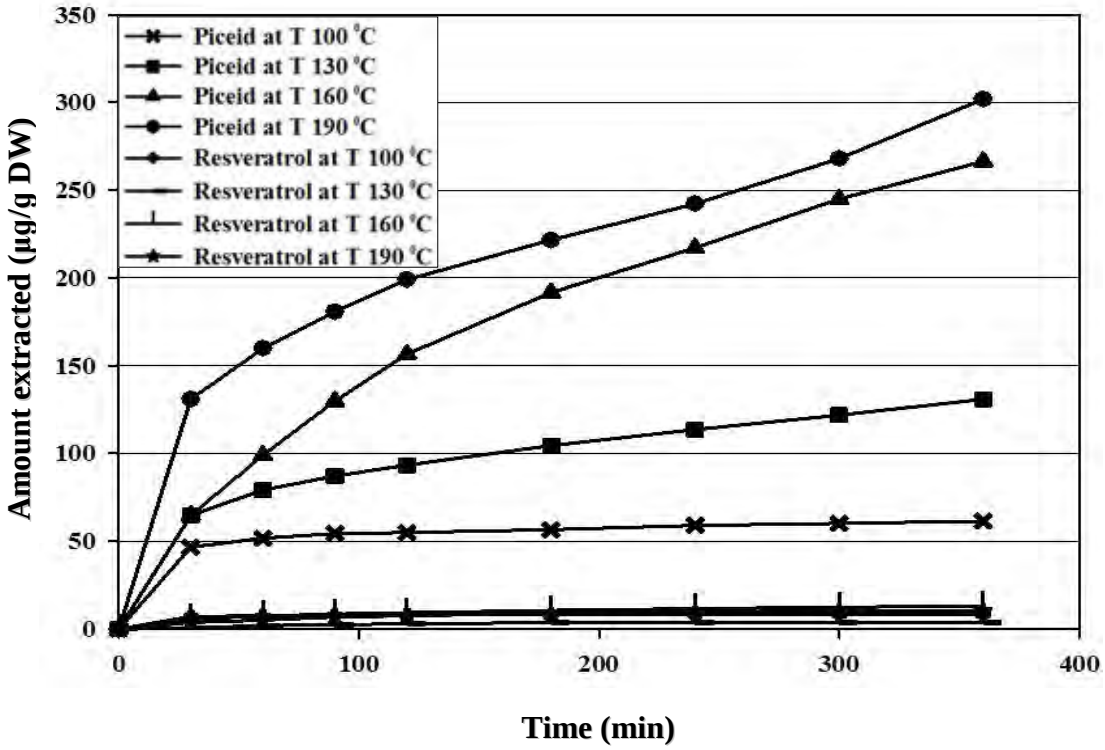


Figure 4.3 Cumulative amount of extracted at various temperatures. Operating condition: flow rate = 2 ml/min, pressure = 10 MPa.

4.2.2 Effect of water flow rate on subcritical water extraction

The effect of water flow rate on the amount of *trans*-piceid extracted was investigated by varying the flow rate from 2 to 4 ml/min at a fixed extraction temperature of 190 °C and a pressure of 10 MPa. The results are presented in Figure 4.4 which shows

the time profile of the extracted *trans*-piceid. Similar to extraction profiles shown in Figure 4.3, Figure 4.4 illustrates that the profiles for various flow rates can be divided into two regions, i.e. (i) the initially high extraction rate region between 0-30 min, and (ii) the subsequent lower mass transfer region. In the first region (0-30 min), the extraction rates of *trans*-piceid were comparable for all flow rates. As the mass transfer rate depends principally on two main factors, mass transfer coefficient (k) and the concentration different driving force (ΔC) (see mass transfer equation of 4.1). During the low flow or laminar flow region which is the case in this work here, the quantity 'k' varies with water flow rate to the power of '*n*' where '*n*' could take the value from 0.1-0.5 [Paterson et al., 2000]. However, the initial rate did not seem to vary with the variation in 'k' which suggested that the initial extraction process was not limited by mass transfer, but perhaps to a more significant level by the driving force or in this case, the concentration difference (ΔC). This is possible as during the initial period, the target compound still stayed inside the solid matrix which resulted in the largest difference between the concentrations in the solid matrix and in the bulk fluid. The extraction at the later time of 30-360 min saw a much clearer in the mass transfer rate, which indicates that the mass transfer coefficient, k, started to play a more important role. As time passed, the solid phase concentration started to fall which rendered the concentration gradient small, and this is when the rate of extracted *trans*-piceid became limited by the mass transfer, and the rate increased with the increasing flow rate. In practice, the proper flow rate must be chosen such that it would result in shorter extraction time and higher concentration of the extract. From the above results, the rate was not significantly increased from the flow rate of 3 to 4 ml/min, thus the flow of 3 ml/min seem to be the most suitable.

$$N = k\Delta C = k\,(Cs - Co) \qquad (4.1)$$

N = Molar flux of component, mole/area.time
k = Mass transfer coefficients, m/s
Cs = Concentration in solid particle, mol/m^3
Co = Concentration in bulk solution, mol/m^3

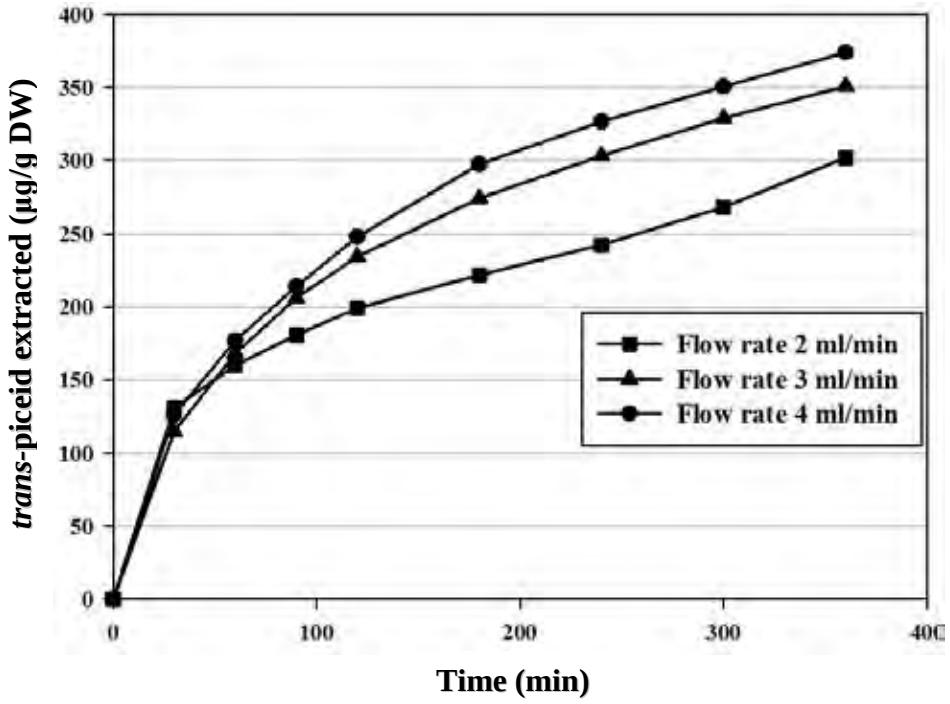


Figure 4.4 Effect of water flow rate on extraction of *trans*-picied. Operating condition: Temperature = 190 °C, pressure = 10 MPa.

4.3 Comparison of subcritical water extraction and other extraction methods

In the present study, comparisons were made between the SCWE and the other extraction methods such as conventional water and ethanol extractions using a stirred vessel and soxhlet apparatus. The results are shown in Table 4.1. Extraction in a stirred vessel at 70 °C and soxhlet extraction at 78 °C indicated that ethanol and 80%ethanol were a better solvent for *trans*-piceid than water. The extraction yield can nevertheless be increased for water extraction using higher temperature as can be seen by the results obtained with soxhlet water extraction which took place at 100 °C. On the other hand, SCWE at 100 °C gave lower amount of *trans*-piceid compared with soxhlet water and stirred vessel ethanol extraction methods carried out at the same temperature. This was possibly because in the system used for SCWE did not allow loading of the bark sample

after the preset extraction temperature was reached, instead the sample must be loaded into the vessel from the beginning. The sample therefore was subject to heating which took approximately (45 min) before actual extraction started, thus leading to the compound degradation. The SCWE at 190 °C, however gave high the amount of *trans*-piceid compound compared with the other extraction methods, since the increase in *trans*-piceid solubility at this condition possibly exceed the negative effect caused by degradation. In addition, the large amount of *trans*-piceid extracted by SCWE was possibly resulted from the hydrolysis of larger aromatic compounds such as lignin in the cell wall of *S.roxburghii*, which is accelerated by a high ion product of water, which thus unlock the sample matrix allowing the release of the compound [Goto et al., 2008].

Table 4.1 Comparison of *trans*-piceid for different extraction methods.

Extraction Methods	Temperature (°C)	Time (min)	*trans*-piceid (μg/g DW)
Extraction with ethanol stirred vessel	70	60	68.37
Extraction with water in stirred vessel	70	60	36.96
Soxhlet extraction with 80%ethanol	78	60	71.12
Soxhlet extraction with 80%ethanol	78	120	92.50
Soxhlet extraction with water	100	60	60.02
Soxhlet extraction with water	100	120	74.87
SWE	100	30	51.71

Extraction Methods	Temperature (°C)	Time (min)	*trans*-piceid (μg/g DW)
SWE	100	120	54.90
SWE	190	30	130.88
SWE	190	120	198.97

4.4 Preliminary results of β-Glucosidase hydrolysis

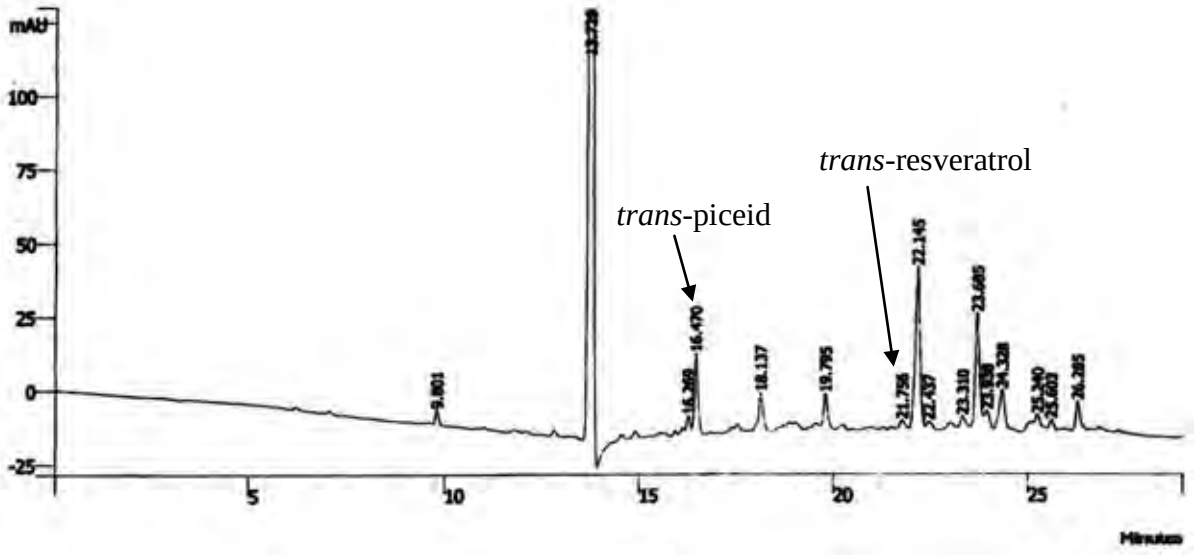
Figure 4.5A and Figure 4.5B show representative chromatograms of *S. roxburghii* samples before and after enzymatic treatment with β-glucosidase. After the treatment, the peak of *trans*-piceid became smaller while that of *trans*-resveratrol became larger. The amounts of *trans*-resveratrol and *trans*-piceid, before and after the hydrolysis with β-glucosidase of the *S. roxburghii* extracts obtained with various extraction methods (i.e., 30 min subcritical water extraction at 10 MPa and at temperature 130 °C and 190 °C, conventional extraction methods such as soxhlet water extraction and stirred vessel water and ethanol extraction are summarized in Table 4.2.

For soxhlet and stirred vessel extraction with water, *trans*-piceid amount decreased after hydrolysis with β-glucosidase, while to the amount of *trans*-resveratrol were increased, indicating the conversion took place as a result of the enzymatic treatment of these water extracts. On the other hand, when the alcohol extract was treated with β-glucosidase, *trans*-piceid could not be transformed into *trans*-resveratrol. This was because alcohol in the extract could denature the enzyme and thus deactivate it [Ogino et al., 2002].

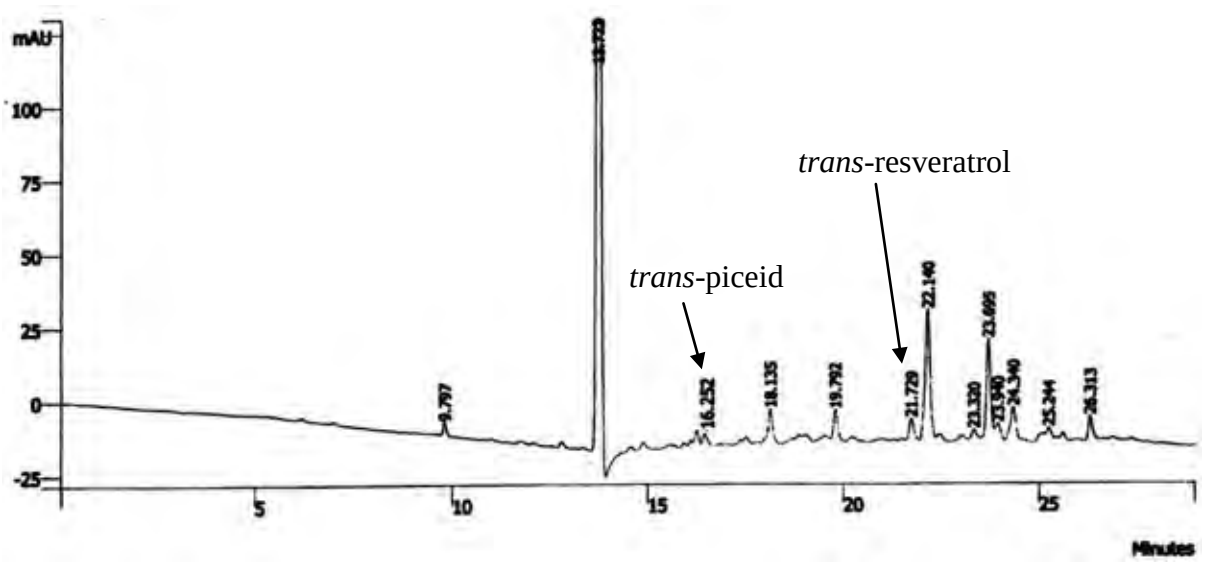
The hydrolysis of the subcritical water extract obtained at 190 °C gave similar results as that of the ethanol extract, that is, *trans*-piceid could not be converted to *trans*-resveratrol, despite that the aqueous extract was previously cooled prior to hydrolysis. A possible reason for this is that the extract obtained at SCWE at this condition could contain certain compounds that inhibit the β-glucosidase activity. Therefore, β-

glucosidase hydrolysis was carried out of the subcritical water extract obtained at lower temperature.

At 130 °C, the result shows that after the enzyme hydrolysis, the amount of *trans*-piceid decreased, however the amount *trans*-resveratrol was not increased, but slightly decreased rather. This result suggests that this extract obtained at lower temperature did not contain the enzyme inhibiting compounds. Since the conditions for hydrolysis with β-glucosidase has not been optimized in this study, further study however is needed to find ways to improve the conversion of trans-piceid into the desired product, *trans*-resveratrol. Furthermore, the temperature of water extraction could be lowered by use of some agents such as surfactants which help increase the solute solubility (Kiathevest et al., 2009).



A)



B)

Figure 4.5 Chromatograms of A) Before the hydrolysis with enzyme B) After the hydrolysis with enzyme of extract from bark of *S. roxburghii* with reflux extraction water

Table 4.2 Comparing hydrolyzed by the enzyme β-glucosidase in extract from the different extraction methods.

Extraction Methods	Temperature (°C)	Extraction time (min)	*trans*-resveratrol (μg/g DW)		*trans*-piceid (μg/g DW)	
			Before	After	Before	After
Soxhlet water extraction	100	120	3.18	8.17	74.87	22.86
Reflux extraction water	70	60	2.53	5.82	36.96	4.75
Reflux extraction ethanol	70	60	3.20	4.50	68.37	64.90
SCWE	130	30	4.51	3.66	75.49	12.37
SCWE	190	30	8.53	6.95	128.38	136.83

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

S.roxburghii bark is a good source of stilbenes including *trans*-resveratrol and *trans*-piceid. The plant extract can be obtained by extraction with subcritical water which is a suitable and environmentally benign solvent. The amount of stilbene contents in the extracts increased as temperature of subcritical water increased. Based on the results of extraction carried out at pressure for 10 MPa for 6 hours in this study, the most suitable condition for subcritical water extraction of *trans*-piceid was found to be at the extraction temperature of 190 ℃ and the water flow rate of 3 ml/min. The extracts obtained by subcritical water under the condition 190 ℃ had higher *trans*-piceid than those obtained by soxhlet extraction with water and stirred vessel extraction with ethanol and water.

For soxhlet and stirred vessel extraction with water, the amount of *trans*-piceid decreased after hydrolysis with β-glucosidase, while to the amount of *trans*-resveratrol were increased, indicating the conversion took place as a result of the enzymatic treatment of these water extracts. The hydrolysis of the subcritical water extract obtained at 190 ℃ gave similar results as that of the ethanol extract, that is, *trans*-piceid could not be converted to *trans*-resveratrol. Conversion of *trans*-piceid to *trans*-resveratrol may further be improved further by decreasing the extraction temperature and optimizing the hydrolysis condition.

5.2 Recommendations

In this study, resveratrol is found as *trans*- forms and its derivative (*trans*-piceid). Generally, resveratrol exists in *cis*- and *trans*- isomeric forms. The isomerization of *trans*-resveratrol to *cis*-resveratrol can occur by exposure to sunlight. *Cis*- isomer has been suggested by a report for its potency as an inhibitor of protein kinases similar to *trans*-resveratrol [Jayatilake et al., 1993]. Moreover, the plant in Dipterocarpaceae family is well known to be the rich source of various coumarins (as shown in Figure 5.1) [Muhtadi et al., 2006] and Temsiririrkkul et al., (2004) also found coumarins compound in part of barks of Pha-Yom. Hence, recovery of *cis*- resveratrol and coumarins compounds from *S.roxburghii* bark could be of particular interest for the future study.

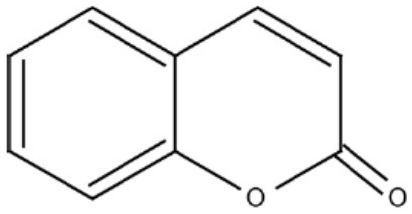


Figure 5.1 Chemical structure of coumarins

As for the effect of extraction temperature, the amount *trans*-piceid increased with the increase in temperature up to 190 °C. Investigation of *trans*-piceid extraction at higher temperature should be carried out to determine the effect in higher range. Excessive heating however may cause degradation of desirable compounds, and from the experimental results subcritical water extracts obtained at high temperature was not effectively hydrolyzed to convert *trans*-piceid to *trans*-resveratrol. Extraction at lower temperature seems to help alleviate such problem. Thus the future study might include extraction at lower temperature by addition of some surfactant to water which helps increase solubility of the solute in water without need of high extraction temperature [Kiathevest et al., 2009]. In addition, optimization of the hydrolysis condition could be carried out in the future study to determine the effects of different factors such as

incubation temperature, incubation time, pH and enzyme concentration. Moreover, direct hydrolysis of the *S.roxburghii* bark sample rather than of the extract, could be carried out prior to extraction, thus these results could be compared to determine the effective way to convert resveratrol glucoside form into its aglycone.

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APPENDICES

APPENDICE A

EXPERIMENTAL DATA FOR ANALYSIS

A-1 Standard calibration curve for HPLC analysis of *trans*-resveratrol and *trans*-piceid

Table A-1.1 Standard calibration curve data of *trans*-resveratrol

Peak area (PDA detector 307 nm)	**Concentration of *trans*-resveratrol (µg/ml)**
253519	0.100
814933	0.500
1200087	0.625
2467882	1.250
5096640	2.500
10615805	5.000
22321628	10.000

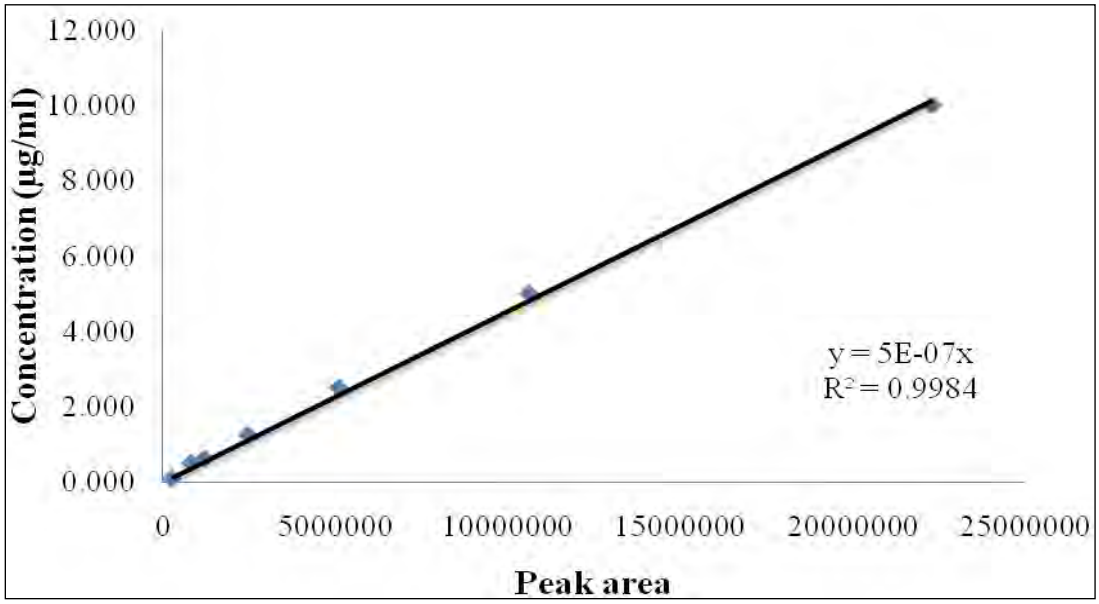

Figure A-1.1 Standard calibration curve of *trans*-resveratrol.

Table A-1.2 Standard calibration curve data of *trans*-piceid.

Peak area (PDA detector 307 nm)	**Concentration of *trans*-piceid (µg/ml)**
860189	1.000
3907326	5.000
8747462	10.000
12672853	15.000
16748786	20.000
20775080	25.000

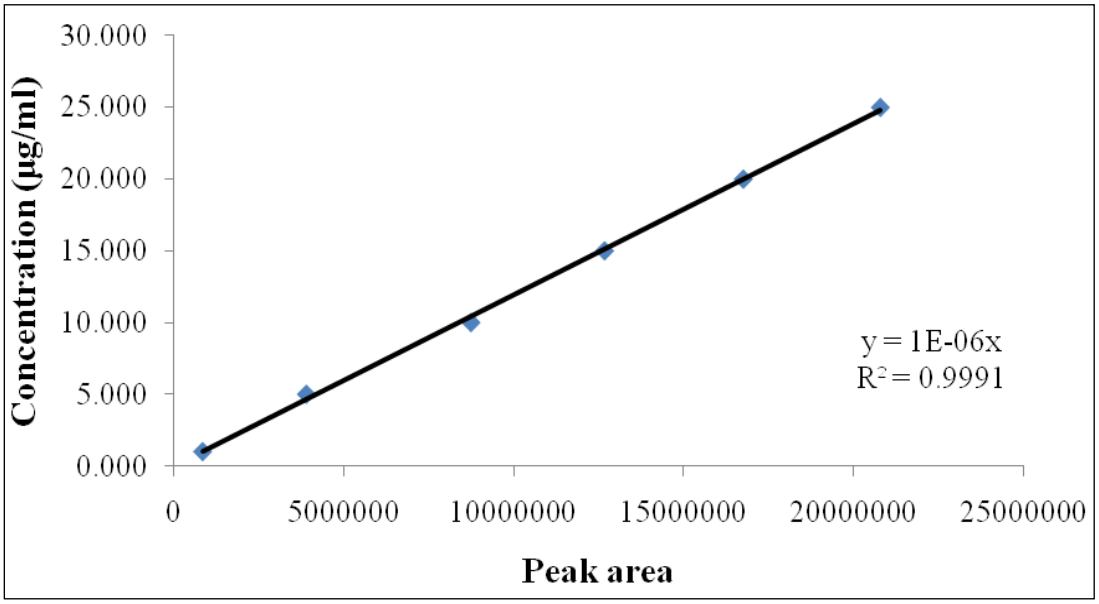

Figure A-1.2 Standard calibration curve of *trans*-piceid.

APPENDICE B

EXPERIMENTAL DATA

B-1 Experimental data of stilbene constituents with subcritical water extraction

Effect of temperature

Table B-1.1 Stilbene constituents of subcritical water extraction at various temperature, flow rate = 2 ml/min, pressure = 10 MPa

Time (min)	Stilbene constituents (μg/g DW)							
	100 °C		130 °C		160 °C		190 °C	
	trans-Resveratrol	*trans*-Piceid	*trans*-Resveratrol	*trans*-Piceid	*trans*-Resveratrol	*trans*-Piceid	*trans*-Resveratrol	*trans*-piceid
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	6.98	46.59	0.68	64.76	5.73	64.66	3.68	130.88
60	7.59	51.71	1.83	79.13	7.34	98.98	4.98	159.78
90	8.10	54.30	2.40	87.12	8.41	129.75	6.85	180.61
120	8.10	54.90	2.89	93.26	9.21	156.56	7.49	198.97
180	8.10	56.57	3.58	104.45	10.50	191.64	8.93	221.48
240	8.10	58.88	3.58	113.57	11.51	217.12	9.75	242.18
300	8.10	60.16	3.58	121.71	12.41	244.85	10.09	267.89
360	8.10	61.37	3.58	130.85	13.01	266.28	10.09	301.70

Effect of water flow rate

Table B-1.2 Stilbene constituents of subcritical water extraction at various water flow rate, temperature = 190 °C, pressure = 10 MPa, volume extraction = 720 ml

Flow rate (ml/min)	Time (min)	Stilbene constituents (μg/g DW)	
		trans-Resveratrol	*trans*-Piceid
2	360	10.098	302.063
3	240	21.444	303.292
4	180	31.232	297.554

VITA

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