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Search for α -glucosidase inhibitors from Indonesian indigenous plants

(インドネシア産植物の α -グルコシダーゼ阻害物質の探索研究)

Hokkaido University Graduate School of Agriculture

Division of Applied Bioscience Doctor Course

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Chapter 1. GENERAL INTRODUCTION

1.1 Overview and structure of thesis

This thesis work consists of five chapters. Chapter 1 (general introduction) brings out the status of diabetes in the world; the usage of medicinal herbs in diabetes medication and the mechanism of antihyperglycemic action through carbohydrate digestive enzymes is also given. Chapter 2, 3, and 4 are the main chapters with distinct background information, methodology, results, discussions and conclusions, and are titled (i) screening of α -glucosidase inhibitors from Indonesian medicinal plants, (ii) intestinal maltase inhibitors from *Pluchea indica* L. leaves, and (iii) antihyperglycemic principles from *Caesalpinia sappan* L. wood. Chapter 5 discusses overall conclusions, recommendations and future perspectives in the use of Indonesian medicinal plant as antihyperglycemic agent.

1.1.1 Overview about diabetes

Diabetes is projected to become the seventh leading cause of death in the world by 2030, and most deaths from diabetes are as a result of cardiovascular complications (World Health Organization [WHO], 2014). It is a chronic disease that occurs when the body cannot produce enough insulin or cannot use insulin effectively. A person with diabetes does not absorb glucose properly, and glucose remains circulating in the blood (a condition known as hyperglycemia) damaging body tissue over time (International Diabetes Federation [IDF], 2013). The possibility that postprandial ‘hyperglycemic spikes’, which are characterized by a rapid and large increase in blood glucose levels, may be related to the onset of cardiovascular complications has recently received much attention. The

evidence connecting diabetes complications to postprandial hyperglycemia is sufficiently compelling to influence the guidelines provided by key professional scientific societies (Ceriello, 2005).

1.1.2 Antihyperglycemia activity via carbohydrate digestive enzymes inhibition

It is widely accepted that the most challenging goal in management of patients with diabetes mellitus is to achieve blood glucose levels as close to normal as possible. One important method of suppressing postprandial hyperglycemia is the reduction or slowing of dietary carbohydrate digestion and absorption through inhibition of carbohydrate digestive enzymes such as pancreatic α -amylase and intestinal α -glucosidase (Figure 1). The initial digestion of starch is carried out by salivary and pancreatic α -amylase. The starch-derived products of α -amylase, maltose, and maltooligosaccharides are further degraded by the combined action of α -glucosidase such as maltase-glucoamylase (MGAM), whereas dietary sucrose is only cleaved by sucrose-isomaltase (SI) (Van Beers, Büller, Grand, Einerhand, & Dekker, 1995).

Treatment with acarbose, a well-known α -amylase and α -glucosidase inhibitor, is reported to decrease the risk of cardiovascular disease in patients with diabetes, though its long-term use is associated with side effects such as flatulence and diarrhea (Godbout and Chiasson, 2007). The gastrointestinal side effects are secondary to the fermentation of undigested carbohydrates in the colon producing gaseous by-products (Riccardi et al., 1999).

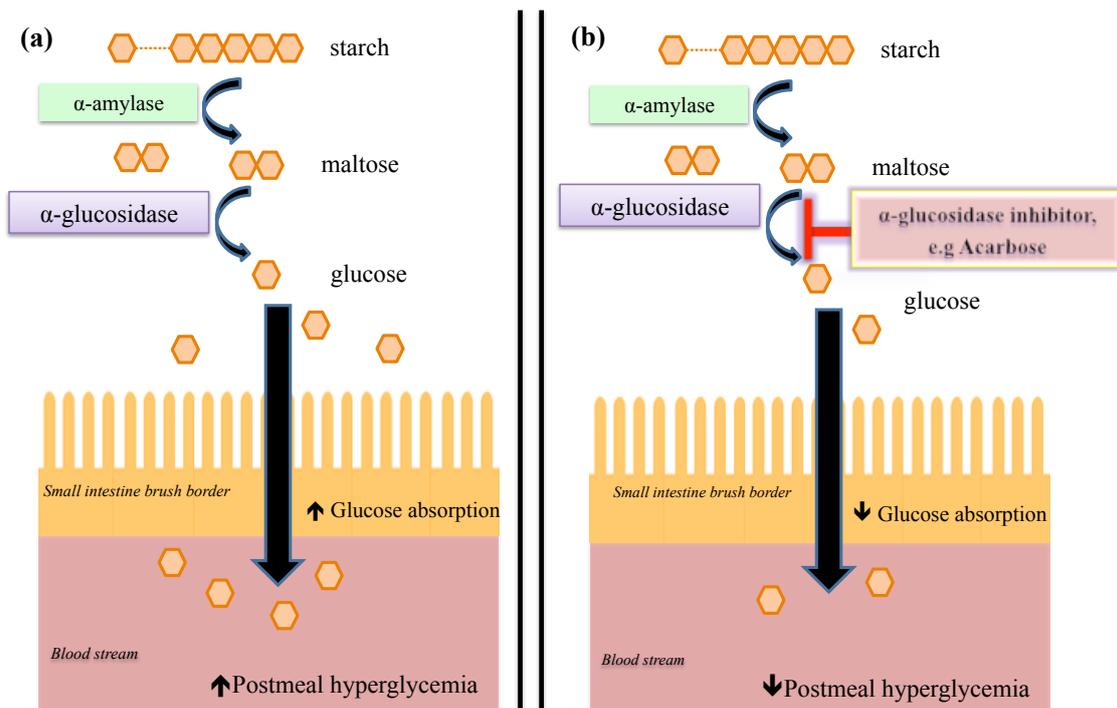


Figure 1. Mechanism of carbohydrate digestion (a) and α -glucosidase inhibition (b).

1.1.3 The usage of indigenous plants in Indonesian traditional medicines.

The area of Indonesian tropical forests covers about 143 million hectares and is home to about 80% of the world's medicinal plants. Zuhud et al. (2001) identified 1845 species with medicinal potential in the forests of Indonesia. These numbers are potentially to be updated due to the continuing inventory and investigation of yet unidentified species. According to the National Agency of Drug and Food Control (NADFC/BPOM), 283 plant species have been officially registered for their medicinal use; the larger remainder is used traditionally (Elfahmi et al., 2014).

Jamu is the Indonesian traditional medicine that has been practiced for many centuries in the Indonesian community to maintain health and to treat disease. Due to this fact, Indonesia has a wealth of biological resources and traditional medicinal knowledge. Traditional medicine, apparently, is perceived as efficient, safe, cost-effective and affordable

(WHO, 2001). Nowadays, the production of *jamu* is also being developed on an industrial scale. The Indonesian government, industry and academia all recognize that to further the development of *jamu*, extensive research is required to establish the safety and efficacy of the many traditional *jamu* preparations.

1.2 Broad objective of the study

Diabetes mellitus (DM) is one of the most common and complex problems of modern societies, which has caused many economic and social problems. Because diabetes has no definite treatment, the use of traditional medicine seems to be an appropriate solution to control and manage it. In this regard, this paper will tend to reveal the active compounds responsible for against α -glucosidase from Indonesian medicinal plants. Our first attempt was to find the active compounds that inhibit intestinal maltase. This is because maltase inhibition is more effective than sucrose inhibition in modulating the increases in postprandial blood glucose upon dietary carbohydrate intake since maltose is the principal disaccharide produced by starch digestion (Van Beers et al., 1995). The revealing can be considered as candidates for the development of functional food, alternative and complementary agent in diabetes prevention and management.

Chapter 2. SCREENING OF α -GLUCOSIDASE INHIBITORS FROM INDONESIAN INDIGENOUS PLANTS

2.1 Introduction

The goal for the treatment of DM is to restore the normal glucose level in blood of diabetes patient. As the first-line treatment for diabetic individuals with postprandial hyperglycemia, commercial α -glucosidase inhibitors such as acarbose and voglibose considered to be used. It would delay the digestion and absorption of carbohydrates and consequently suppress hyperglycemia. However, these α -glucosidase inhibitors have prominent gastrointestinal side effects like flatulence, diarrhea, and abdominal discomfort. This warrants the search for alternative natural herbal medicines that have fewer side effects than the available commercial inhibitors.

Efforts have been made to identify α -amylase and α -glucosidase inhibitors that are derived from plants and use them to develop functional foods and discover new diabetes treatments (Kumar et al., 2011). As described in Chapter 1, Indonesia has a high biodiversity in medicinal plants. Various kinds of Indonesian herbs were thus screened for intestinal α -glucosidase inhibitors.

2.1.1 Specific objective

In this regard, this paper identifies potential Indonesian medicinal plants as antihyperglycemic agent via α -glucosidase, in particular intestinal maltase inhibition.

2.2 Materials and methods

2.2.1 Materials

Indonesian medicinal plant materials were collected from Central Java, East Java, and Irian Jaya in 2007. An intern herbalist of Merapi Farma Plantation Yogyakarta Indonesia identified the plant materials.

2.2.2 Reagents

Rat intestinal acetone powder (Sigma Aldrich Japan Co.) was used as the enzyme source. Cosmosil 75C₁₈-OPN (Nacalai Tesque, Inc) as reverse phase ODS gel. All chemicals used were analytical grade and were purchased from Wako Pure Chem. Co. unless otherwise stated.

2.2.3 Preparation of plant extracts

Dried plant materials (5 g) were extracted with 50% aqueous methanol (50 mL) in room temperature for 24 hr. The crude extracts were filtered and concentrated under reduced pressure at 40 °C. The dried residues were re-dissolved in 50% aqueous dimethyl sulfoxide (DMSO) with a final concentration of the extracts obtained from 0.1 g dried plant material in one mL solution, which is designated as 1 n. These solutions were subjected to rat intestinal maltase inhibitory activity assays.

2.2.4 Rat intestinal α -glucosidase inhibitory activity assay

Rat intestinal maltase inhibitory activity was determined using the method of Jong-Anurakkun et al. (2007), with minor modifications. Rat intestinal acetone powder was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM

ethylenediaminetetraacetic acid (EDTA), homogenized, and centrifuged at $10,600 \times g$ for 30 min at 4°C . The supernatant was collected and used as the crude enzyme solution. The maltose-hydrolyzing activity of the crude enzyme solution (2.33 U/mg protein) was designated as maltase activity.

Inhibition of maltose hydrolysis was measured using the following procedure: Test tubes containing 350 μL maltose solution (3.5 mM) in potassium phosphate buffer (0.1 M, pH 6.3) were preincubated at 37°C for 5 min. Plant extracts were dissolved in 50% dimethyl sulfoxide (DMSO, 100 μL) and added to preincubated tubes. Control tubes contained 50% DMSO alone (100 μL). The reaction was started by the addition of crude rat intestinal glucosidase solution (50 μL). Test tubes containing 400 μL potassium phosphate buffer (0.1 M, pH 6.3) served as blanks. After incubation for 15 min at 37°C , the reaction was stopped by adding 0.75 mL of 2 M Tris HCl buffer (pH 7.0). The reaction mixture was passed through a short ODS column (Cosmosil 75C₁₈-OPN, ϕ 5 \times 55 mm) to remove phenolic compounds that might interfere with the glucose quantification. The amount of liberated glucose was measured by the glucose oxidase method using a commercial test kit (Glucose CII-test Wako, Wako Pure Chem. Co.) in 96-well microplates at 37°C for 30 min. The optical density (OD) of the wells was measured at 490 nm using SynergyTM MX microplate reader (Biotek Instruments, Inc.). Inhibitory activity was calculated using the following equation (1):

$$\text{Inhibitory activity (\%)} = [1 - (\text{OD}_{\text{sample}} - \text{OD}_{\text{sample blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{control blank}})] \times 100 \dots\dots (1)$$

All experiments were performed in duplicate. Acarbose, which had 50% inhibitory activity against maltase at 0.5 μM concentration, was used as the positive control.

2.3 Results

To reveal the potential α -glucosidase inhibitors from natural sources, 28 Indonesian medicinal plants (Table 1) were randomly selected and evaluated for their intestinal maltase inhibitory activity by *in vitro* assay. As shown in Figure 2, some of the tested plants showed potential in inhibiting intestinal maltase in 1 n concentration. Among them, seven plants showed high inhibitory activity (above 60%). These are *Syzgium polyanthum* (6, 64%), *Pluchea indica* (8, 79%), *Blumea balsamifera* (12, 77%), *Melaleuca leucadendra* (16, 62%), *Abrus precatorius* (17, 63%), *Starchytarpheta jamaicensis* (23, 61%), and *Caesalpinia sappan* (28, 63%).

Table 1. List of tested Indonesian medicinal plants.

Number	Scientific name	Traditional name	Part of used
1	<i>Andropogon nardus</i>	Sere minyak	Leaves
2	<i>Artocarpus altilis</i>	Kluwih	Leaves
3	<i>Zephyranthes candida</i>	Daun bawang	Leaves
4	<i>Stevia rebaudiiana</i>	Stevia	Leaves
5	<i>Digitalis purpurea</i>	Digitalis	Leaves
6	<i>Syzgium polyanthum</i>	Salam	Leaves
7	<i>Artocarpus communis</i>	Sukun	Leaves
8	<i>Pluchea indica</i>	Beluntas	Leaves
9	<i>Vitex trifolia</i>	Legundi	Leaves
10	<i>Sauropus androgynous</i>	Katuk	Leaves
11	<i>Coleus artipurpureus</i>	Iler	Leaves
12	<i>Blumae balsamifera</i>	Sembung	Leaves
13	<i>Piper betle</i>	Sirih	Leaves
14	<i>Sonchus arvensis</i>	Tempuyung	Leaves
15	<i>Usnea barbarata</i>	Kayu angin	Root
16	<i>Melaleuca leucadendra</i>	Kayu putih	Leaves
17	<i>Abrus precatorius</i>	Saga	Leaves
18	<i>Orthosiphon stamineus</i>	Kumis kucing	Leaves
19	<i>Psidium guajava</i>	Jambu biji	Leaves
20	<i>Henslowia frutescence</i>	Benalu	Leaves
21	<i>Barleria prioritis</i>	Daun madu	Leaves
22	<i>Murraya paniculata</i>	Kemuning	Leaves
23	<i>Starchytarpheta jamaicensis</i>	Pecut kuda	Leaves

Table 1. Continued

24	<i>Artemisia vulgaris</i>	Sudomolo	Leaves
25	<i>Carica papaya</i>	Papaya	Leaves
26	<i>Plantago major</i>	Daun sendok	Leaves
27	<i>Myristica fragrans</i>	Pala	Fruits
28	<i>Caesalpinia sappan</i>	Secang	Wood

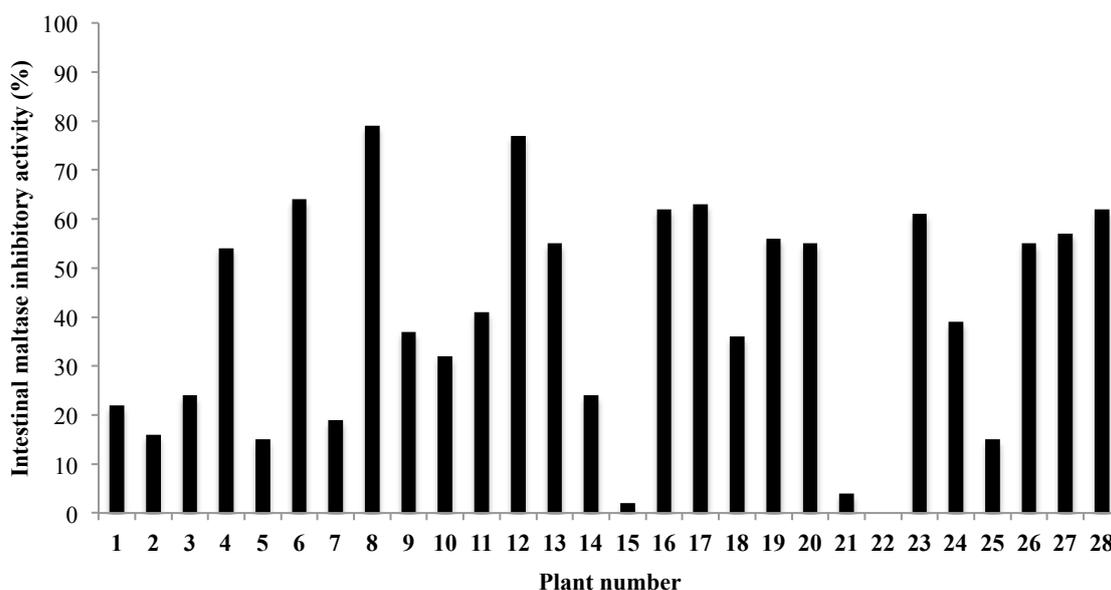


Figure 2. Intestinal maltase inhibitory activity of Indonesian medicinal plants. Concentration was adjusted at 1 n in final reaction solution

2.4 Discussion

Based on the intestinal maltase inhibitory assay screening experiment of well known 28 Indonesian medicinal plants, seven plants showed high maltase inhibitory activity (above 60% inhibition against maltase). These are *Syzygium polyanthum* (6), *Pluchea indica* (8), *Blumea balsamifera* (12), *Melaleuca leucadendra* (16), *Abrus precatorius* (17), *Starchytarpheta jamaicensis* (23), and *Caesalpinia sappan* (28).

Syzygium polyanthum (Myrtaceae), known as Indonesian bay leaf or “daun salam”, is used as popular culinary additive and also used for diabetes, diarrhea and skin infection (Kusuma et al., 2011). It grows wild and is also usually planted in yards and around most houses in Asia (Widyawati et al., 2015). Saraswaty (2010) reported for the α -glucosidase inhibitory activity from *S. polyanthum* extract.

Pluchea indica (Asteraceae), also known as “beluntas”, showed the highest intestinal maltase inhibitory activity among screened Indonesian herbs. Numbers of chemical principles have been isolated from *P. indica* (Basnet et al., 1996; Biswas et al., 2007). Pramanik et al. (2006) reported the hypoglycemic and antihyperglycemic activity of leaf extract of *P. indica* leaves.

Blumea balsamifera (Asteraceae), known as “daun sembung,” has been noted to contain flavonoids (Ali et al., 2005; Barua and Sharma, 2003). In Seberida Riau, decoction drink of *B. balsamifera* leaf is used for stomachaches, while fresh leaves are eaten to purify the blood of postpartum (Mahyar et al., 1991). *B. balsamifera* extract has been reported for the anti-obesity effect (Hiroaki et al., 2009) and apigenin isolated from *B. balsamifera* reported for the aldose reductase inhibitory activity (Lee et al., 2012).

Melaleuca leucadendra (Myrtaceae), known as “kayu putih”, leaves are rich in essential oils and are used for medicinal purposes, insect repellents and cosmetics (Amin, 2010). *Melaleuca leucadendra* is original Indonesian plant is a relatively underutilized species and important source of honey. (Brophy et al., 2013).

Abrus precatorius (Fabaceae), known as “saga”, has been reported for its anti-inflammatory activity (Anam, 2001). A triterpene isolated from *A. precatorius* has been reported to exert α -amylase inhibitory activity (Yonemoto et al., 2014). Concurrently with result of screening experiment, *A. precatorius* has antihyperglycemic activity through the carbohydrate digestive enzymes inhibition.

Starchytarpheta jamaicensis (Verbenaceae), known as “pecut kuda”, has been used for digestive problems, allergies, and respiratory treatment (Idu et al., 2007). It has also reported to have significant antimicrobial and insecticidal activity (Chariandy et al., 1999). It also one of the plant that frequently used against diabetes mellitus in Suriname (Mans, 2012).

Caesalpinia sappan (Fabaceae), is one of the popular plant that is not only used as traditionally as medicinal use, but also as dying agents (Badami, et al., 2004). It is traditionally used for skin care, especially on Sumbawa Island and the wood of *C. sappan* is used to obtain pink pigment for drinks such as Bir Pletok, a Batavian spice drink (Batubara et al., 2010). Antidiabetic activity of *C. sappan* wood have been documented (Moon et al., 1990) and it is one of the ingredient of functional drink for antihyperglycemic purpose in Indonesia (Indriani et al., 2014).

2.5 Conclusions

Indonesian medicinal plants provide potential plants contained α -glucosidase inhibitor agents. This fact is supported by the screening experiment and resulted in seven plants that showed potential intestinal maltase inhibitory activity. The use of medicinal plants in the society, concerning as cost-effective way and improve the quality of life, can be used as a complimentary way in the diabetes management and treatment. Bioassay-guided fractionation becomes essential to isolate the active principles from the potential plants as the lead compound for finding the α -glucosidase inhibitors.

Chapter 3. α -GLUCOSIDASE INHIBITORS FROM *PLUCHEA INDICA* L. LEAVES

3.1 Introduction

Pluchea indica (L.) Less (Asteraceae) is widely distributed in Southeast Asia (Raharjo and Horsten, 2001). Extracts of *P. indica* parts have anti-oxidant, anti-ulcer, anti-nociceptive, anti-diuretic, and anti-inflammatory properties (Sen and Chaudhuri, 1991; Choi and Hwang, 2005; Biswas et al., 2007; Buapool et al., 2013). In Thailand, *P. indica* leaves are used as tea because they are believed to have an indigenous remedy due to their anti-diabetic properties. It has been reported for its anti-diuretic and anti-diabetic pharmacological effects in streptozocin-induced rats (Pramanik et al., 2006). Prior chemical investigations of *P. indica* have led to the isolation of several terpenes, lignin glycosides and terpenic glycosides from the aerial part (Uchiyama et al., 1991; Raharjo and Horsten, 2001). The methanolic extract of the *P. indica* roots led to the isolation of an alkynylthiophene derivative, 2-(prop-1-ynyl)-5(5,6-dihydroxyhexa-1,3-diynyl) thiophene, for its anti-amoebic activity (Biswas et al., 2007).

Based on screening experiments (Chapter 2), *P. indica* was found to be one of promising source of α -glucosidase inhibitors. However, *P. indica* is not used as an anti-diabetic agent in Indonesia. The compounds in *P. indica* responsible for α -glucosidase inhibition are unknown. Therefore, this study was performed to discover the compounds responsible for the inhibition of α -glucosidase, specifically of intestinal maltase, using an *in vitro* assay-guided approach.

3.1.1 Specific objective

In this regard this paper will identify intestinal maltase inhibitors of *P. indica* leaves that delay postprandial hyperglycemia by using *in vitro* enzyme-assay guided isolation and to investigate the intestinal maltase inhibitory activity of *P. indica* isolates.

3.2 Materials and methods

3.2.1 Materials

Pluchea indica leaves were purchased from Merapi Farma Traditional Herbs Distributor, Yogyakarta, Indonesia, in January 2007. A voucher specimen (batch number B0107.002) was deposited with the Merapi Farma Herbal Traditional Herbs Distributor.

3.2.2 Reagents

The reagents used were same as mentioned in Section 2.2.2.

3.2.3 Rat intestinal α -glucosidase inhibitory activity assay

α -Glucosidase inhibitory activity was determined based on inhibition of maltose hydrolysis using the method described in Section 2.2.4. All experiments were performed in duplicate, and the results were presented as IC₅₀ values. The IC₅₀ values were determined by constructing a dose-response curve between the logarithm of the concentration of substances on the *x*-axis and the inhibitory activity on the *y*-axis.

3.2.4 Instrumental analysis

NMR spectra were recorded using Bruker AMX500 spectrometer (^1H , 500 MHz; ^{13}C , 125 MHz, Bruker Biospin Co.). Chemical shifts were calculated from the residual solvent signal (methanol- d_4 , δ_{H} 3.30, δ_{C} 49.5). Mass spectrum was obtained using Thermo Scientific Exactive spectrometer (Thermo Fisher Scientific K.K) or Waters LCT Premier XE (Waters Co.). High-pressure liquid chromatography (HPLC) was performed with a JASCO 802-SC system (JASCO Co.).

3.3 Results

3.3.1 Isolation of intestinal maltase inhibitors from *P. indica* leaves

Dried *P. indica* leaves (94 g) were extracted at room temperature for 24 h with 50% aqueous methanol. The crude extract was filtered through a filter paper (Whatman No. 5C, 110 mm) and evaporated under reduced pressure below 40 °C to obtain the 50% methanol extract (PI, 14.5 g, yield 14% based on dried leaves). The dried residue was partitioned between ethyl acetate and water to give water-soluble part (PIW, 11.2 g), and ethyl-acetate soluble part (PIE, 3.0 g). As shown in Table 2, PIE (57%) had higher maltase inhibitory activity than PIW (35%) in 0.2 n concentration.

Table 2. Maltase inhibitory activity of *P. indica* extracts.

Extracts	Maltase inhibitory activity (%)		
	0.2 n	1 n	5 n
Crude 50% methanolic extract (PI)	85	73	*
Water-soluble layer (PIW)	35	85	97
Ethyl acetate-soluble layer (PIE)	57	83	93

*not tested.

Concerning its high inhibitory activity, PIE was selected for further investigation. PIE (3.0 g) was fractionated by silica gel (Silica Gel 60 N, Kanto Chemical Co. Inc.) column chromatography (200 g, 3 × 42 cm) using a chloroform-methanol gradient. Eight collected fractions of PIE were subjected to maltase inhibitory assay and the result is shown in Figure 3. PIE 8 showed the highest activity (80% in 1 n). The high yield (1.51 g) of PIE 8 indicated that the silica gel column chromatography could not give good separation. PIE 8 was further fractionated using ODS column chromatography (Cosmosil 75C₁₈ OPN, Nacalai Tesque, 3x30 cm). PIE 8 (1.51 g dry weight) dissolved in methanol-water (75:25) was put on the column and eluted with a methanol-water gradient. The summary of fractionation of *P. indica* leaves is shown in Figure 4.

Nine obtained fractions were monitored by maltase inhibitory activity assay. The result is shown in Figure 5. Among nine fractions, PIE 8-4 showed the highest maltase inhibitory activity (71%) followed by PIE 8-5 (59%) in 1 n concentration. Since PIE 8-4 and 8-5 were thought to contain the active principles, these two fractions were chosen for further purification.

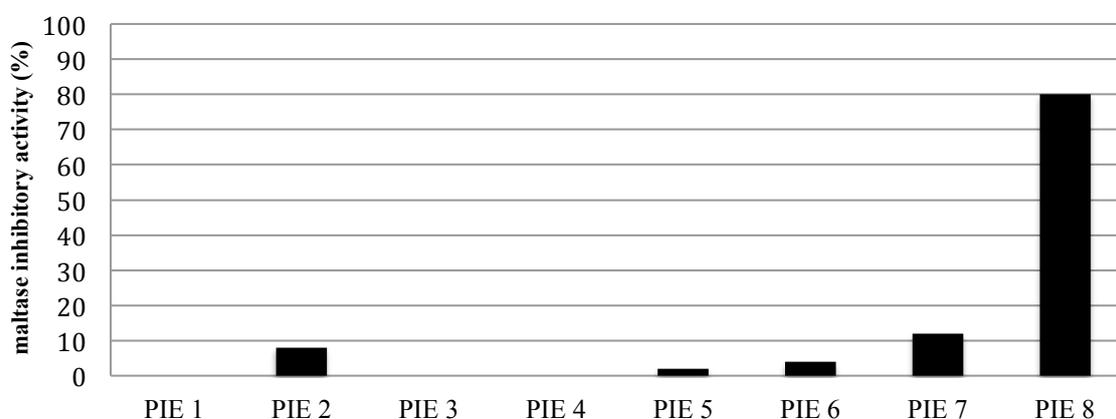


Figure 3. Maltase inhibitory activity of fractions obtained from silica gel column chromatography of PIE. Concentration of each fraction was adjusted to 1 n in reaction mixture.

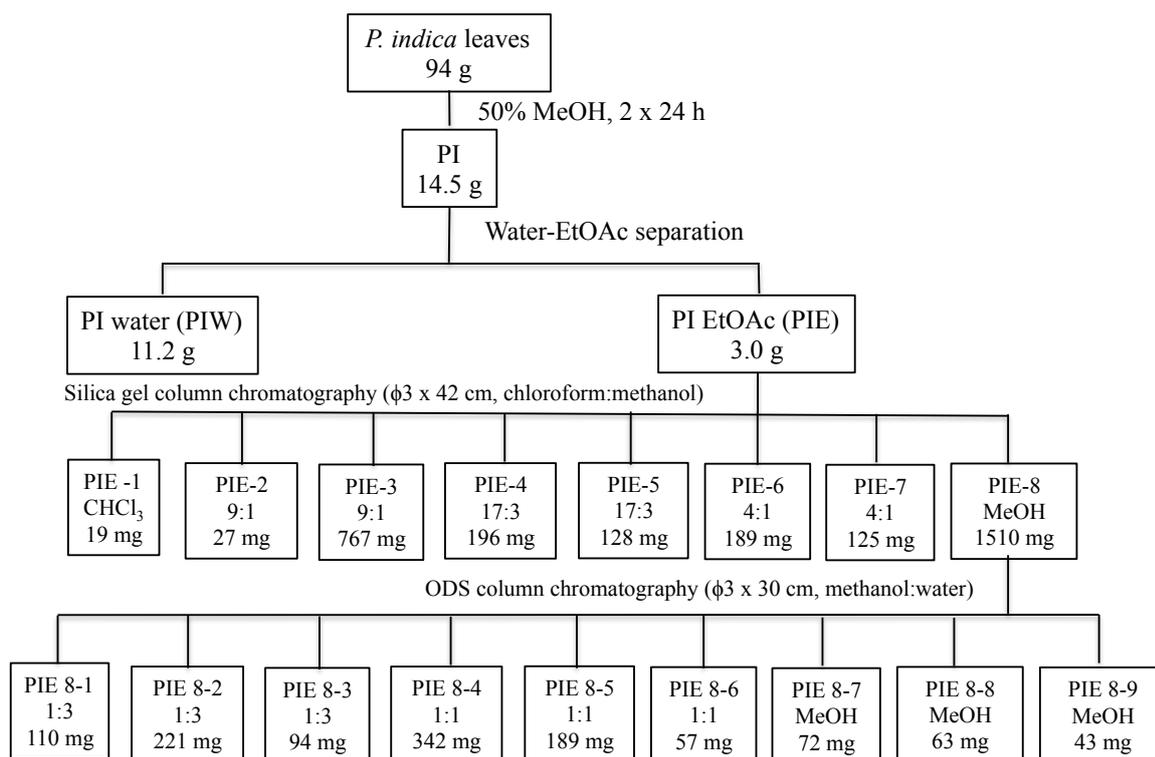


Figure 4. Summary of fractionation of *P. indica* leaves.

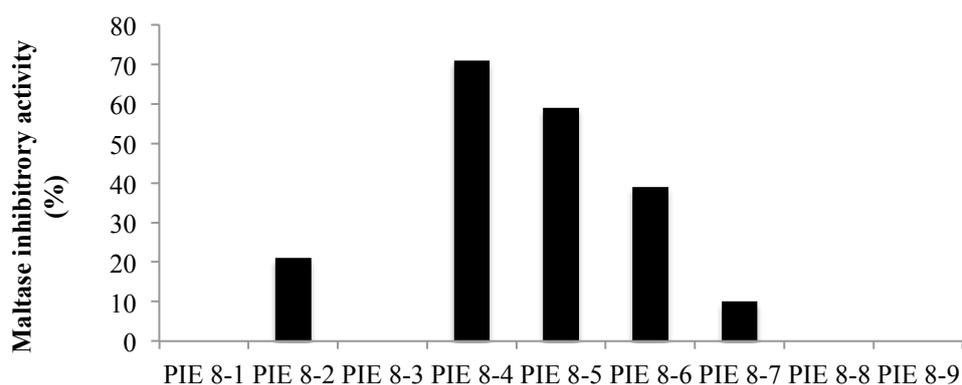


Figure 5. Maltase inhibitory activity of fractions obtained from ODS column chromatography of PIE 8. Concentration of each fraction was adjusted to 1 n.

Fractionation of PIE 8-4

PIE 8-4 was further purified using preparative HPLC. HPLC profile of PIE 8-4 is shown in Figure 6. According to the chromatogram, 5 major peaks, PIE 8-4-1 (t_R 32 min, 10 mg), PIE 8-4-2 (t_R 35 min, 75 mg), PIE 8-4-3 (t_R 40 min, 44 mg), PIE 8-4-4 (t_R 47 min, 42 mg), and PIE 8-4-5 (t_R 50 min, 41 mg) were collected together with two non-peak fractions (PIE-8-4-6 and 7).

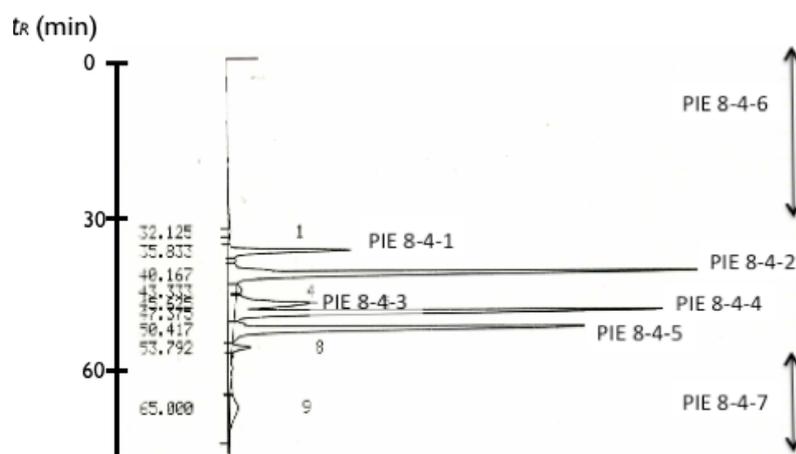


Figure 6. HPLC profile of PIE 8-4.

Column: Inertsil PREP-ODS (ϕ 20 \times 250 mm; mobile phase: methanol-water containing 0.1% formic acid (0–50 min: 40–60% methanol; 50–70 min: methanol); flow rate: 5 mL/min; detection wavelength: UV 254 nm.

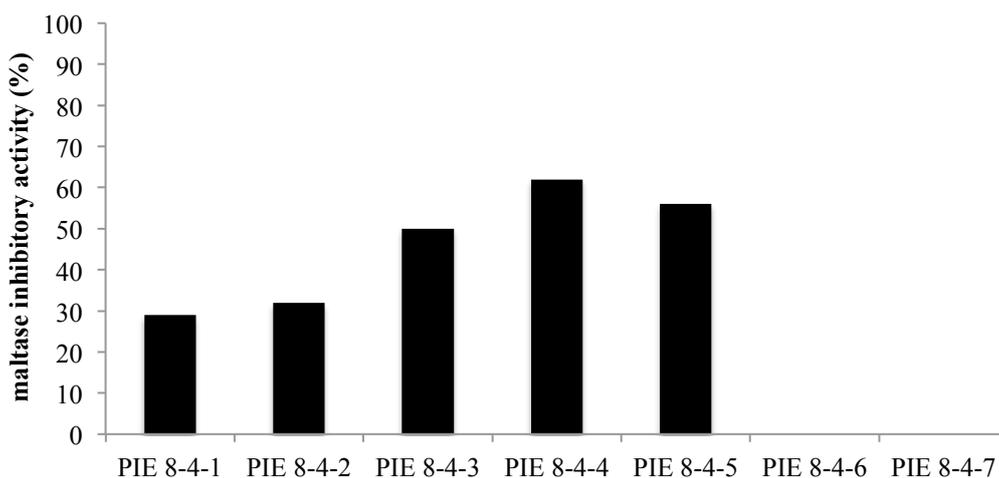


Figure 7. Intestinal maltase inhibitory activity of fractions obtained from preparative-ODS HPLC of PIE 8-4. Concentration of each fraction was adjusted to 5 n.

Seven obtained fractions were determined for maltase inhibitory activity. As shown in Figure 7, PIE 8-4-4 (62%) showed the highest inhibitory activity, followed by PIE 8-4-5 (56%), PIE 8-4-3 (50%), PIE 8-4-2 (32%), and PIE 8-4-1 (29%). Out of seven obtained fractions, PIE 8-4-1 and 8-4-5 were isolated as a single compound without further purification and thus submitted for compound identification. PIE 8-4-2 and PIE 8-4-4 were further purified using preparative HPLC, although PIE 8-4-3 could not be purified because of the difficulty in separation.

Preparative HPLC of PIE 8-4-2 (Figure 8) resulted in two major peaks, PIE 8-4-2-1 (t_R 7 min, 75 mg) and PIE 8-4-2-2 (t_R 9 min, 2 mg). Only PIE 8-4-2-1 was isolated as a single compound without further purification, and thus it was submitted for compound identification.

Preparative HPLC of PIE 8-4-4 (Figure 9) resulted in three major peaks. PIE 8-4-4-1 (t_R 39 min, 5 mg), PIE 8-4-4-2 (t_R 45 min, 3 mg) and PIE 8-4-4-3 (t_R 51min, 40 mg) were collected. Only PIE 8-4-4-3 was isolated as a single compound without further purification, so that it was submitted for compound identification.

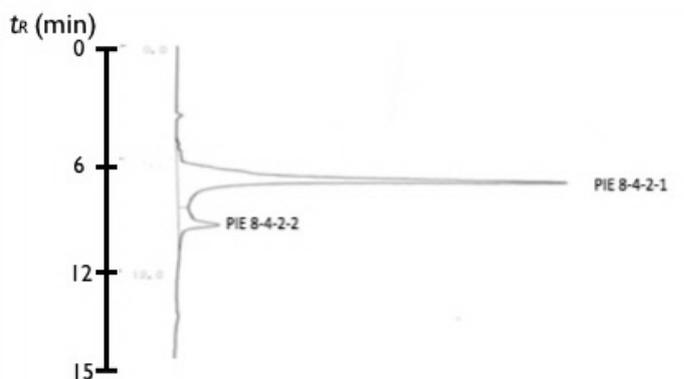


Figure 8. HPLC profile of PIE 8-4-2.

Column: Inertsil PREP-ODS (ϕ 20 \times 250 mm); mobile phase: water-methanol containing 0.1% formic acid (0–30 min: 50% methanol); flow rate: 5 mL/min; detection wavelength: UV 254 nm.

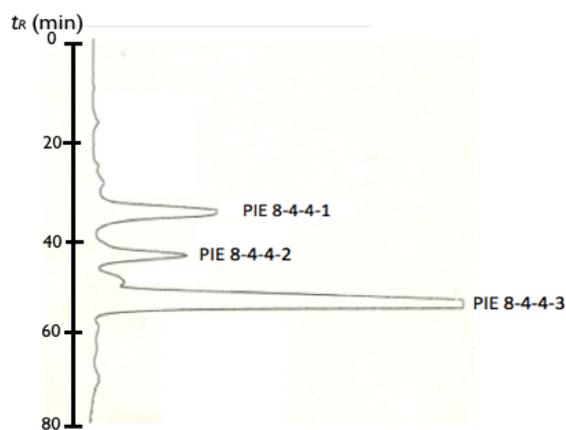


Figure 9. HPLC profile of PIE 8-4-4.

Column: Inertsil ODS-3 (ϕ 20 \times 250 mm); mobile phase: water-methanol containing 0.1% formic acid (0–30 min: 50–60% methanol; 30–70 min: methanol); flow rate: 5 mL/min; detection wavelength: UV 254 nm.

Fractionation of PIE 8-5

The HPLC profile of fraction PIE 8-5 in water methanol system (Figure 10) showed three dominant peaks, PIE 8-5-1 (t_R 7 min, 1 mg), 8-5-2 (t_R 13 min, 19 mg), and 8-5-3 (t_R 17 min, 3 mg). Only PIE 8-5-2 was isolated as a single compound without further purification, so that it was submitted for compound identification.

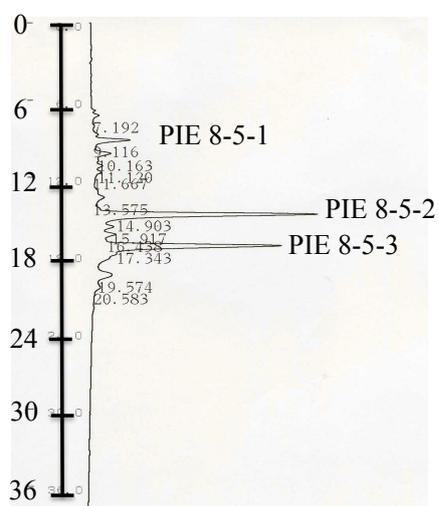


Figure 10. HPLC profile of PIE 8-5.

Column: Inertsil ODS-3 (ϕ 4.6 \times 250 mm); mobile phase: methanol-water containing 0.1% formic acid (0–30 min: 50–80% methanol; 30–50 min: MeOH); flow rate: 1 mL/min; detection wavelength: 254 nm.

3.3.2 Structure determination of isolates from *P. indica* leaves

3.3.2.1 PIE 8-4-1 (3,5-di-*O*-caffeoylquinic acid)

PIE 8-4-1 was obtained as off-white powder. ESI mass and ¹H NMR spectra of PIE 8-4-1 are shown in Figures 11 and 12, respectively, and the physicochemical properties are shown in Table 3. Based on the pseudomolecular ion at *m/z* 515 [M-H]⁻ in ESI-mass (negative) spectrum, molecular weight was determined as 516. The ¹H NMR spectrum showed a characteristic pattern of caffeoylquinic acid. Signals for quinic acid protons were found at δ 2.12-2.33, 3.96, and 5.41. Two sets of caffeoyl residues at δ 6.26 and 6.34 (2 \times 8'-H), 6.77 and 6.88 (2 \times 5'-H), 6.95 and 6.96 (2 \times 6'-H), 7.05 and 7.06 (2 \times 2'-H), 7.57 and 7.61 (2 \times 7'-H) were also observed. The low field shift of H-3 and H-5 of the quinic acid moiety indicated that caffeoyl groups are esterified to 3-OH and 5-OH of the quinic acid. The NMR data was consistent with those reported for 3,5-di-*O*-caffeoylquinic acid (Basnet, et al., 1996) and thus, PIE 8-4-1 was confirmed as 3,5-di-*O*-caffeoylquinic acid.

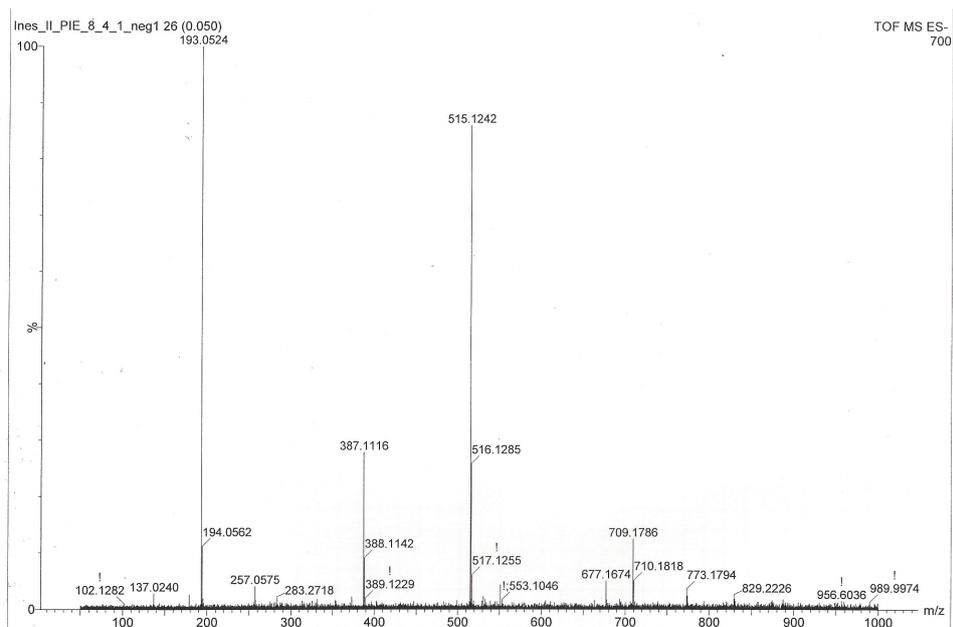


Figure 11. ESI-TOF mass spectrum (negative) of PIE 8-4-1.

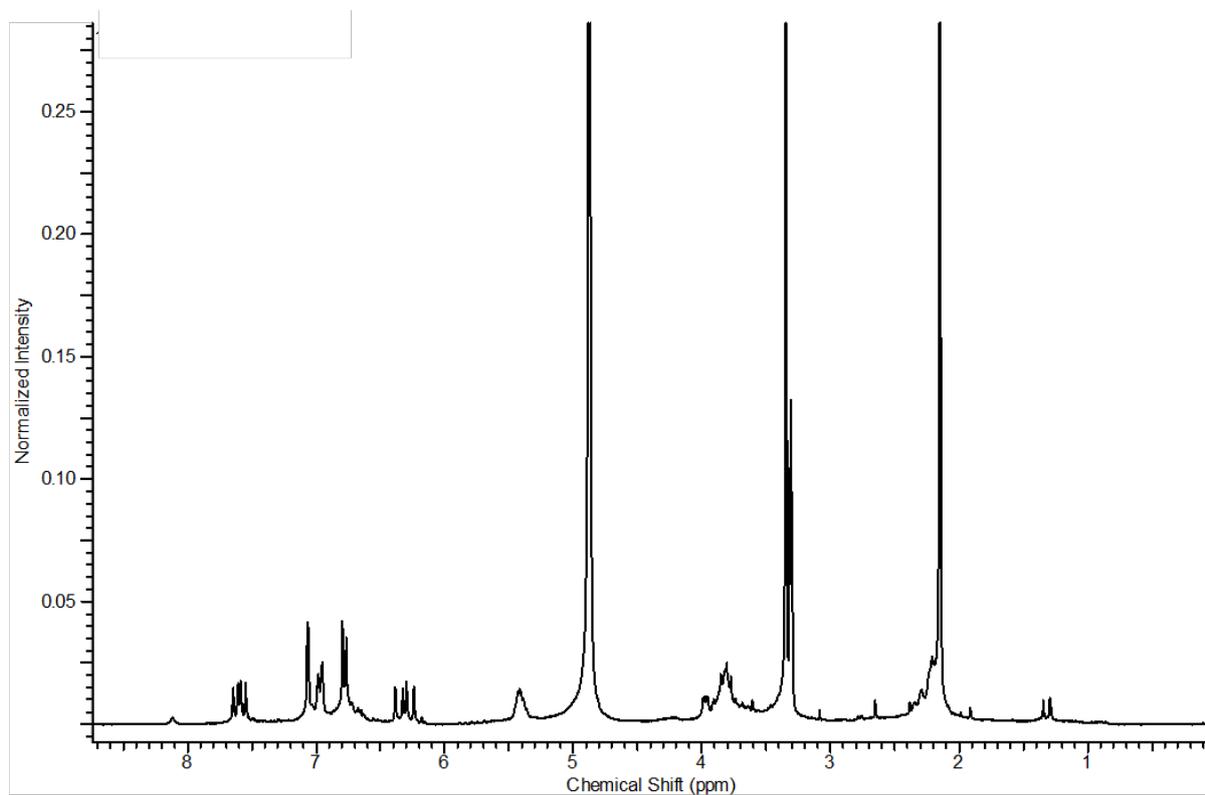
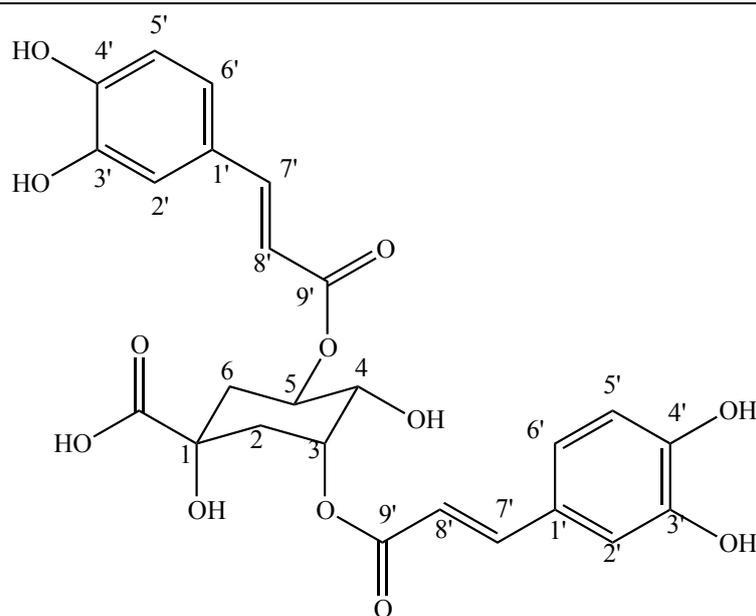


Figure 12. ^1H NMR spectrum of PIE 8-4-1 (270 MHz, methanol- d_4).

Table 3. Physicochemical properties of PIE 8-4-1 (3,5-di-*O*-caffeoylquinic acid).



Appearance: off white powder

ESI-TOF-MS (negative) m/z : 515.17

$^1\text{H-NMR}$ (500 MHz, methanol- d_4) δ (ppm), J (Hz)

PIE 8-4-1	Reference*
2.12-2.33 (4H, m, 2 and 6-H)	2.12-2.30
3.96 (1H, dd, $J = 7.3$ Hz, 3.5 Hz, 4-H)	3.94
5.41 (1H, m, 5-H),	5.41
5.42 (1H, m, 3-H)	5.47
6.26 and 6.34 (each 1H, d, $J = 16.0$ Hz, $2 \times 8'$ -H)	6.28 and 6.38
6.77 and 6.88 (each 1H, d, $J = 8.0$ Hz, $2 \times 5'$ -H)	6.77 and 6.78
6.95 and 6.96 (each 1H, brd, $J = 8.1$ Hz, $2 \times 6'$ -H)	6.95 and 6.96
7.05 and 7.06 (each 1H, d, $J = 2.2$ Hz, $2 \times 2'$ -H)	7.06 and 7.08
7.57 and 7.61 (each 1H, d, $J = 16.0$ Hz, $2 \times 7'$ -H)	7.58 and 7.61

* Basnet et al. (1996).

3.3.2.2 PIE 8-4-2-1 (4,5-di-*O*-caffeoylquinic acid methyl ester)

PIE 8-4-2-1 was obtained as a light yellowish powder. ESI mass, ^1H NMR and COSY spectra of PIE 8-4-2-1 are shown in Figures 13–15, respectively, and the physicochemical properties are shown in Table 4. Based on the pseudomolecular ions at m/z 529 $[\text{M-H}]^-$ (negative mode) and m/z 553 $[\text{M}+\text{Na}]^+$ (positive mode) in the ESI-mass spectra, molecular

weight was determined as 530. The NMR profile was similar to that of PIE 8-4-1. In the ^1H NMR spectrum, a singlet (δ 3,71, 3H) was assigned to methyl ester protons. Signals for quinic acid protons were found at δ 2.07, 2.28, 2.32, 3.71, 4.40, 5.12, and 5.54. Two sets of caffeoyl residues at δ 6.16 and 6.28 ($2 \times 8'$ -H), 6.75 ($2 \times 5'$ -H), 6.90 and 6.91 ($2 \times 6'$ -H), 7.00 and 7.02 ($2 \times 2'$ -H), 7.50 and 7.60 ($2 \times 7'$ -H) were also observed. The low field shift of H-4 and H-5 of the quinic acid moiety indicated that caffeoyl groups are esterified to 4-OH and 5-OH of the quinic acid. The NMR data was consistent with those reported for 4,5-di-*O*-caffeoylquinic acid methyl ester (Gao et al., 2008) and thus, PIE 8-4-2-1 is confirmed as 4,5-di-*O*-caffeoylquinic acid methyl ester.

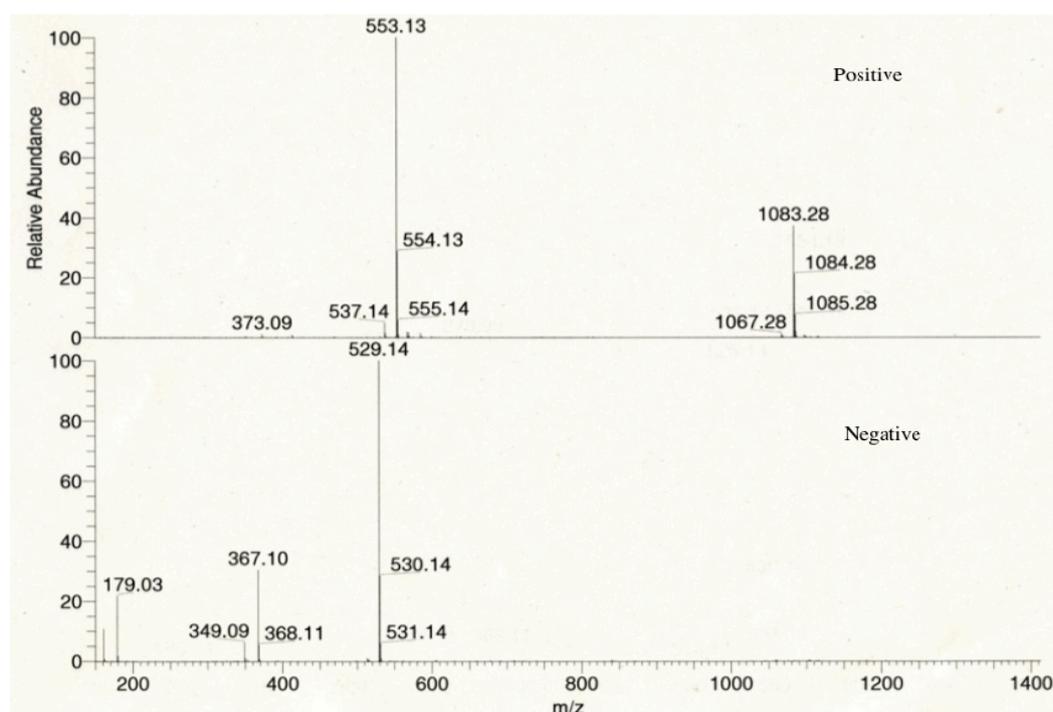


Figure 13. ESI mass spectra of PIE 8-4-2-1.

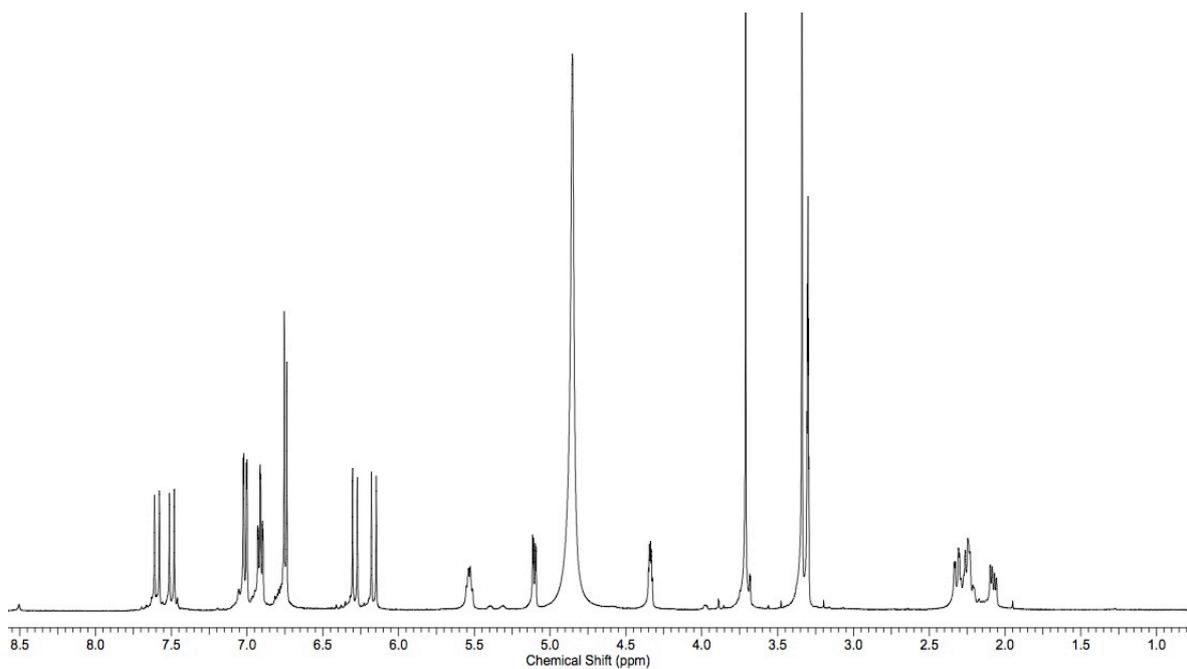


Figure 14. ^1H NMR spectrum of PIE 8-4-2-1 (500 MHz, methanol- d_4).

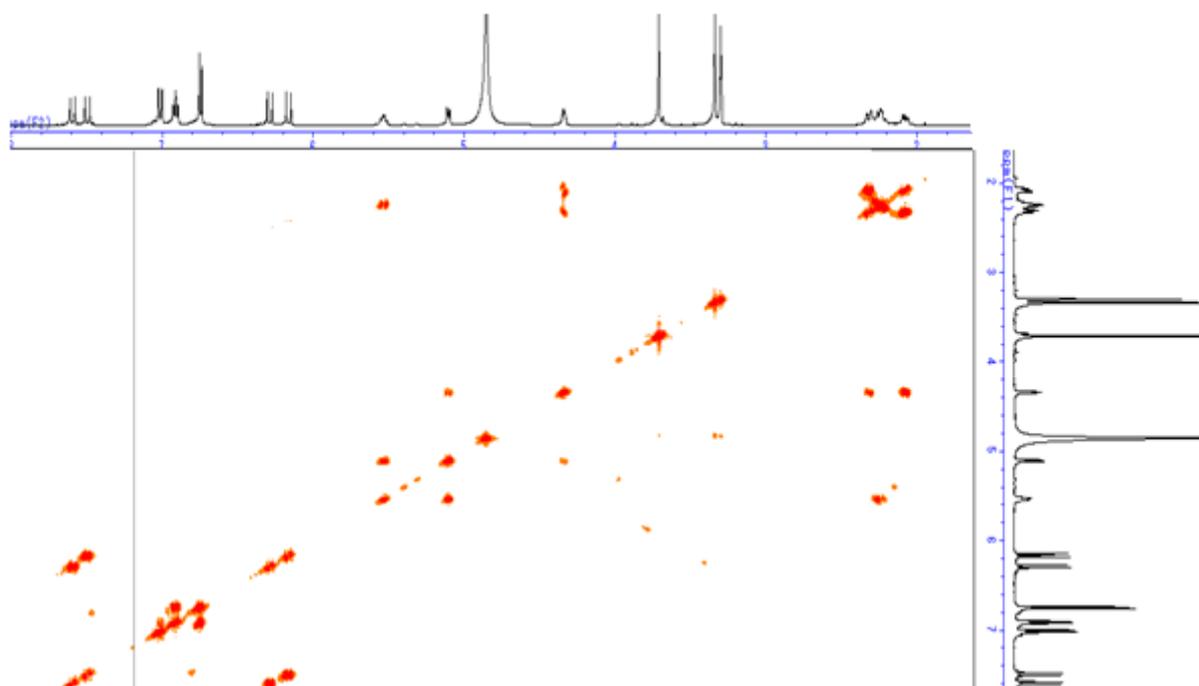
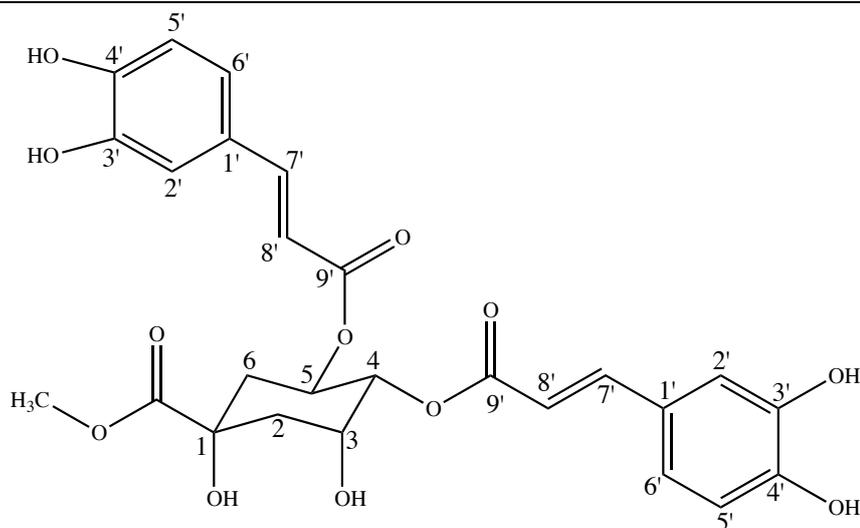


Figure 15. COSY spectrum of PIE 8-4-2-1 (500 MHz, methanol- d_4).

Table 4. Physicochemical properties of PIE 8-4-2-1 (4,5-di-*O*-caffeoylquinic acid methyl ester).



Appearance: Light yellow powder

ESI-MS m/z : 529.14 $[M-H]^-$ (negative), m/z : 553.13 $[M+Na]^+$ (positive)

1H -NMR (500 MHz, methanol- d_4), δ (ppm, J in Hz)

PIE 8-4-2-1	Reference*
2.07 (1H, dd, $J=14.0, 5.0$ Hz, 2eq-H)	2.12
2.28 (2H, m, 6-H)	2.27
2.32 (1H, dd, $J=5.0, 3.5$ Hz, 2ax-H)	2.32
3.71 (3H, s, OCH ₃)	3.74
4.40 (1H, m, 3-H)	4.38
5.12 (1H, dd, $J=8.2, 3.2$ Hz, 4-H)	5.15
5.54 (1H, dd, $J=13.0, 8.2$ Hz, 5-H)	5.59
6.16 and 6.28 (2H, d, $J=16.0$ Hz, 8'-H)	6.19 and 6.31
6.75 (2H, d, $J=8.20$ Hz, 5'-H)	6.77
6.90 and 6.91 (2H, dd, $J=8.2, 2.2$ Hz, 6'-H)	6.92 and 6.93
7.00 and 7.02 (2H, d, $J=2.0$ Hz, 2'-H)	7.04 and 7.05
7.50 and 7.60 (2H, d, $J=16.0$ Hz, 7'-H)	7.52 and 7.62

*Gao et al. (2008)

3.3.2.3 PIE 8-4-4-3 (3,4,5-tri-*O*-caffeoylquinic acid methyl ester)

PIE 8-4-4-3 was a light yellowish powder. ESI mass, 1H NMR and COSY spectra of PIE 8-4-4-3 are shown in Figures 16–18, respectively, and the physicochemical properties are

shown in Table 5. ESI-mass spectrum showed the pseudomolecular ion at m/z 691 $[M-H]^-$ (negative mode) and m/z 715 $[M+Na]^+$ (positive mode), in which molecular weight was determined as 692. In the 1H NMR spectrum, a singlet (δ 3,73, 3H) was assigned to methyl ester protons. The NMR profile also contained characteristic of caffeoylquinic acid. Signals of quinic acid moiety were found at δ 2.13, 2.39, 2.45, 5.33, 5.57 and 5.64. Three sets of caffeoyl residues at δ 6.19, 6.23, and 6.29 (d, $3 \times 8'$ -H); 6.71, 6.75, and 6.75 (d, $3 \times 5'$ -H) 6.86, 6.91, and 6.91 (dd, $3 \times 6'$ -H); 7.00, 7.02, and 7.02 (d, $3 \times 2'$ -H); 7.52, 7.52, and 7.58 (d, $3 \times 7'$ -H), were also observed. The low field shift of H-3, H-4, and H-5 of the quinic acid moiety indicated three caffeoyl groups are esterified to 3-OH, 4-OH, and 5-OH of the quinic acid. The NMR data was consistent with those reported for 3,4,5-tri-*O*-caffeoylquinic acid methyl ester (Merfort, 1992) and thus, PIE 8-4-4-3 is confirmed as 3,4,5-tri-*O*-caffeoylquinic acid methyl ester.

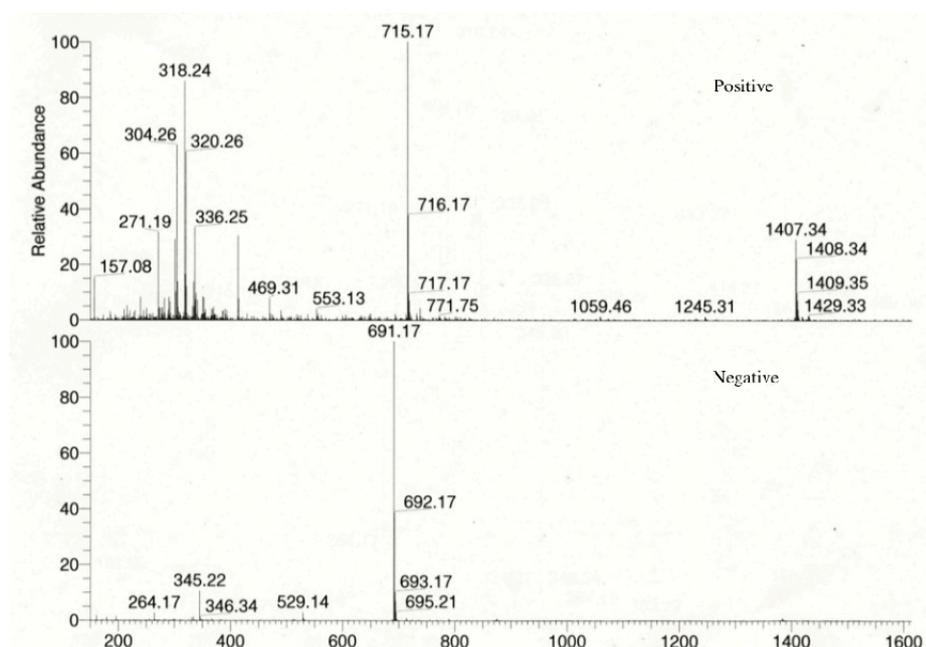


Figure 16. ESI mass spectrum PIE 8-4-4-3.

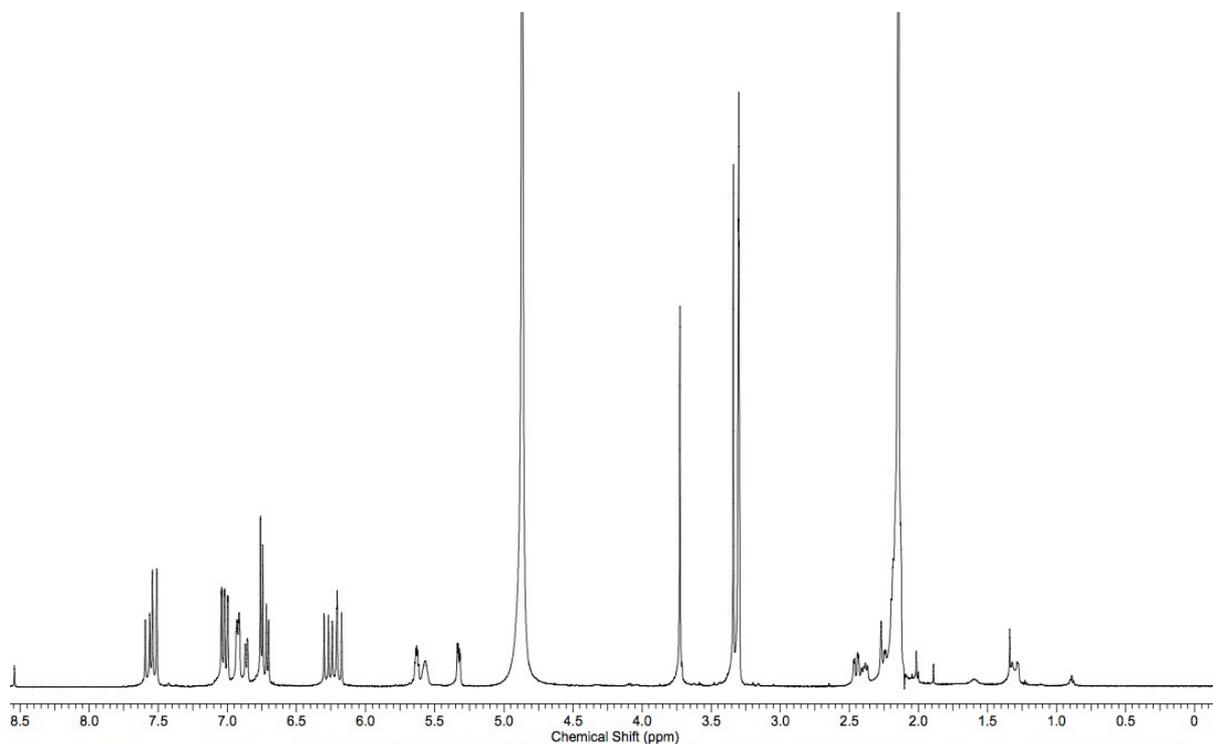


Figure 17. ^1H NMR spectrum PIE 8-4-4-3 (500 MHz, methanol- d_4).

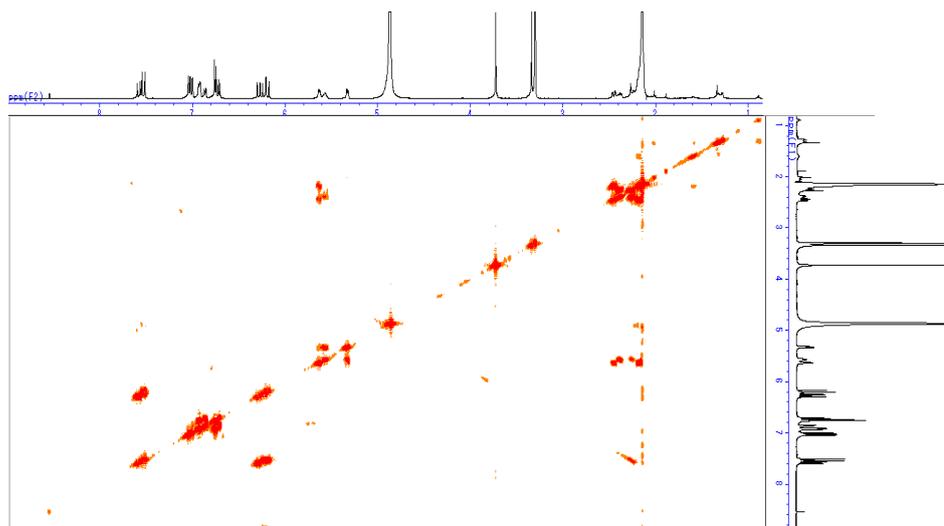
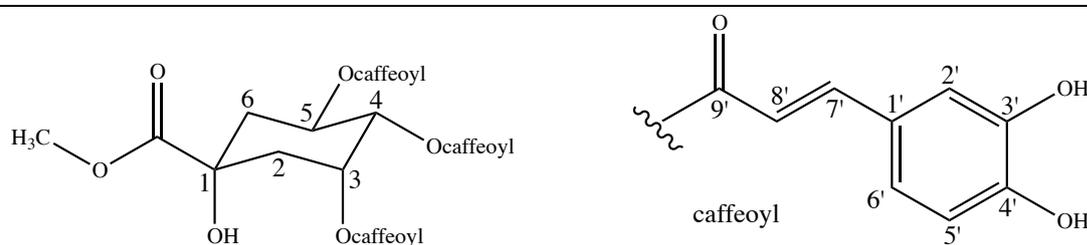


Figure 18. COSY spectrum PIE 8-4-4-3 (500 MHz, methanol- d_4).

Table 5. Physicochemical properties of PIE 8-4-4-3 (3,4,5-tri-*O*-caffeoylquinic acid methyl ester)



Appearance: Light yellow powder

ESI-MS m/z : 691.17 $[M-H]^-$ (negative), m/z : 715.17 $[M+Na]^+$ (positive)

1H -NMR (500 MHz, methanol- d_4), δ (ppm, J in Hz)

PIE 8-4-4-3	Reference*
2.13 (1H, m, 6ax-H)	2.15
2.39 (1H, dd, $J=13.5, 8.0$ Hz, 2ax)	2.59
2.45 (1H, dd, $J=13.5, 3.8$ Hz, 2eq-H)	2.67
3.73 (3H, s, OMe)	3.62
5.33 (1H, dd, $J=7.6, 3.0$ Hz, 4-H)	5.30
5.57 (1H, m, 3-H)	5.68
5.64 (1H, m, 5-H)	5.71
6.19, 6.23 and 6.29 (each 1H, d, $J=16.0$ Hz, 8'-H)	6.16, 6.22 and 6.42
6.71, 6.75 and 6.75 (each 1H, d, $J=8.20$ Hz, 5'-H)	6.73, 6.74 and 6.81
6.86, 6.91 and 6.91 (each 1H, dd, $J=8.2, 1.9$ Hz, 6'-H)	6.91, 6.91 and 7.04
7.00, 7.02 and 7.02 (each 1H, d, $J=1.9$ Hz, 2'-H)	7.00, 7.00 and 7.13
7.52, 7.52 and 7.58 (each 1H, d, $J=16.0$ Hz, 7'-H)	7.54, 7.55 and 7.64

* Mertfort (1992).

3.3.2.4 PIE 8-4-5 (1,3,4,5-tetra-*O*-caffeoylquinic acid)

PIE 8-4-5 was a light yellowish-white amorphous powder. ESI mass, 1H NMR and COSY spectra of PIE 8-4-5 are shown in Figures 19–21, respectively, and the physicochemical properties are shown in Table 6. ESI mass spectrum (negative mode) showed the pseudomolecular ion at m/z 839 $[M-H]^-$, in which molecular weight was determined as 840. In the 1H NMR spectrum, characteristic signals of quinic acid moiety

protons in caffeoylquinic acid were found at δ 2.17, 2.6, 2.73, 2.94, 5.37, 5.73, and 5.86. Four sets of caffeoyl residues at δ 6.15, 6.24, 6.37, and 6.37 (d, $4 \times 8'$ -H); 6.05, 6.66, 6.73, and 6.73 (d, $4 \times 5'$ -H); 6.78, 6.78, 6.92, and 6.92 (dd, $4 \times 6'$ -H); 6.67, 6.94, 7.01, and 7.07 (d, $4 \times 2'$ -H); 7.47, 7.49, 7.57, 7.65 (d, $4 \times 7'$ -H) also appeared. The low field shift of H-3, H-4, H-5 of the quinic acid moiety indicated that three caffeoyl groups were esterified to 3-OH, 4-OH, and 5-OH of the quinic acid. The remaining caffeoyl group was esterified to 1-OH of the quinic acid indicated by the low field shift of 2-H and 6-H of the quinic acid. The NMR data was consistent with those reported for 1,3,4,5-tetra-*O*-caffeoylquinic acid (Scholz et al., 1994) and thus, PIE 8-4-5 is confirmed as 1,3,4,5-tetra-*O*-caffeoylquinic acid.

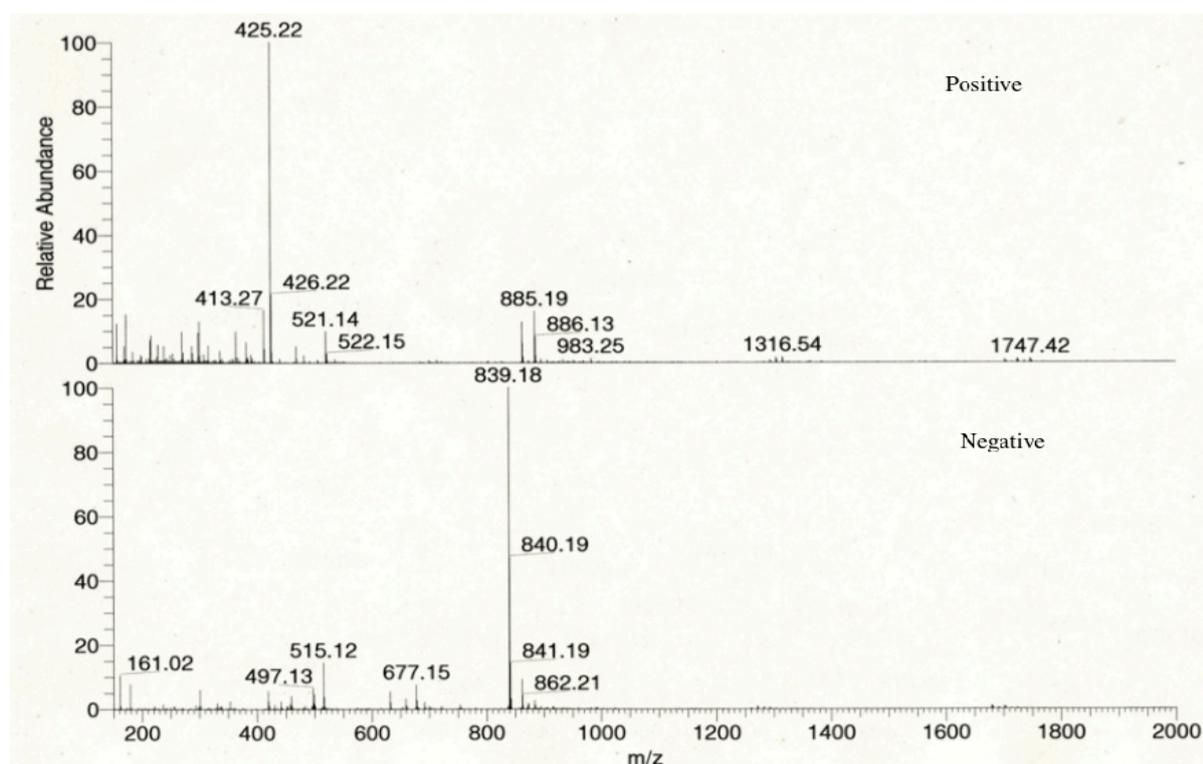


Figure 19. ESI mass spectra of PIE 8-4-5.

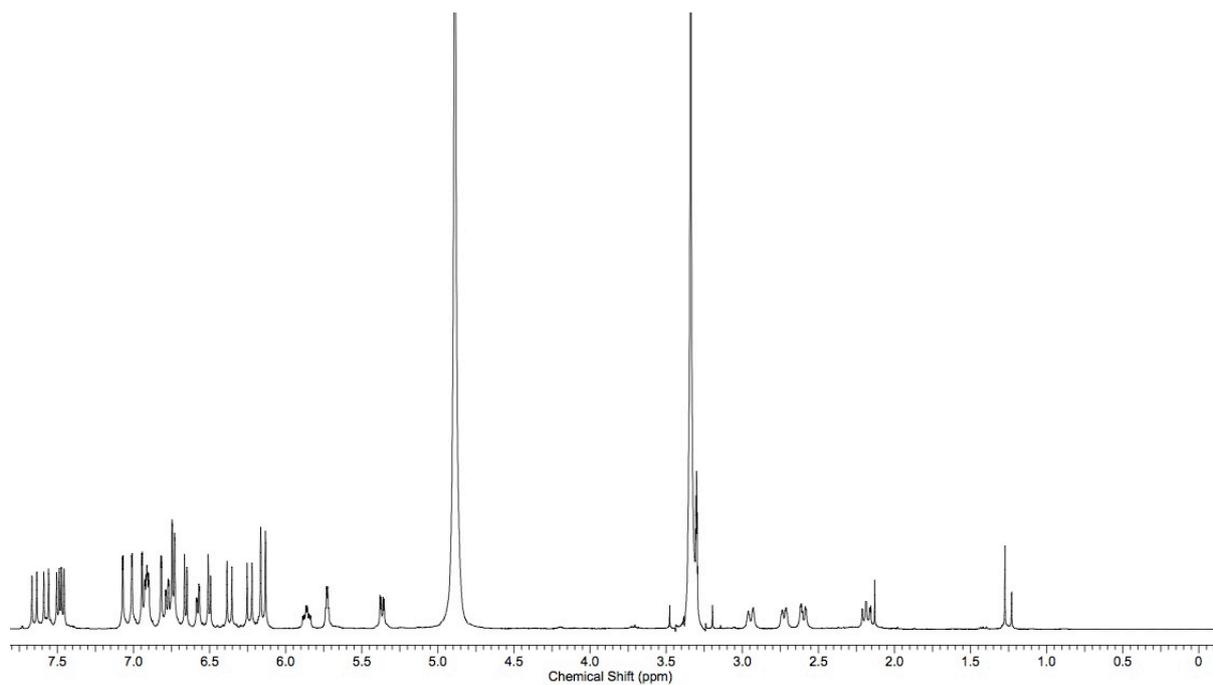


Figure 20. ^1H NMR spectrum of PIE 8-4-5 (500 MHz, methanol- d_4).

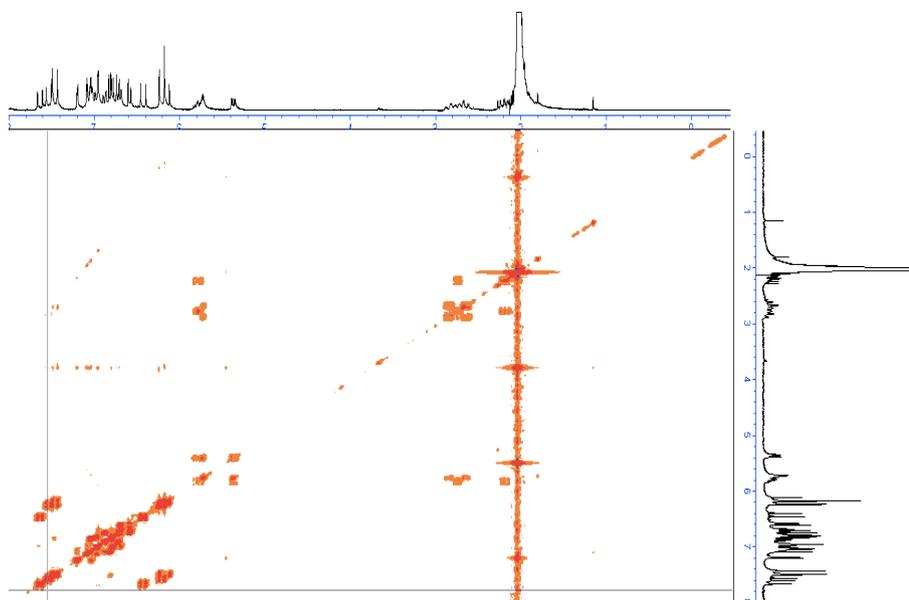
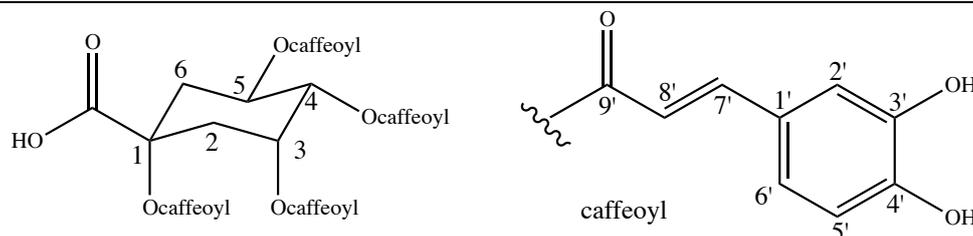


Figure 21. COSY spectrum of PIE 8-4-5 (500 MHz, methanol- d_4).

Table 6. Physicochemical properties of PIE 8-4-5 (1,3,4,5-tetra-*O*-caffeoylquinic acid).



Appearance: yellowish-white, amorphous powder

ESI-MS m/z : 840.19 $[M-H]^-$ (negative)

1H -NMR (500 MHz, methanol- d_4), δ (ppm, J in Hz)

PIE 8-4-5	Reference*
2.17 (1H, br d, $J=13.0$, 6ax-H)	2.14
2.6 (1H, br dd, $J=16.0, 2.0$, 2ax-H)	2.61
2.73 (1H, m, 6eq-H)	2.73
2.94 (1H, m, 2eq-H)	3.05
5.37 (1H, dd, $J=10.3, 3.6$, 4-H)	5.33
5.73 (1H, m, 3-H)	5.72
5.86 (ddd, $J=11.5, 10.3, 4.5$, 5-H)	5.90
6.15, 6.24, 6.37 and 6.37 (each 1H, d, $J=15.7$ Hz, 8'-H)	6.12, 6.14, 6.22 and 6.39
6.05, 6.66, 6.73 and 6.73 (each 1H, d, $J=8.2$ Hz, 5'-H)	6.47, 6.65, 6.72 and 6.73
6.78, 6.78, 6.92 and 6.92 (each 1H, dd, $J=8.2, 2.2$ Hz, 6'-H)	6.55, 6.76, 6.87 and 6.89
6.67, 6.94, 7.01 and 7.07 (each 1H, d, $J=2.2$ Hz, 2'-H)	6.80, 6.93, 7.00 and 7.06
7.47, 7.49, 7.57 and 7.65 (each 1H, d, $J=15.7$ Hz, 7'-H)	7.44, 7.46, 7.55 and 7.62

*Scholz et al. (1994).

3.3.2.5 PIE 8-5-2 (3,4,5-tri-*O*-caffeoylquinic acid)

PIE 8-5-2 was obtained as a yellowish white powder. ESI mass and 1H NMR spectra of PIE 8-5-2 are shown in Figures 22 and 23, respectively, and the physicochemical properties are shown in Table 7. ESI mass spectrum showed the pseudomolecular ions at m/z 677 $[M-H]^-$ (negative mode) and m/z 701 $[M+Na]^+$ (positive mode), in which molecular weight was determined as 678. In the 1H NMR spectrum, signals of quinic acid moiety were found at δ 2.06-2.47, 5.31, and 5.67. Three sets of caffeoyl residues at δ 6.22, 6.23, and 6.29 (d, $3 \times 8'$ -H); 6.68-6.85 (m, $3 \times 5'$ -H); 6.85, 6.92 (m, $3 \times 6'$ -H); 6.98, 7.02, and 7.06 (m, $3 \times 2'$ -H); 7.51, 7.53, and 7.60 (d, $3 \times 7'$ -H), were also observed. The low field shift of H-3, H-4,

and H-5 of the quinic acid moiety indicated three caffeoyl groups are esterified to 3-OH, 4-OH, and 5-OH of the quinic acid. The NMR data was consistent with those reported for 3,4,5-tri-*O*-caffeoylquinic acid (Islam et al., 2002) and thus, PIE 8-5-2 was confirmed as 3,4,5-tri-*O*-caffeoylquinic acid.

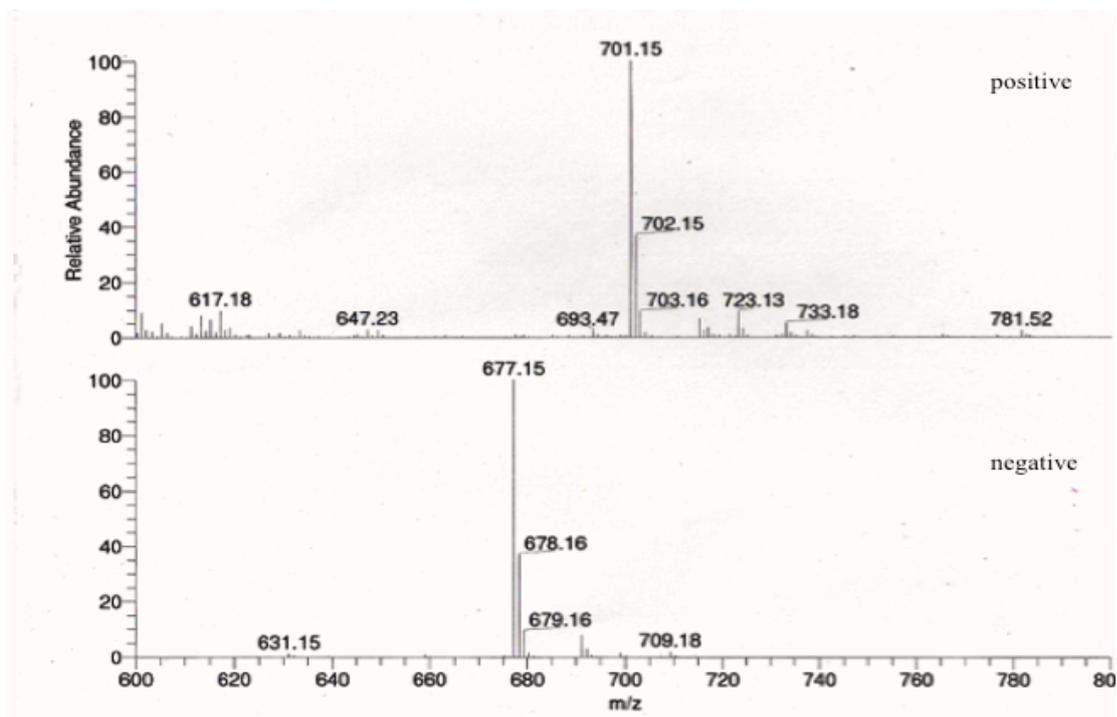


Figure 22. ESI mass spectra of PIE 8-5-2.

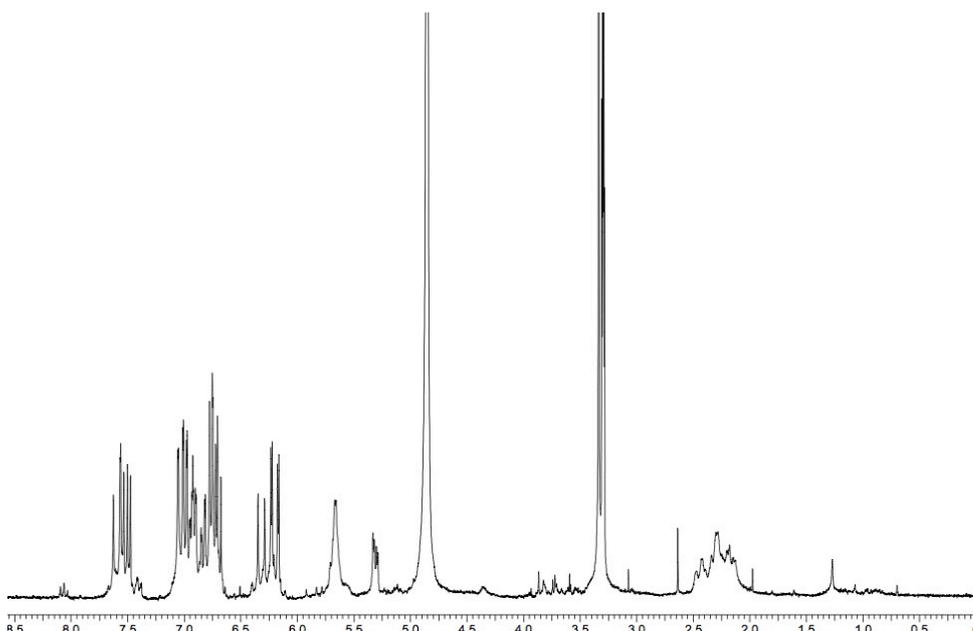
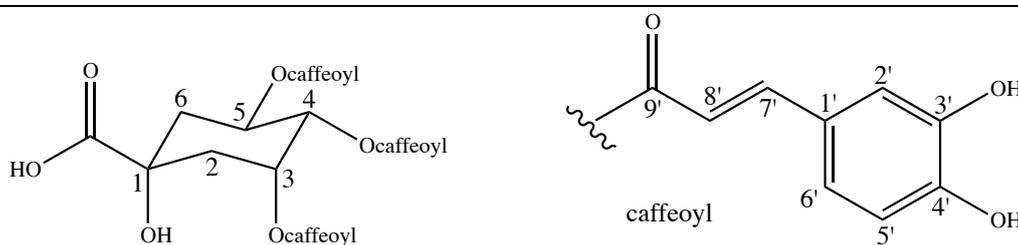


Figure 23. ^1H -NMR spectrum of PIE 8-5-2 (500 MHz, methanol- d_4).

Table 7. Physicochemical properties of PIE 8-5-2 (3,4,5-tetra-*O*-caffeoylquinic acid)



Appearance: yellowish white powder

ESI-MS: m/z 677.12 $[\text{M}-\text{H}]^-$ (negative), m/z : 701 $[\text{M}+\text{Na}]^+$ (positive)

^1H NMR δ (500 MHz, methanol- d_4)

Reference*

2.06-2.47 (4H, m, 2,6-H)	2.06–2.47
5.31 (1H, dd, $J = 8.4$ Hz, 3.5 Hz, 4-H)	5.34
5.67 (2H, m, 3,5-H)	5.69
6.22, 6.23, and 6.29 (each 1H, d, $J = 16$ Hz, $3 \times 8'$ -H)	6.22, 6.23, and 6.35
6.68-6.85 (4H, m, $3 \times 5'$ -H, $6'$ -H)	6.72–6.81
6.92 (2H, m, $2 \times 6'$ -H)	6.93
6.98, 7.02, and 7.06 (each 1 H, br s, $3 \times 2'$ -H)	7.01, 7.05, and 7.09
7.51, 7.53, and 7.60 (each 1H, d, $J = 16$ Hz, $3 \times 7'$ -H)	7.52, 7.55, and 7.62

*Islam et al. (2002).

3.3.3 Intestinal maltase inhibitory activity of isolated compounds

Five isolated compounds (**1–5**) from *P. indica* leaves were examined for their maltase inhibitory activity (Figure 24). All the isolates showed the increasing dose-dependent effect against intestinal maltase inhibition. Among the isolated compounds, compound **3** ($IC_{50} = 0.002$ mM) showed the highest inhibitory activity followed by **5** ($IC_{50} = 0.011$ mM), **4** ($IC_{50} = 0.013$ mM), **2** ($IC_{50} = 0.208$ mM), and **1** ($IC_{50} = 1.16$ mM) (Table 8).

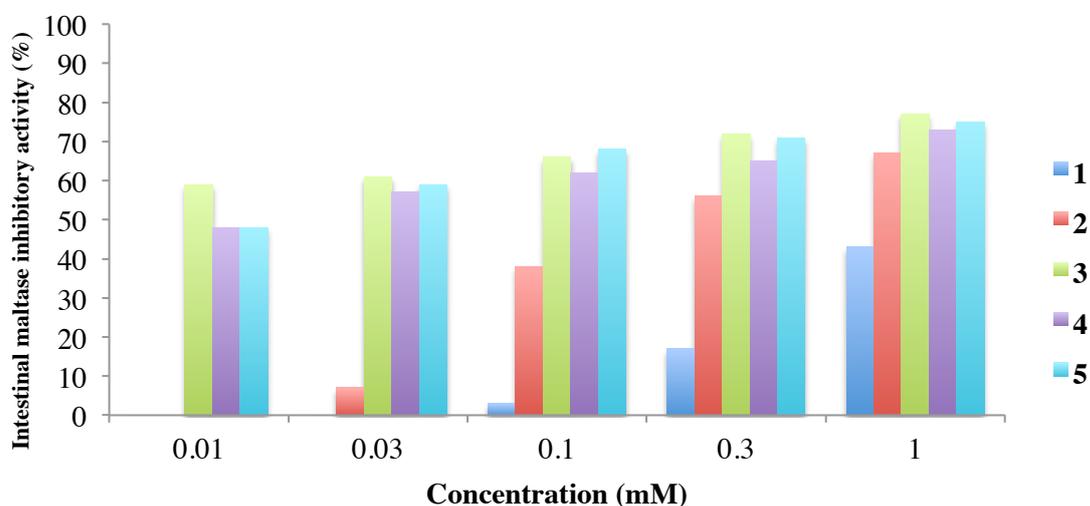


Figure 24. Rat intestinal maltase inhibitory activity of isolated compounds from *P. indica* leaves; 3,5-di-*O*-caffeoylquinic acid (**1**), 4,5-di-*O*-caffeoylquinic acid methyl ester (**2**), 3,4,5-tri-*O*-caffeoylquinic acid methyl ester (**3**), 1,3,4,5-tetra-*O*-caffeoylquinic acid (**4**), and 3,4,5-tri-*O*-caffeoylquinic acid (**5**).

Table 8. IC₅₀ values for the inhibition of intestinal maltase by isolates (1–5) and acarbose

Compounds	IC ₅₀ (μM)	Yield (mg) *
3,5-di- <i>O</i> -caffeoylquinic acid (1)	1166	10
4,5-di- <i>O</i> -caffeoylquinic acid methyl ester (2)	208	75
3,4,5-tri- <i>O</i> -caffeoylquinic acid methyl ester (3)	2	42
1,3,4,5-tetra- <i>O</i> -caffeoylquinic acid (4)	13	41
3,4,5-tri- <i>O</i> -caffeoylquinic acid (5)	11	19
Acarbose	0.5	-

*Yield is calculated from 94 g leaves.

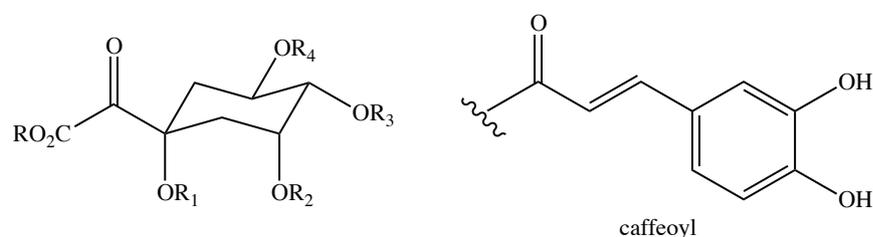
3.4 Discussion

3.4.1 Intestinal maltase inhibitors of *P. indica* leaves

P. indica leaves showed the potential intestinal maltase inhibition. The 50% aqueous methanol extract of *P. indica* leaves was partitioned between ethyl acetate (EtOAc) and water. The EtOAc fraction was subjected to silica gel column chromatography using chloroform-methanol gradient and the active fraction was further fractionated using ODS column chromatography. The active ODS fractions were further purified using preparative HPLC resulting in the isolation of five caffeoylquinic acid derivatives: 3,5-di-*O*-caffeoylquinic acid (1), 4,5-di-*O*-caffeoylquinic acid methyl ester (2), 3,4,5-tri-*O*-caffeoylquinic acid methyl ester (3), 1,3,4,5-tetra-*O*-caffeoylquinic acid (4), and 3,4,5-tri-*O*-caffeoylquinic acid (5).

The structure of all isolates (1–5) was determined using ESI mass and ¹H-NMR spectra. The NMR pattern of all compounds showed characteristic signals of caffeoylquinic acids and their structures were finally confirmed by comparing with published spectral data: 1 (Basnet et al., 1996; Gao et al., 2008), 2 (Gao et al., 2008), 3 (Mertfort, 1992), 4 (Scholz et al., 1994) and 5 (Islam et al., 2002). The structures of the isolated compounds are shown in Figure 25. This finding is consistent with the

hypoglycemic effect of this plant extract on streptozotocin-induced diabetic rats (Pramanik et al., 2006).



- 3,5-Di-*O*-caffeoylquinic acid (**1**) : R = R₁ = R₃ = H, R₂ = R₄ = caffeoyl
 4,5-Di-*O*-caffeoylquinic acid methyl ester (**2**) : R = Me, R₁ = R₂ = H, R₃ = R₄ = caffeoyl
 3,4,5-Tri-*O*-caffeoylquinic acid methyl ester (**3**) : R = Me, R₁ = H, R₂ = R₃ = R₄ = caffeoyl
 1,3,4,5-Tetra-*O*-caffeoylquinic acid (**4**) : R = H, R₁ = R₂ = R₃ = R₄ = caffeoyl
 3,4,5-Tri-*O*-caffeoylquinic acid (**5**) : R = R₁ = H, R₂ = R₃ = R₄ = caffeoyl

Figure 25. Intestinal maltase inhibitors isolated from *P. indica* leaves (**1 – 5**).

Caffeoylquinic acid derivatives are richly contained in Asteraceous plants such as, *Chrysanthemum morifolium*, *Artemisia annua*, *Chrysanthemum coronarium* (Lai et al., 2007), *Liguralia stenocephala* (Lee et al., 2010), and *Articum lappa* (Maruta et al., 1995). Caffeoylquinic acid derivatives have also been reported for their biological activities including hepatoprotective (Purusotam et al., 1996; Kim et al., 2008) antioxidant, neuroprotective (Nakajima et al., 2007), antiviral (Hak et al., 2000), antibacterial (Scholz et al., 1994) activities.

This is the first report for the identification of compounds **1–3** from *P. indica*, whereas compounds **4** and **5** were recently isolated as constituents for this plant (Ohtsuki et al., 2008). Interestingly, the 3,5-isomer of dicaffeoylquinic acid was isolated as a free form (**1**), whereas the 4,5-isomer was isolated only as a methyl ester (**2**). This suggests that methyl esterification selectively occurs in plant tissues.

4,5-Di-*O*-caffeoylquinic acid methyl ester isolated from *Chrysanthemum sinense* possessed hypouricemic and rat liver xanthine oxidase inhibitory activities (Nguyen et al., 2005). It has also been isolated from propolis for its hepatoprotective activity in experimental liver injury models. 3,4,5-Tri-*O*-caffeoylquinic acid methyl ester was obtained from flowers of *Arnica chamissonis* ssp. *foliosa* var. *incana*. There are few reports on the physiological functions of tri-*O*-caffeoylquinic acid methyl ester. It is supposed that tri-*O*-caffeoylquinic acid normally occur in the free form, as in *Chrysothamnus paniculatus*, and the methyl ester is thought to be formed by esterification with methanol during preparation of the extract (Mertfort, 1992). It has been reported that 1,3,4,5-tetra-*O*-caffeoylquinic acid isolated from *P. indica* exerted collagenase inhibitory activity (Ohtsuki et al., 2008) and from *P. symphytifolia* leaves possessed antibacterial and anthelmintic activity (Scholz et al., 1994). There was little documentation on biological activities of the tetracaffeoylquinic acid derivatives because it is less common than mono-, di- and tri-caffeoylquinic acids.

3.4.2 Intestinal maltase inhibitory activity of *P. indica* leaves isolates

The isolated caffeoylquinic acid derivatives were compared for their rat intestinal maltase inhibitory activity (Table 8). Half maximal inhibitory concentration (IC₅₀) values were used as measures of the effectiveness of each compound to inhibit maltase function. On the basis of IC₅₀ values, compound **3** had the highest inhibition level among the caffeoylquinic acid derivatives isolated from *P. indica* leaves, followed by **5**, **4**, **2**, and **1** respectively. Compounds **1** and **5** have been previously reported as maltase inhibitors (Matsui et al., 2004). However, compounds **2**, **3**, and **4** have not been evaluated for their intestinal maltase inhibitory activity.

This suggests that, when comparing the inhibitory activity of compounds **1**, **4**, and **5**, increasing numbers of caffeoyl groups attached to quinic acid moiety enhanced maltase inhibitory activity. This is consistent with a previous study in which the caffeoyl group played an important role in intestinal α -glucosidase inhibitory activity (Matsui et al. 2004). Among the compounds with the same number of caffeoyl groups, compounds **2** and **5** had five-fold higher inhibitory activities than compounds **1** and **3**, respectively. This suggests that methyl esterification of the carboxylic group in quinic acid has an additional effect of enhancing maltase inhibitory activity. This is the first report for the importance of the methyl esterification of caffeoylquinic acids with respect for their α -glucosidase inhibitory activity, although the mechanism still remains undetermined.

In the present study, increases in maltase inhibitory activity were due to both the number of caffeoyl groups attached to the quinic moiety and to the presence of methyl esterification on the carboxyl group. Considering the yields and IC_{50} values of the caffeoylquinic acid derivatives isolated, the inhibitory activity of *P. indica* leaves is likely to be due to compounds **3**, **4**, and **5**, although compounds **1** and **2** may also contribute to the activity. According to the intestinal maltase inhibitory effect of acarbose, IC_{50} value of compound **3** is 4-fold higher compared to acarbose.

Caffeoylquinic acid derivatives from this plant may be important medicinal substances, which may delay postprandial hyperglycemia. *Pluchea indica* is an indigenous plant that may potentially be used as a functional food or as a medicinal supplement for DM treatment and prevention. Further studies are needed to address the potential function of di-, tri-, and tetra -*O*-caffeoylquinic acid.

3.5 Conclusions

In conclusion, intestinal maltase inhibition of *Pluchea indica* leaves extract may be physiologically useful for suppressing postprandial hyperglycemia by ingesting it or using its extract in the diet. Thus, this study provides basic information for further examination of *Pluchea indica*'s suitability as a functional food and medicinal supplement that contributes for treatment and prevention of diabetes.

Chapter 4. ANTIHYPERGLYCEMIC PRINCIPLES FROM *CAESALPINIA*

SAPPAN L. WOOD

4.1 Introduction

Much attention has been focused on the use of *Caesalpinia sappan* L. (Leguminosae) wood as an ingredient in a functional drink with antihyperglycemic properties (Indriani et al., 2014). The wood of *C. sappan* is native to many parts of Southeast Asia and has been cultivated in Africa and the U.S.A (Utomo, 2001). Owing to its pleasing fragrance and color (Jansen, 2005), *C. sappan* has been used in many traditional cultures; for example, it is used as an ingredient of a refreshing beverage called *Ya-u-Tai* in Thailand (Det-anand, 1975), and *Bir Pletok* (Batubara et al., 2010) in Indonesia. Recent scientific studies have also shown that it is valuable for use in foods. *C. sappan* wood extract can be used as an additive to prevent lipid oxidation in beef patties (Han and Rhee, 2005), to inhibit bacterial growth for up to 6 months in chilli paste (Saraya et al., 2009), and as a food coloring agent (Sinsawasdi et al., 2014). In addition to its use in beverages and foods, a decoction of the bark and wood is used as a natural astringent for the treatment of tuberculosis, diarrhea, and dysentery (Zerrudo, 1991). An important compound that is isolated from *C. sappan* wood is brazilin, and this has been reported to exert antihyperglycemic effect by regulating glucose uptake, hepatic glucose output, and adipocyte differentiation (Moon et al., 1990; Moon et al., 1993; Kim et al., 1995).

Screening experiments of Indonesian herbs have shown that *C. sappan* wood potentially exerts inhibitory activity against intestinal maltase (Chapter 2). Although brazilin's antihyperglycemic activities have been reported (Moon et al., 1993; Kim et al., 1995), the use of *C. sappan* wood to delay postprandial hyperglycemia via

inhibition of carbohydrate digestive enzymes and muscle glucose uptake has not yet been investigated.

4.1.1 Specific objective

This chapter deals with identification of compounds that can delay postprandial hyperglycemia through inhibition of intestinal α -glucosidases, in particular, intestinal maltase. In addition, the isolated compounds were evaluated for their ability to inhibit intestinal sucrase and pancreatic α -amylase (PPA), and to enhance glucose uptake on skeletal muscle.

4.2 Materials and methods

4.2.1 Materials

Dried *C. sappan* wood was purchased from Merapi Farma Traditional Herbs Distributor, Yogyakarta, Indonesia, in April 2014 and a voucher specimen (batch number CS1314.17) was deposited in the Merapi Farma Traditional Herbs Distributor, Yogyakarta, Indonesia.

4.2.2 Reagents

Rat intestinal acetone powder and α -amylase, type VI-B from porcine pancreas were supplied by Sigma-Aldrich Co. Cosmosil 75C₁₈-OPN was purchased from Nacalai Tesque, Inc. All chemicals were of analytical grade and purchased from Wako Pure Chem. Co., unless otherwise stated.

4.2.3 Rat intestinal α -glucosidase inhibitory activity assay

α -Glucosidase inhibitory activity was determined based on inhibition of maltose or sucrose hydrolysis using the method described in Section 2.2.4. Procedure for inhibitory activity against sucrose hydrolysis were basically the same as in Section 2.2.4 except for replacing maltose solution (3.5 mM, 350 μ L) with sucrose solution (56 mM, 200 μ L) and for increasing the amount of enzyme solution from 50 μ L to 200 μ L. The sucrose-hydrolysing activity of the crude enzyme solution (0.44 U/mg protein) was designated as sucrase activity.

4.2.4 Pancreatic α -amylase (PPA) inhibitory activity assay

Porcine pancreatic α -amylase (PPA) inhibitory activity was determined using the method previously described by Yonemoto et al. (2014). Test samples (100 μ L) in 50% DMSO and PPA solution (150 μ L; 0.5 U/mL) were added to each assay sample, and phosphate buffer (100 μ L; pH 6.9) was used in place of the plant extract for the blank sample. The mixtures were then pre-incubated at 37 °C for 15 min. Each mixture was added to soluble starch solution (250 μ L; 25 mg/mL) as a substrate. The mixtures were incubated at 37 °C for 15 min. Boiling for 1 min stopped the reactions.

The reaction mixtures were passed through an ODS column (ϕ 0.5 \times 2 cm) as for the α -glucosidase inhibitory assay. A portion (100 μ L) of each sample was added to dinitrosalicylic acid (DNS) coloring agent, and the mixture was boiled for 15 min. The reactions were stopped by cooling in ice. The mixtures were added to water (450 μ L), and a portion of this mixture (200 μ L) was then added to a 96-well plate. The optical density (OD) of the wells was measured at 540 nm. PPA inhibitory activity was calculated using the following formula (2):

$$\text{Inhibitory activity (\%)} = [1 - (\text{OD}_{\text{sample}} - \text{OD}_{\text{sample blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{control blank}})] \times 100$$

(2)

All experiments were performed in duplicate, and the results were presented as IC₅₀ values. The IC₅₀ values were determined by constructing a dose-response curve between the logarithm of the concentration of substances on the *x*-axis and the inhibitory activity on the *y*-axis.

4.2.5 Cell cultures

L6 cells (JCRB9081, Lot10102000) were provided by the JCRB Cell Bank through the National Institute of Biomedical Innovation, Japan. L6 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum at 37 °C in a humidified atmosphere with 10% CO₂. Cells were reseeded in 48-well plates for glucose uptake assay. After the cells reached confluence, the medium was switched to DMEM containing 2% horse serum for differentiation. The medium was replaced every second day, and experiments were performed 6–8 d after initiating differentiation.

4.2.6 Glucose uptake enhancement assay

A glucose uptake assay was performed according to the reported method (Yamamoto et al., 2006) with modifications. The medium was switched to serum-free DMEM and the cells were serum starved for 15 h. The cells were then incubated with the corresponding concentration of a sample diluted in serum-free DMEM for 4 h. After treatment, the cells were rinsed twice with Krebs-Ringer phosphate-HEPES (KRPH) buffer (20 mM HEPES, 136 mM NaCl, 4.7 mM KCl, 1 mM NaH₂PO₄,

1 mM MgSO₄, and 1 mM CaCl₂, pH 7.4) and incubated in KRPH buffer containing 1 mM 2-deoxyglucose for 20 min. After washing the cells thrice with KRPH buffer, 0.05 M NaOH aq was added to lyse the cells. The lysate was frozen, thawed, and heated at 85 °C for 60 min. A mixture of 0.1 M HCl aq and 50 mM triethanolamine buffer (pH 8.1, with 0.5 mM MgCl₂ and 50 mM KCl) was added and adjusted to pH 8.1. For quantitation of 2-deoxyglucose, 50 μL of the cell lysate, or standards containing various concentrations of 2-deoxyglucose, and 50 μL of the assay cocktail [1.3 mM ATP, 20 μM NADP⁺, 50 μM resazurin sodium salt, 12 units/mL hexokinase (Sigma–Aldrich Co.), 32 units/mL glucose 6-phosphate dehydrogenase (from *Leuconostoc mesenteroides*), and 4 units/mL diaphorase dissolved in triethanolamine buffer] were mixed in 96-well plates. After 1 h of incubation at 37 °C, the fluorescence was measured ($\lambda_{\text{ex}} = 530 \text{ nm}$, $\lambda_{\text{em}} = 590 \text{ nm}$). Background fluorescence (performed without 2-deoxyglucose incubation) was subtracted, and the values are represented relative to the control.

Glucose uptake enhancement assays were performed independently; each experiment was repeated twice, with $n = 4$ for each, and the representative values are shown on the graphs. Student's t-test was used to assess the significant differences between the two mean values. $P < 0.01$ was considered statistically significant.

4.2.7 Instrumental analysis

¹H-NMR spectra were recorded using JEOL JNM-EX-270 (¹H, 270 MHz; ¹³C, 67.5 MHz, JEOL Ltd.) and Bruker AMX500 spectrometers (¹H, 500 MHz; ¹³C, 125 MHz, Bruker Biospin Co.). Chemical shifts were calculated from the residual solvent signal (DMSO-*d*₆, $\delta_{\text{H}} 2.49$, $\delta_{\text{C}} 39.5$; methanol-*d*₄, $\delta_{\text{H}} 3.30$; acetone-*d*₆, $\delta_{\text{H}} 2.05$, $\delta_{\text{C}} 29.8$). Mass spectra were obtained using a Thermo Scientific Exactive

spectrometer (Thermo Fisher Scientific K.K.) or a Waters LCT Premier XE spectrometer (Waters Co.). High-pressure liquid chromatography (HPLC) was performed with a JASCO 802-SC system (JASCO Co.).

4.3 Results

4.3.1 Isolation of intestinal maltase inhibitors from *C. sappan* L. wood

Dried *C. sappan* wood (200 g) was twice extracted at room temperature with 50% aqueous methanol for 24 hr per extraction. The crude extract was filtered through a filter paper (Whatman No. 5C, 110-mm) and evaporated under reduced pressure below 40 °C to obtain the methanol extract (CS, 14.5 g, yield 7.23% based on dried wood). This residue was partitioned between ethyl acetate (EtOAc) and water. Each layer was evaporated under reduced pressure to yield an EtOAc-soluble fraction (11.6 g, CSE) and water-soluble fraction (2.4 g, CSW). CSE was found to have maltase inhibitory activity (Figure 26).

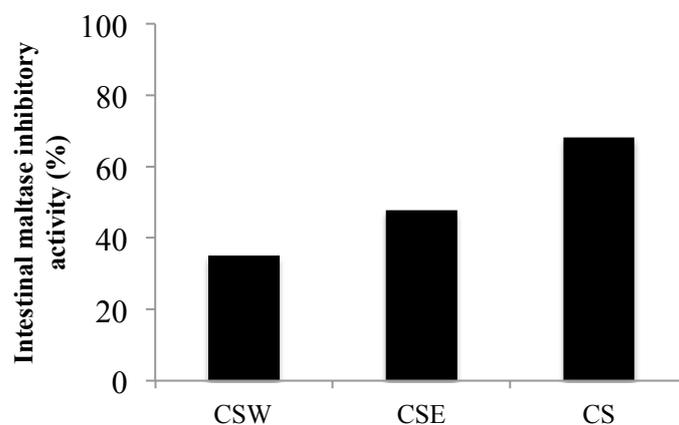


Figure 26. Intestinal maltase inhibitory activity of CS, CSE and CSW. Concentration was adjusted at 1 n.

Part of CSE (1 g) was separated on a silica gel (Silica Gel 60 N, Kanto Chemical Co., Inc.) column (ϕ 3 \times 16 cm) to yield five fractions. The fractions were obtained using the following solvent systems (chloroform:methanol): 19:1 (CSE-1), 9:1 (CSE-2), 4:1 (CSE-3), 1:1 (CSE-4), and methanol (CSE-5). These five fractions were evaluated for their intestinal maltase inhibitory activity in 1 n concentration and CSE-2 (61%) and CSE-3 (56%) showed intestinal maltase inhibitory activity (Figure 27). Hence, CSE-2 and CSE-3 were further fractionated.

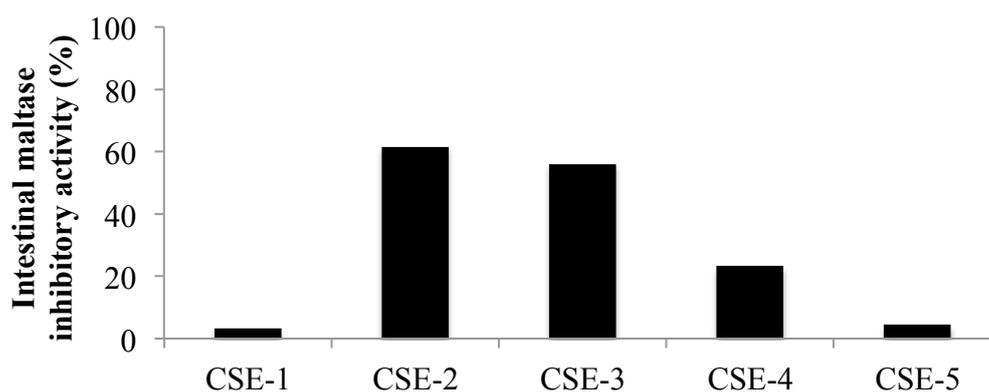


Figure 27. Intestinal maltase inhibitory activity of CSE-1 to CSE-5. Concentration of each reaction was adjusted to 1 n.

Fractionation of CSE-2

CSE-2 (370 mg) was further separated on a silica gel column (ϕ 3 \times 9 cm). Eluting fraction 2 with a 1:1 mixture of EtOAc and hexane yielded three fractions (CSE 2-1 to 2-3), eluting with EtOAc yielded three fractions (CSE 2-4 to 2-6), and eluting with methanol yielded one fraction (CSE 2-7). The summary of fractionation of *C. sappan* wood is shown in Figure 28.

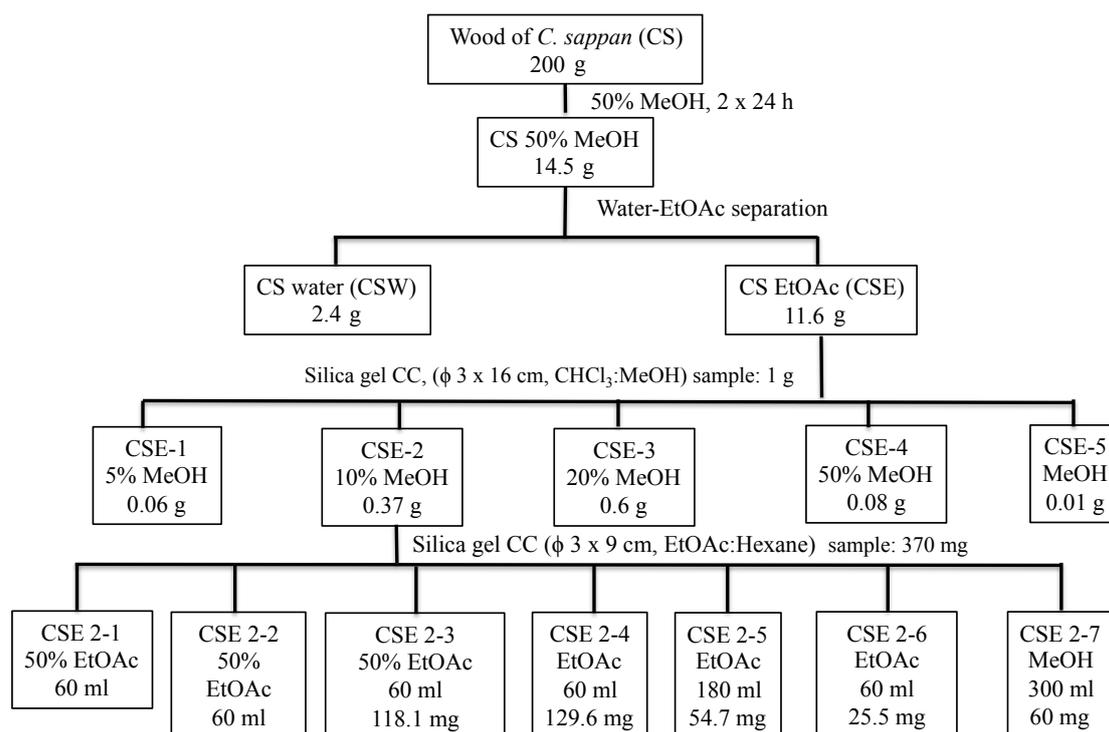


Figure 28. Summary of fractionation of *C. sappan* wood.

Seven collected fractions from CSE-2 were subjected to intestinal maltase inhibitory activity assay. From the results (Figure 29), CSE 2-4 (46% in 1 n concentration) showed the highest inhibitory activity among collected fractions. Therefore, CSE 2-4 was chosen for further fractionation using preparative HPLC to yield ten fractions (Figure 30).

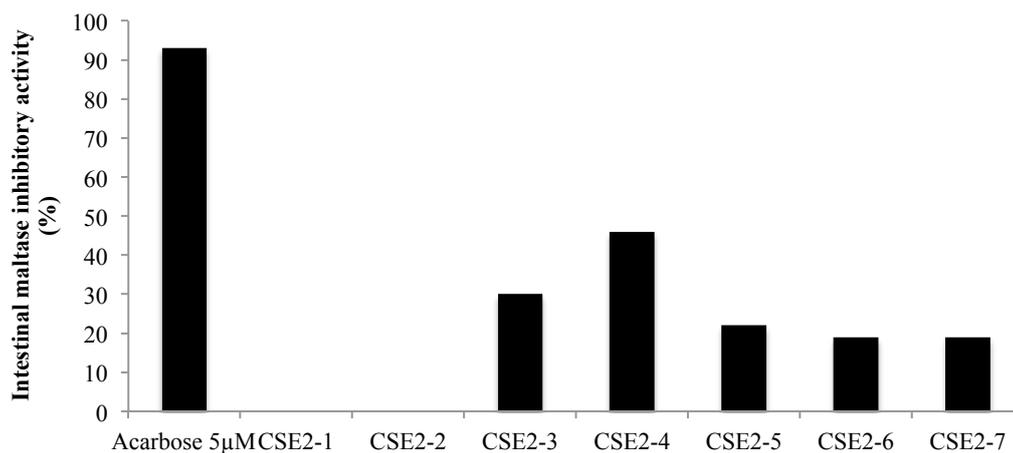


Figure 29. Intestinal maltase inhibitory activity of fractions obtained from silica gel column chromatography of CSE-2. Concentration of each fraction was adjusted to 1 n.

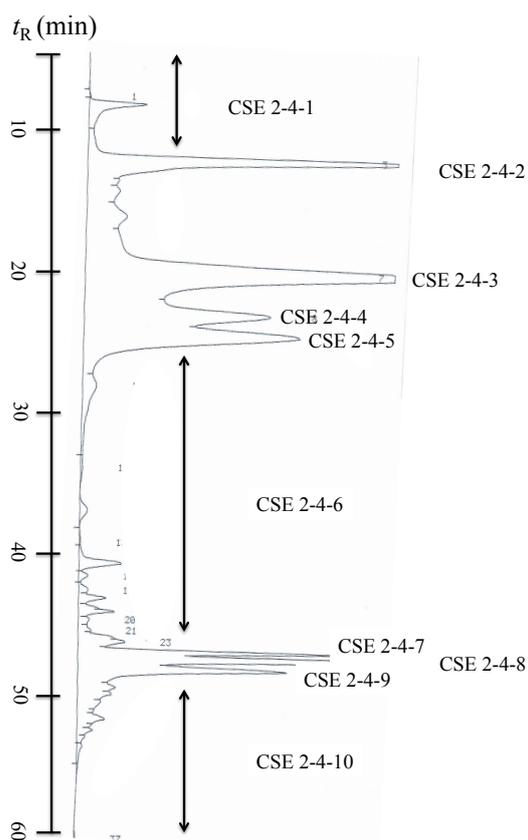


Figure 30. HPLC analysis profile of CSE 2-4. Column: Inertsustain C18 (ϕ 4.6 \times 250 mm); mobile phase: water-methanol (0-30 min: 30% methanol, 30-60 min: 30–100% methanol); flow rate: 1 mL/min; detection wavelength: 220 nm.

HPLC analysis of CSE-2 with water-methanol system yielded single peak of CSE 2-4-2 (t_R 12 min, 10 mg), CSE 2-4-3 (t_R 20 min, 25 mg), inseparable CSE 2-4-4 and 2-4-5 (t_R 21-26 min, 40 mg), CSE 2-4-7 (t_R 79 min, 4 mg), CSE 2-4-8 (t_R 47 min, 3 mg), CSE 2-4-9 (t_R 48 min, 7 mg), and were collected together non-peak fractions (CSE 2-4-1, -6 and 10). Except for non-peak fraction CSE 2-4-1, nine fractions of CSE 2-10 were subjected to intestinal maltase inhibitory activity assay in 10 n concentration (Figure 31). Fractions in single peak (CSE 2-4-2, 2-4-3, and 2-4-9) and inseparable peaks (CSE 2-4-4 and 2-4-5) showed moderate intestinal maltase inhibitory activity. The single peaks, CSE 2-4-2, 2-4-3 and 2-4-9, were further investigated for the structure determination.

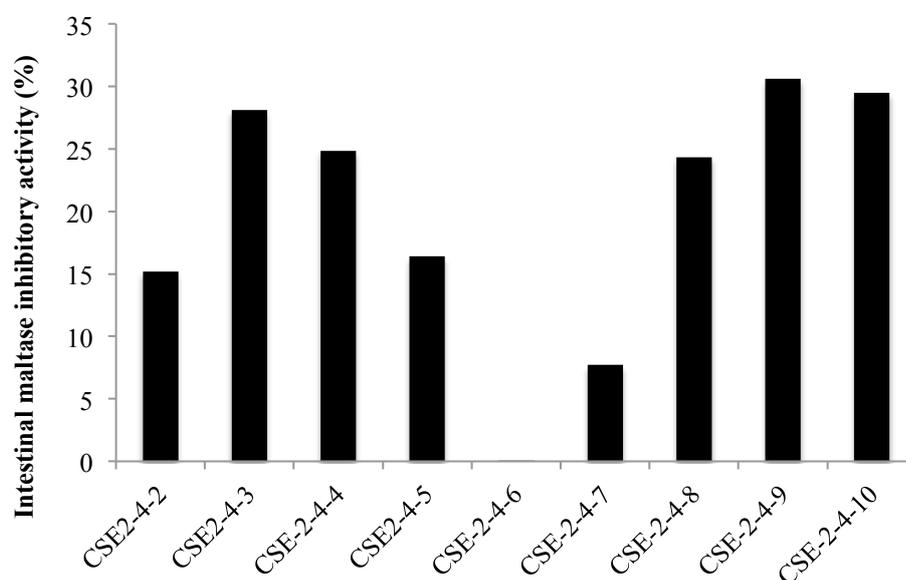


Figure 31. Intestinal maltase inhibitory activity of fractions obtained from preparative-ODS HPLC of CSE 2-4. Concentration of each fraction is adjusted in 10 n in the reaction mixture.

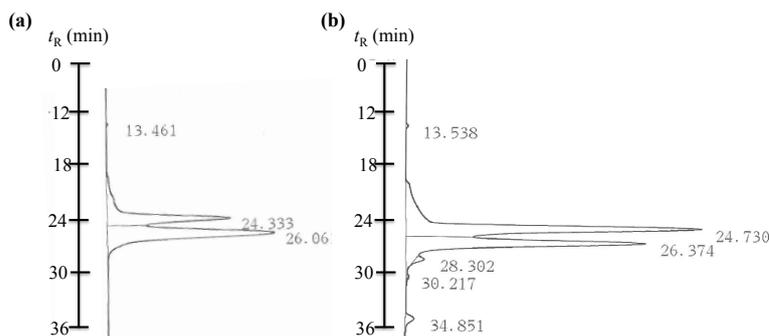


Figure 32. Preparative HPLC profile of CSE 2-4-4 (a) and 2-4-5 (b) after HPLC separation.

Column: Inertsustain C18 (ϕ 20 \times 250 mm); mobile phase water-methanol (0-30 min: 30% methanol, 30-60 min: 30-100% methanol); flow rate: 5 mL/min; detection wavelength: 220 nm.

Although CSE 2-4-4 and 2-4-5 were separately collected by HPLC, HPLC analysis of the collected peaks by the same condition gave a similar two-peak profile again (Figure 32). This result indicates 2-4-4 and 2-4-5 may be isomeric forms interchangeable in the HPLC condition. CSE 2-4-4 and 2-4-5 were thus investigated directly for the structure determination.

Fractionation of CSE-3

CSE-3 was fractionated using the same method as CSE-2 to give CSE 3-1 to 3-10 (Figure 33). Eluting CSE-3 with a 1:1 mixture of EtOAc and hexane yielded three fractions (CSE 3-1 to 3-3), eluting with EtOAc yielded six fractions (CSE 3-4 to 3-9), and eluting with methanol yielded one fraction (CSE 3-10). Ten collected fractions were further subjected to intestinal maltase inhibitory activity assay (Figure 34). The active fractions were found in CSE 3-3 (64%), 3-4 (59%), and 3-5 (41%) in 4 n concentration. Thus, these fractions were further investigated for their HPLC profile. Since CSE 3-3 showed complex peaks profile (data not shown), CSE 3-3 was

not chosen for further fractionation. CSE 3-4 and 3-5 showed similar single peak elution in t_R 9.83 min and t_R 9.42 min, respectively (Figure 35). These fractions might contain the same principle. Since CSE 3-4 and 3-5 showed relatively high intestinal maltase inhibitory activity, their active principle was subjected to the structure determination.

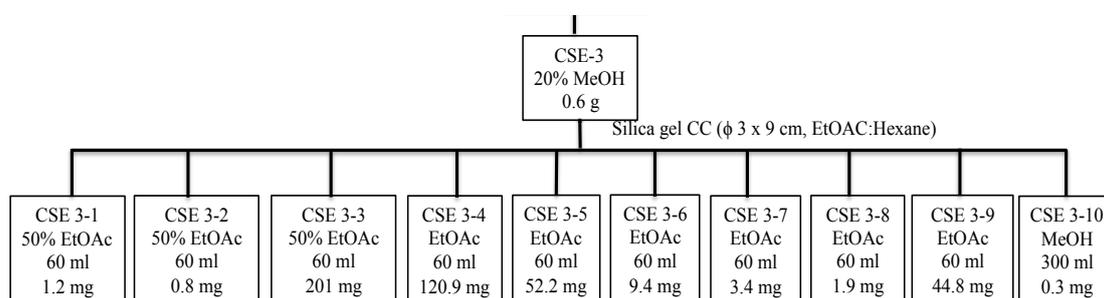


Figure 33. Scheme fractionation of CSE-3.

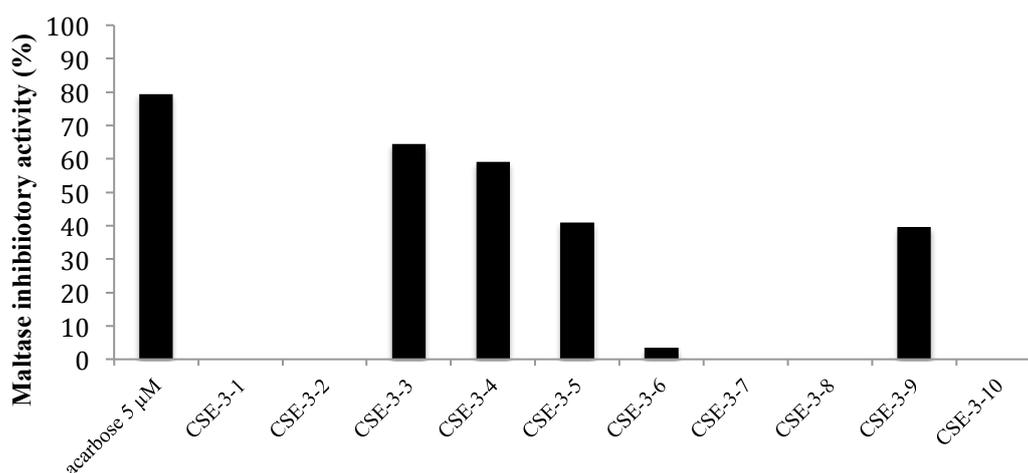


Figure 34. Intestinal maltase inhibitory activity of CSE 3-1 to 3-10. Concentration was adjusted at 4 n.

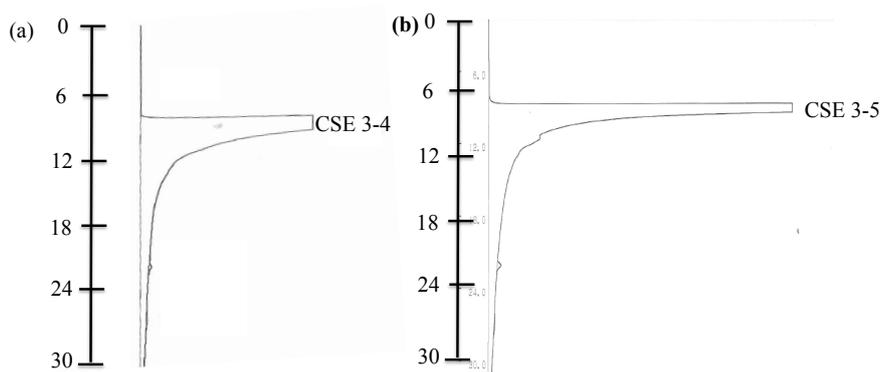


Figure 35. HPLC profile of CSE 3-4 (a) and CSE 3-5 (b).
 HPLC condition: column Inertsil ODS-3 (ϕ 4.6 \times 250 mm); mobile phase water-methanol (0-30 min: 30% methanol, 30-60 min: 30-100% methanol, 60-90 min: methanol); flow rate: 1 mL/min; detection wavelength: 220 nm.

4.3.2 Structure determination of isolates from *C. sappan* wood

4.3.2.1 CSE 2-4-2 (*brazilin*)

CSE 2-4-2 was obtained as a dark brown crystal. FD mass and ^1H NMR spectra of CSE 2-4-2 are shown in Figures 36 and 37, respectively, and the physicochemical properties are shown in Table 9. The FD mass spectrum showed the molecular ion at m/z 286 $[\text{M}]^+$. In the ^1H NMR spectrum, signals for the region of the five hydrogens were found in aliphatic region at δ 2.5–4.0 ppm, δ 2.76 and 2.92 (H-7), 3.69 and 3.92 (H-6), and 3.96 (H-12). Aromatic proton signals were found in δ 6.29 (H-4), 6.47 (H-2), 6.60 (H-11), 6.71 (H-8), and 7.18 (H-1). Compound **1** was identified as brazilin by comparison of these data with those in the literature (Batubara et al., 2010).

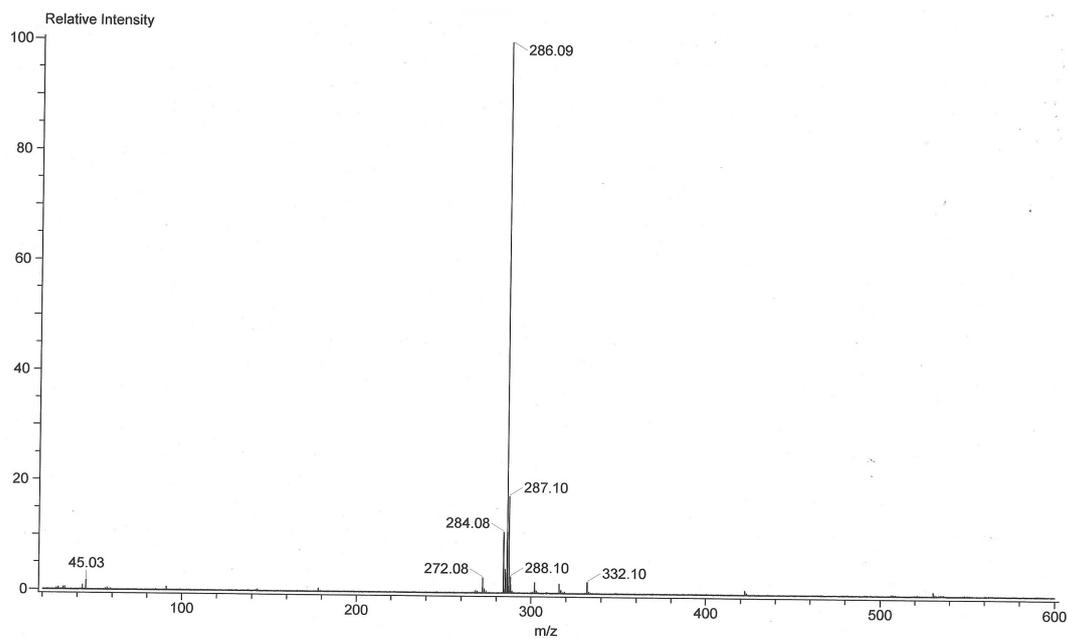


Figure 36. FD mass spectrum of CSE 2-4-2.

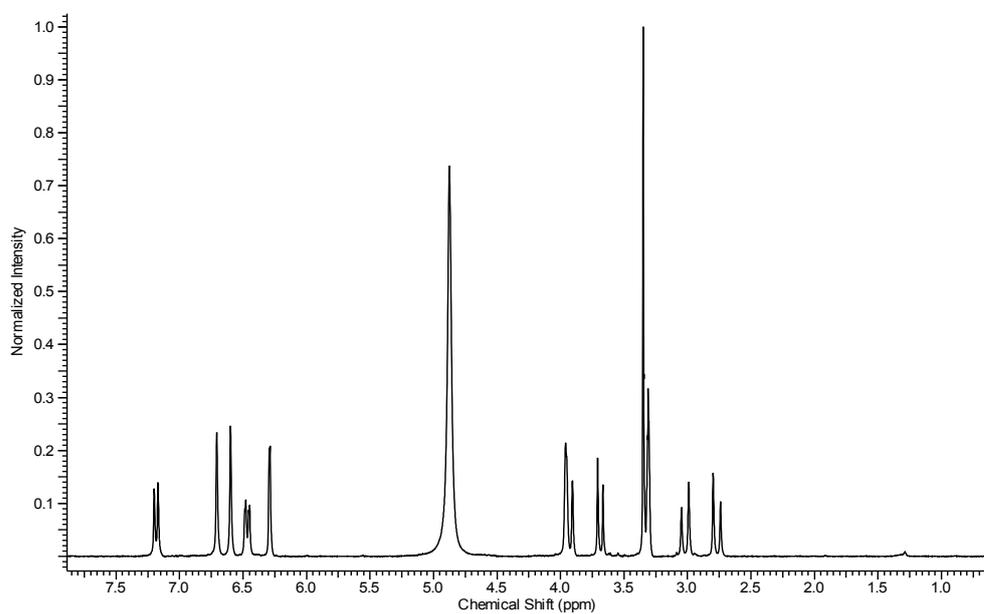
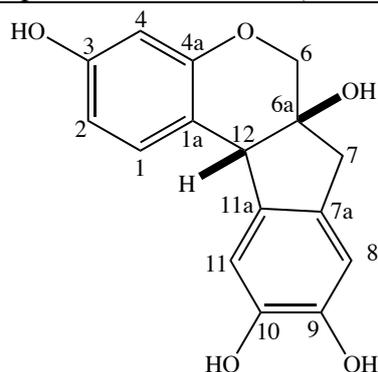


Figure 37. ¹H NMR spectrum of CSE 2-4-2 (500 MHz, methanol-*d*₄).

Table 9. Physicochemical properties of CSE 2-4-2 (brazilin).



Appearance: dark brown crystal

FD-MS m/z : 286.09 $[H]^+$

1H -NMR (500 MHz, methanol- d_4), δ (ppm), J (in Hz)

CSE 2-4-2	Reference*
2.76 (1H, d, $J = 15.8$, H-7)	2.73
2.92 (1H, d, $J = 15.8$, H-7)	2.97
3.69 (1H, d, $J = 11.3$, H-6)	3.67
3.92 (1H, d, $J = 11.3$, H-6)	3.89
3.96 (1H, s, H-12)	3.93
6.29 (1H, d, $J = 2.5$, H-4)	6.26
6.47 (1H, dd, $J = 8.3, 2.5$, H-2)	6.44
6.60 (1H, s, H-11)	6.58
6.71 (1H, s, H-8)	6.68
7.18 (1H, d, $J = 8.3$, H-1)	7.15

*Batubara et al. (2009).

4.3.2.2 CSE 2-4-3, 2-4-4, and 2-4-5 (protosappanin C)

Although the analytic HPLC profile CSE 2-4-3 exhibited a single peak (data not shown), CSE 2-4-4/2-4-5 existed as interchangeable peaks (Figure 32). Based on this fact, CSE 2-4-3, 2-4-4 and 2-4-5 are presumed as to be easily equilibrated conformational isomers and/or chemical derivatives. The 1H NMR spectral measurements of CSE 2-4-3, 2-4-4, and 2-4-5 in room temperature showed the similar broadened peak profile (Figure 38). The 1H NMR spectra of CSE 2-4-3, 2-4-4, and 2-4-5 were then measured at 90 °C in DMSO- d_6 after complete removal of alcoholic

solvents (Figure 39). The high temperature measurements gave similar sharp peak profile for CSE 2-4-3, 2-4-4 and 2-4-5. Hence structure determination was carried out using analysis data of CSE 2-4-3. The relationship of CSE 2-4-3, 2-4-4 and 2-4-5 will be discussed in Section 4.4.

The FD-mass, ^1H NMR and ^{13}C NMR spectra of CSE 2-4-3 are shown in Figure 40–42, respectively, the physicochemical properties are shown in Table 10. The FD-mass spectrum showed M^+ at m/z 302.12. A carbonyl resonating at δ_{C} 202.7 and one proton singlet at δ_{H} 9.67 ppm indicated the presence of aldehyde group in CSE 2-4-3. These data and other physicochemical properties of CSE 2-4-3 were identical with those of protosappanin C isolated from *C. sappan* wood (Nagai and Nagumo, 1987).

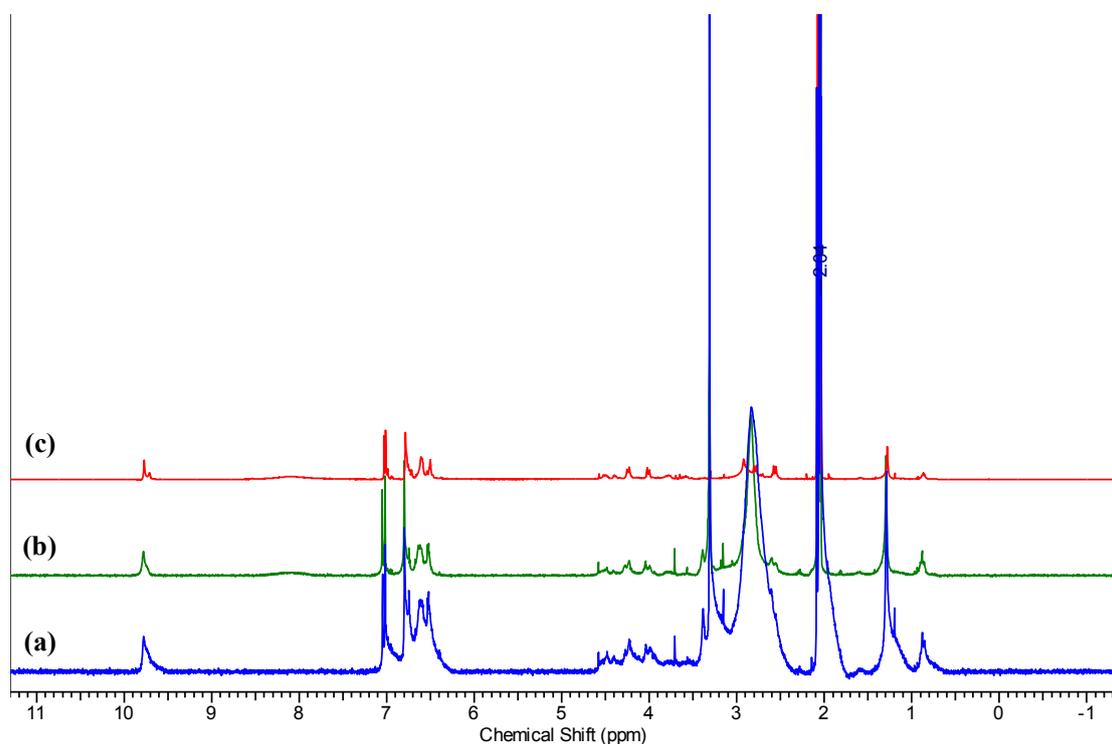


Figure 38. ^1H NMR (270 MHz, methanol- d_4) profile of CSE 2-4-3 (a), 2-4-4 (b), 2-4-5 (c) at room temperature.

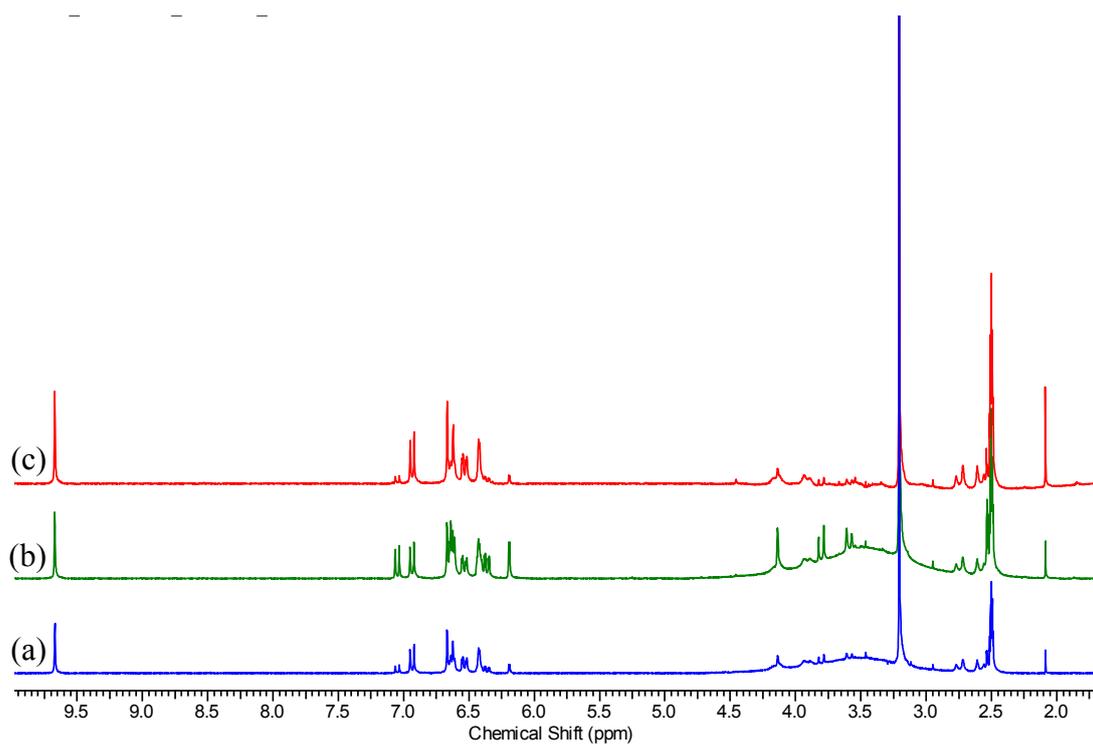


Figure 39. ¹H NMR (270 MHz, DMSO-*d*₆, 90 °C) spectra of CSE 2-4-3 (a), 2-4-4 (b), and 2-4-5 (c).

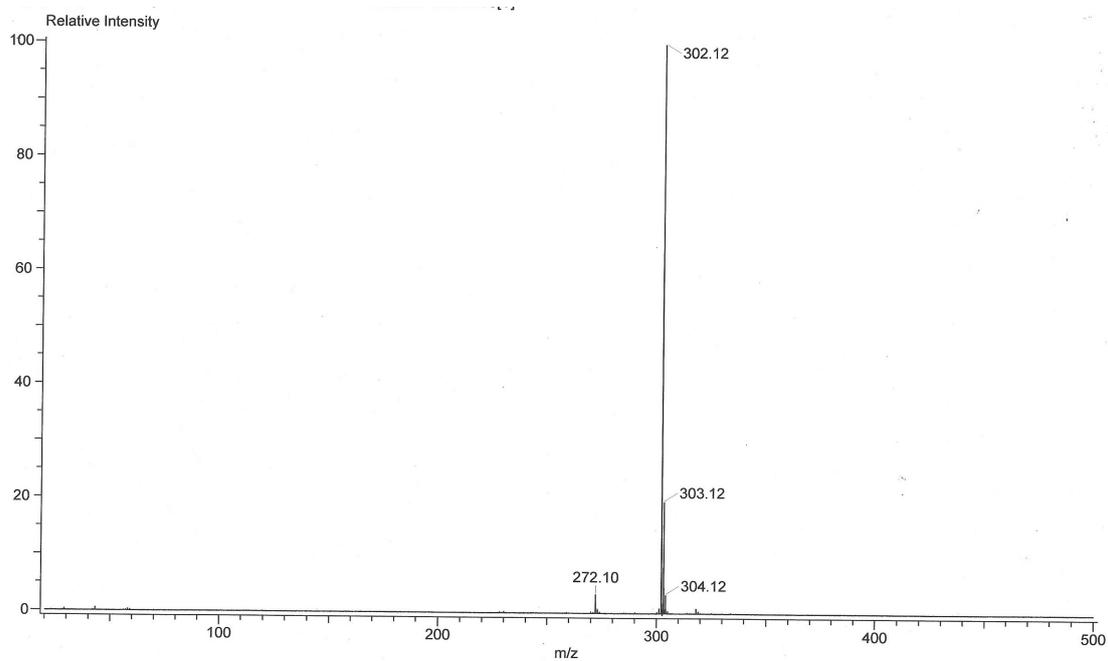


Figure 40. FD-mass spectrum of CSE 2-4-3.

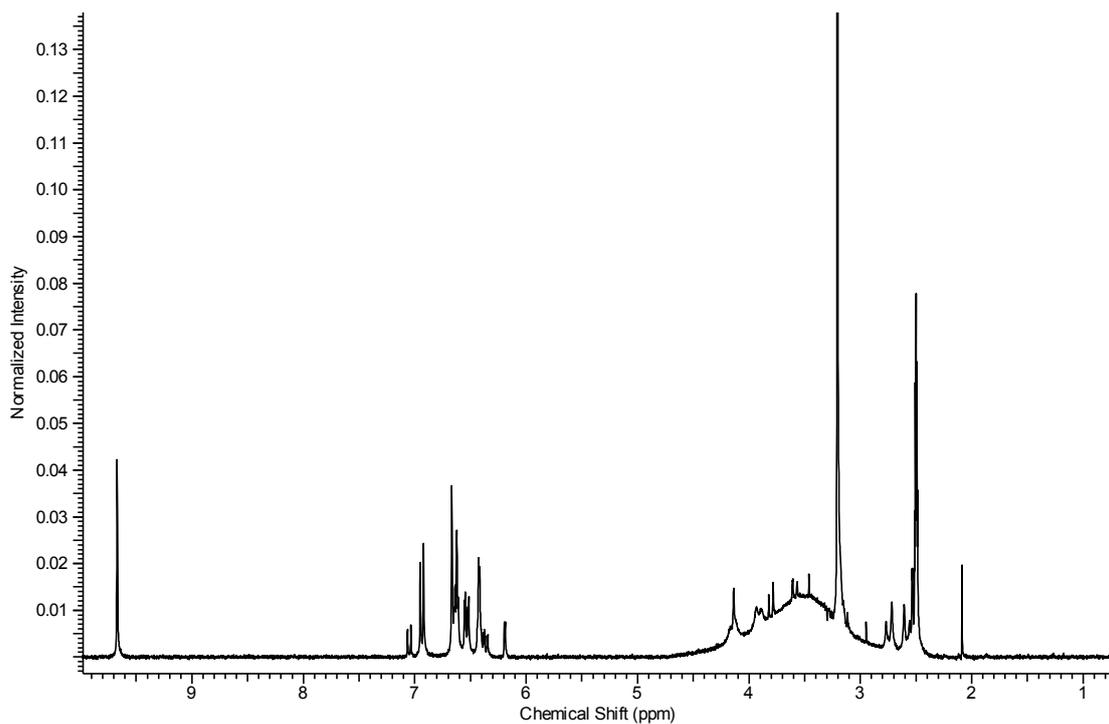


Figure 41. ^1H NMR spectrum of CSE 2-4-3 (270 MHz, $\text{DMSO-}d_6$, 90 °C).

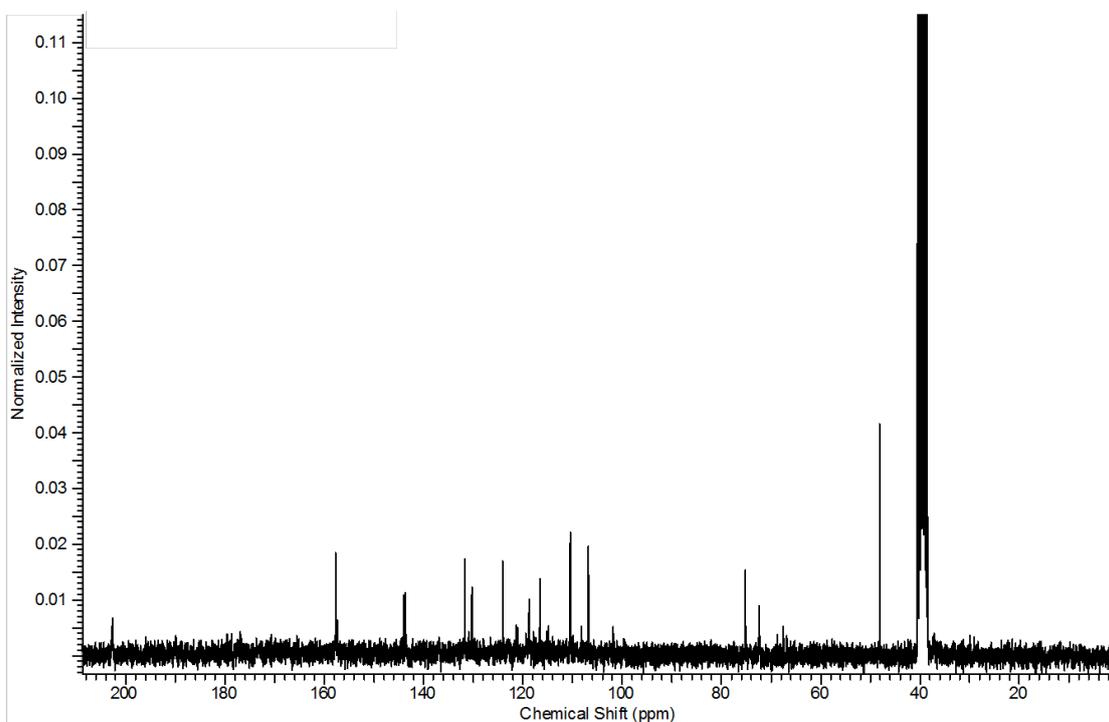
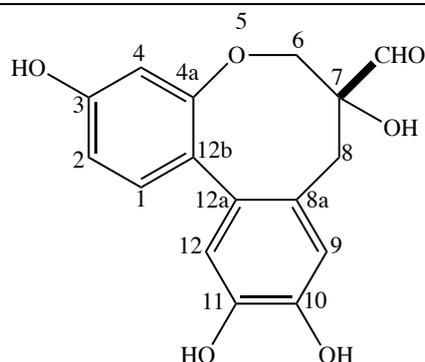


Figure 42. ^{13}C NMR spectrum of CSE 2-4-3 ($\text{DMSO-}d_6$, 90 °C).

Table 10. Physicochemical properties of CSE 2-4-3 (protosappanin C).



Appearance: red brown oil

FD-MS m/z : 303.12 [M]⁺

¹H NMR (270 MHz, DMSO-*d*₆, 90°C) δ (ppm, *J* in Hz)

CSE 2-4-3	Reference*
3.18 (2H, s, H-8)	3.18
3.46–4.14 (2H, m, H-6)	3.45 – 4.5
6.94 (1H, d, <i>J</i> =8.3, H-1)	6.94
9.67 (1H, s, -CHO)	9.67

¹³C NMR (67.5 MHz, DMSO-*d*₆, 90 °C) δ (ppm)

CSE 2-4-3	Reference*
39.8 (C-8)	39.4
72.4 (C-6)	72.4
75.2 (C-7)	75.1
106.8 (C-4)	106.7
110.5 (C-2)	110.4
116.6 (C-12)	116.4
118.8 (C-9)	118.6
121.3 (C-8a)	121.2
124.0 (C-12b)	123.9
130.3 (C-12a)	130.2
131.7 (C-1)	131.5
143.4 (C-10)	143.4
144.0 (C-11)	143.7
157.4 (C-3)	157.2
157.7 (C-4a)	157.5
202.7 (-CHO)	202.3

*Nagai and Nagumo (1987).

4.3.2.3 CSE 2-4-9 (*sappanchalcone*)

CSE 2-4-9 was isolated as a yellow oil. ESI mass and ^1H NMR spectra of CSE 2-4-9 are shown in Figures 43 and 44, respectively, and the physicochemical properties are shown in Table 11. Based on the pseudomolecular ions at m/z 285 $[\text{M}-\text{H}]^-$ (negative mode) and m/z 309 $[\text{M}-\text{Na}]^+$ (positive mode), molecular weight was determined as 286. The ^1H NMR spectrum of CSE 2-4-9 exhibited an aromatic methoxy singlet at δ 3.90, characteristic two 1,2,4-trisubstituted benzene proton signals at δ 6.50, 6.56, and 7.58 ppm and at δ 6.83, 7.02, and 7.18 ppm. In addition, a pair of olefinic methines with *trans*-coupling each other at δ 7.37 and 7.49 (each d, $J = 15.8$ Hz) were observed. These data and other physicochemical properties of CSE 2-4-9 were identical with those of sappanchalcone isolated from *C. sappan* wood (Baek et al., 2000).

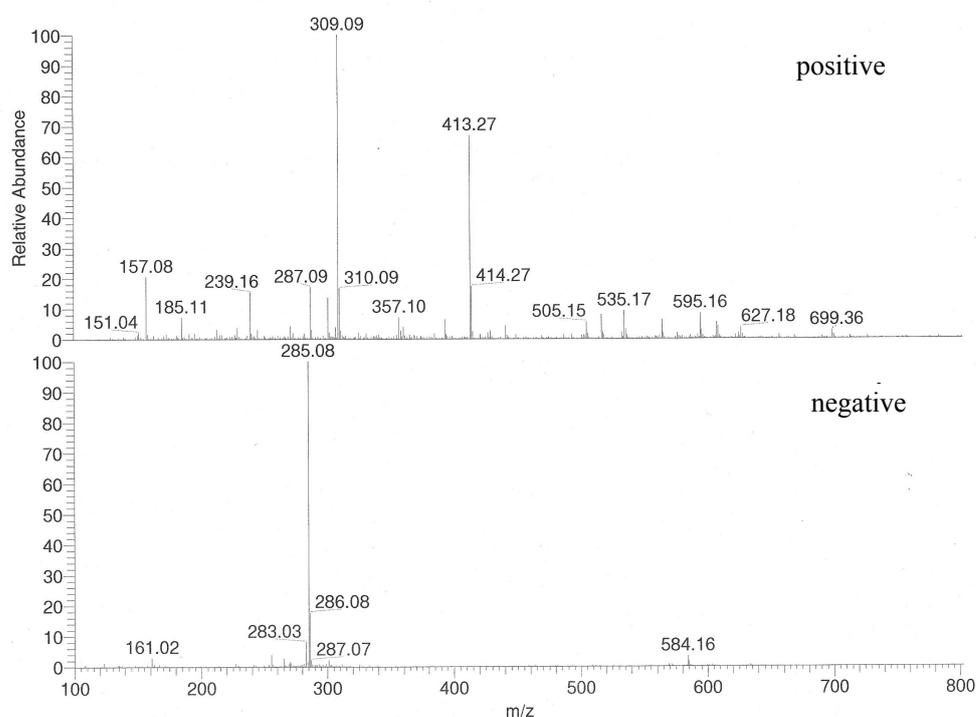


Figure 43. ESI mass spectrum of CSE 2-4-9.

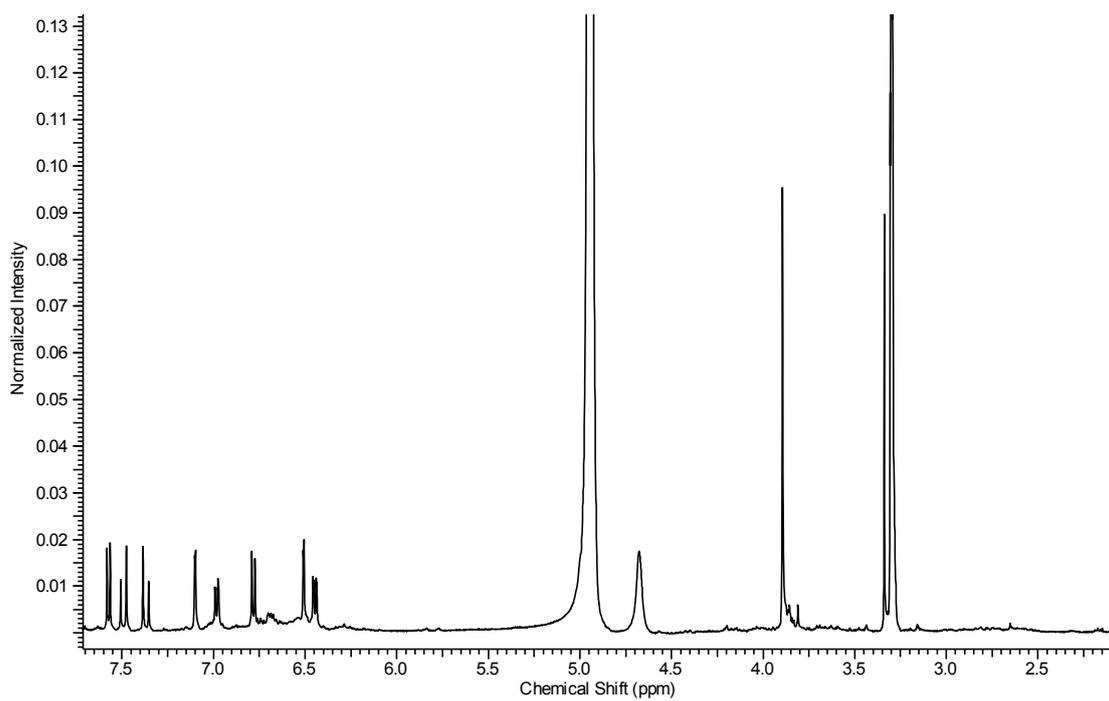
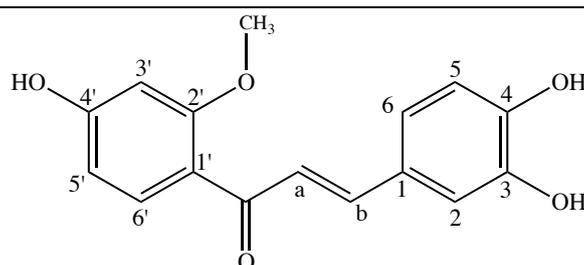


Figure 44. ^1H NMR spectrum of CSE 2-4-9 (500 MHz, methanol- d_4)

Table 11. Physicochemical properties of CSE 2-4-9 (sappanchalcone).



Appearance: yellow crystal

ESI-MS (negative) m/z : 285.08 [M-H]⁻

¹H NMR (500 MHz, methanol-*d*₄) δ (ppm), J (in Hz)

CSE 2-4-9	Reference*
3.90 (2'-OMe)	3.77
6.50 (1H, dd, $J = 2.2$ Hz, 8.5 Hz, H-5')	6.35
6.56 (1H, d, $J = 2.2$ Hz, H-3')	6.40
6.83 (1H, d, $J = 8.2$ Hz, H-5)	6.69
7.02 (1H, dd, $J = 2.2, 8.1$, H-6)	6.88
7.18 (1H, d, $J = 2.2$, H-2)	7.01
7.37 (1H, d, $J = 15.8$, H- α)	7.25
7.49 (1H, d, $J = 15.8$, H- β)	7.39
7.58 (1H, d, $J = 8.5$, H-6')	7.47

* Baek et al. (2000).

4.3.2.4 CSE 3-4 and 3-5 (protosappanin B)

Based on the HPLC profile (Figure 35), CSE 3-4 and 3-5 were considered as the same compound. The careful inspection of ¹H NMR and ¹³C NMR spectra, CSE 3-4/3-5 would be a mixture of two inseparable mixtures similar to CSE 2-4-3 (Section 4.3.2.2). CSE 3-4/3-5 were obtained as a yellow oil. FD-mass, ¹H NMR and ¹³C NMR spectra are shown in Figures 45–47, respectively, and the physicochemical properties are shown in Table 12. CSE 3-4 showed M⁺ at m/z 304 in the FD-mass spectrum. The ¹H NMR spectrum showed 1,2,4-trisubstituted benzene signals at δ 6.50–6.58 (2H, d, $J = 2.5$ Hz), 6.60–6.65 (2H, dd, $J = 8.0, 2.5$ Hz), and 7.02 ppm (2H, d, $J = 8.0$) and two singlets of 1,2,4,5-tetrasubstituted benzene at δ 6.73 – 6.87 ppm. The ¹³C NMR spectrum of CSE 3-4 showed a quaternary carbon bearing oxygen (δ 72.8/72.3), a

methylene (δ 42.7/40.0 ppm) and two oxygenated methylenes (δ 65.8/68.2 and 77.3/76.1 ppm). These data and other physicochemical properties of CSE 3-4 were identical with those of protosappanin B isolated from *C. sappan* wood (Namikoshi, Nakata, Nuno, Ozawa & Saitoh, 1987 and Fu et al., 2008).

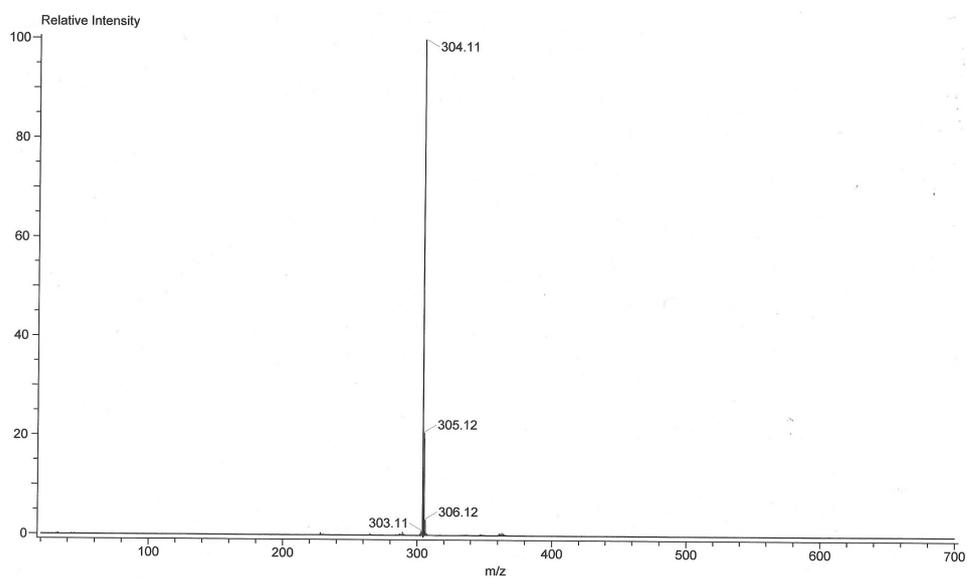


Figure 45. FD-MS of CSE 3-4.

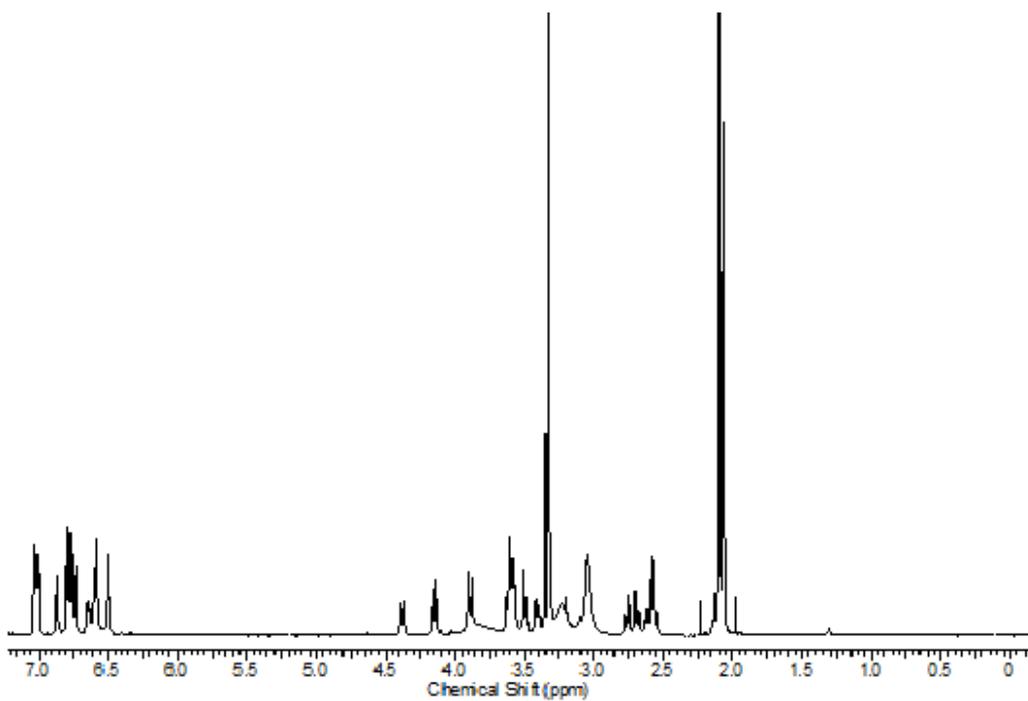


Figure 46. ^1H NMR spectrum of CSE 3-4 (500 MHz, acetone- d_6).

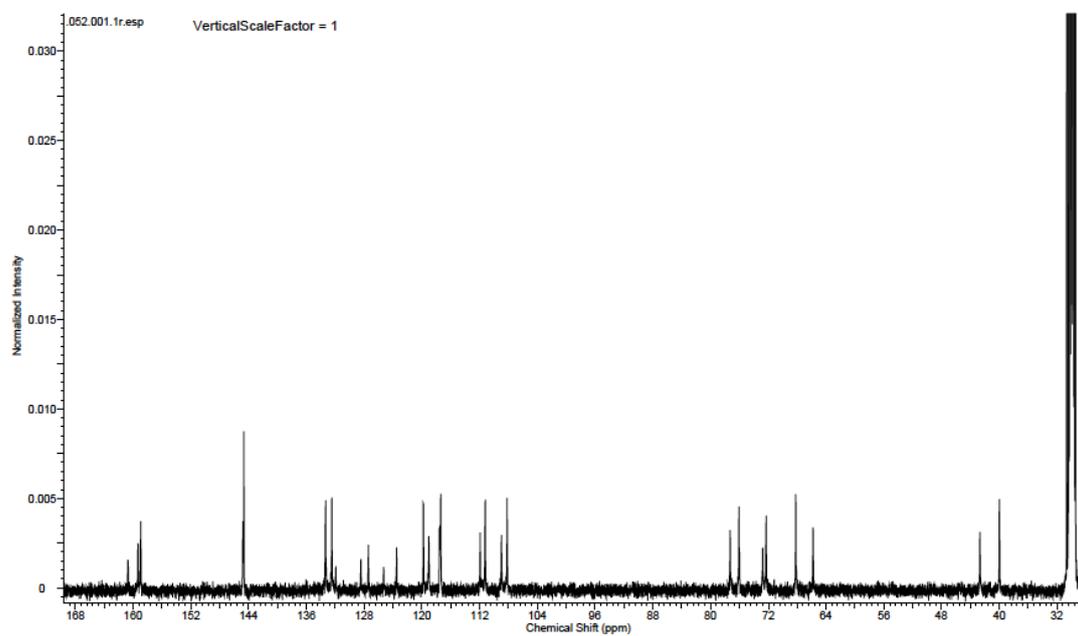
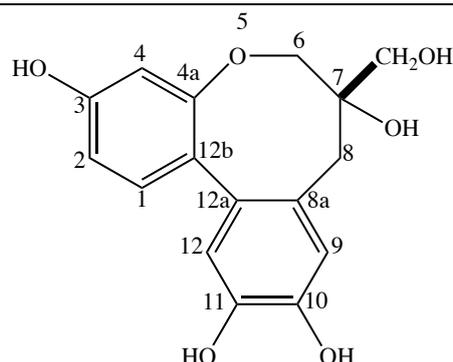


Figure 47. ^{13}C NMR spectrum of CSE 3-4 (acetone- d_6).

Table 12. Physicochemical properties of CSE 3-4 (protosappanin B).



Appearance: Yellow oil

FD-MS m/z : 304 $[M]^+$

^1H NMR (500 MHz, acetone- d_6) δ (ppm. J in Hz)

CSE 3-4	Reference*
2.58–2.78 (4H, each H J = 13 Hz, H-8)	2.54 – 2.74
3.20–4.38 (2H, each H J = 11.19 Hz, 7-CH ₂ OH)	3.16 – 4.35
3.20–4.38 (4H, each J = 12 Hz, H-6)	3.16 – 4.35
6.50–6.58 (2H, d, J = 2.5 Hz, H-4)	6.48 – 6.56
6.60–6.65 (2H, dd, J = 8.0, 2.5 Hz, H-2)	6.56 – 6.62
6.73–6.87 (4H, s, H-9 & H-12)	6.71 – 6.84
7.02 (2H, d, J = 8.0, H-1)	7.00

^{13}C NMR (125 MHz, acetone- d_6) δ ppm

CSE 3-4	Reference**
42.7/40.0 (C-8)	43.6/40.9
65.8/68.2 (7-CH ₂ OH)	66.7/69.1
72.8/72.3 (C-7)	73.7/73.2
77.3/76.1 (C-6)	78.2/76.9
109.0/108.2 (C-4)	109.9/109.1
111.9/111.2 (C-2)	112.8/112.1
117.4/117.5 (C-9)	118.4/118.3
119.0/119.8 (C-12)	119.9/120.7
125.3/123.5 (C-8a)	125.9/124.3
128.5/127.4 (C-12b)	129.4/128.3
131.9 (C-12a)	131.1/132.6
134.3/132.5 (C-1)	134.2/133.3
144.7/144.7 (C-11)	145.5/145.5
144.8 (C-10)	145.5/145.7
158.9/159.3 (C-3)	159.8/159.8
160.68 (C-4a)	159.8/160.2

* Namikoshi et al. (1987); ** Fu et al. (2008).

4.3.3 α -glucosidase inhibitory activity of isolated compounds

Four isolated compounds from *C. sappan* wood were evaluated for intestinal maltase inhibitory activity (Figure 49). The assay was carried under a series of concentration from 0.2 mM to 5 mM for brazilin (**6**), protosappanin B (**8**) and C (**9**) and to 2 mM for sappanchalcone (**7**). In 1 mM concentration, compound **8** (58%) showed the highest maltase inhibitory activity, followed by **7** (42%), **9** (37%), and **6** (18%).

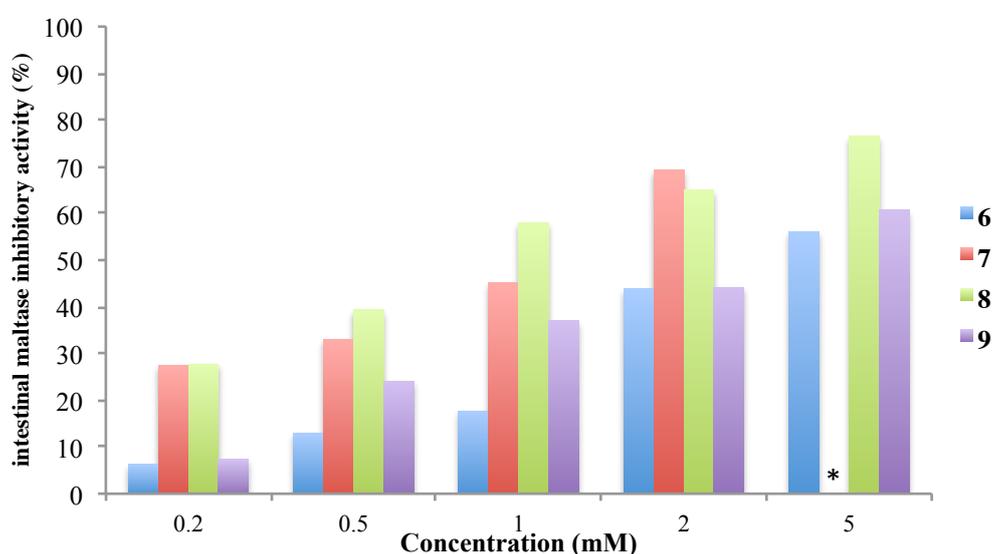


Figure 49. Intestinal maltase inhibitory activity of brazilin (**6**), sappanchalcone (**7**), protosappanin B (**8**) and C (**9**) in designated concentration. *not tested

Four isolated compounds (**6–9**) were further evaluated for their intestinal sucrase inhibitory activity (Figure 50). Except for brazilin (78% inhibition), three other compounds did not show significant inhibitory activity in 2 mM.

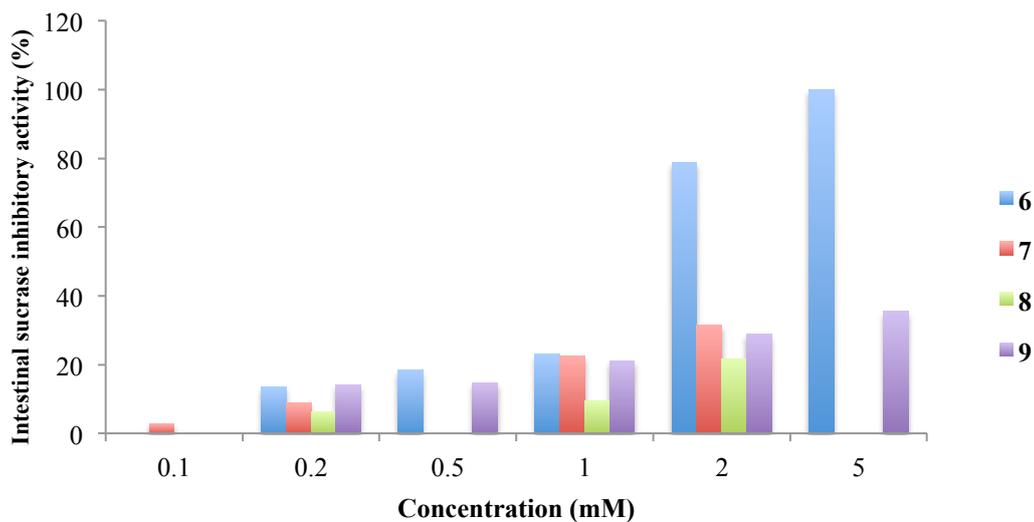


Figure 50. Intestinal sucrase inhibitory activity of brazilin (6), sappanchalcone (7), protosappanin B (8) and C (9) in designated concentration.

4.3.4 PPA inhibitory activity of isolated compounds

Four isolated compounds were evaluated for their PPA inhibitory activity (Figure 51). Brazilin (6) was not tested due to strong color of the solution. Only compound 7 showed weak inhibitory activity against PPA dose dependently. Compound 7 showed 45% inhibition at 2 mM concentration. Compound 8 and 9 showed no significant inhibition at tested concentration (below 20%).

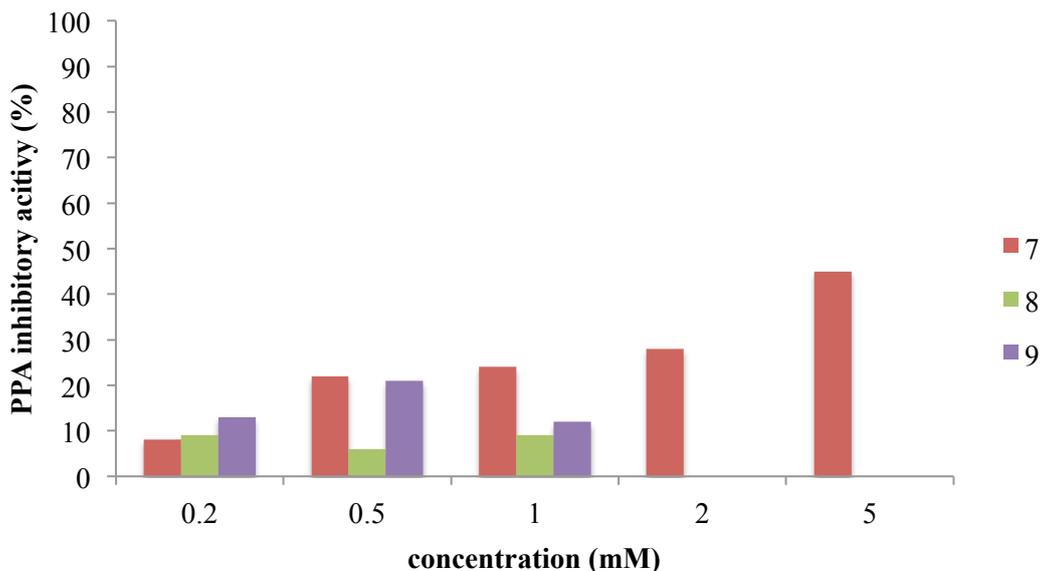


Figure 51. PPA inhibitory activity of sappanchalcone (**7**), protosappanin B (**8**) and C (**9**) in designated concentration.

4.3.5 Summary of inhibitory activity of isolated compounds against carbohydrate digestive enzymes

The inhibitory activities of the isolates are presented as IC_{50} values as a measure of their effectiveness at inhibiting carbohydrate digestive enzymes, intestinal maltase, sucrase and pancreatic α -amylase. The intestinal maltase inhibitory activity was in the order of compound **8** (IC_{50} = 0.81 mM), **7** (0.96 mM), **9** (2.59 mM), and **6** (3.83 mM) (Table 15). Compounds **8** and **7** showed higher maltase inhibitory activity than compounds **9** and **6**.

The isolated compounds were also tested for their inhibition of rat intestinal sucrase (Table 15). Although **6** showed moderate inhibition of intestinal sucrase (IC_{50} = 1.12 mM) compounds **7**, **8**, and **9** did not significantly inhibit sucrase at the tested concentrations. In contrast, **7** showed weak inhibitory activity against PPA, whereas **6**, **8**, and **9** showed no inhibition.

Table 13. Summary of yield and IC₅₀ values of *C. sappan* isolates.

Compounds	IC ₅₀ value (mM)			Yield* (mg)
	α-glucosidase		α-amylase	
	maltase	sucrase		
Brazilin (6)	3.83	1.12	>5	60
Sappanchalcone (7)	0.96	>5	>5	20
Protosappanin B (8)	0.81	>5	>5	1000
Protosappanin C (9)	2.59	>5	>5	240
Acarbose	5×10^{-4}	1.4×10^{-2}	5×10^{-3}	

*From 200 g *C. sappan* wood.

4.3.6 Glucose uptake activity of isolated compounds

Four isolated compounds were tested for their glucose uptake activity on skeletal muscle L6 cell line. Although compound **6** and **7** showed significant glucose uptake activity compared to control at 50 μM (Figure 52), compound **8** and **9** did not show the activity above 100 μM (data not shown).

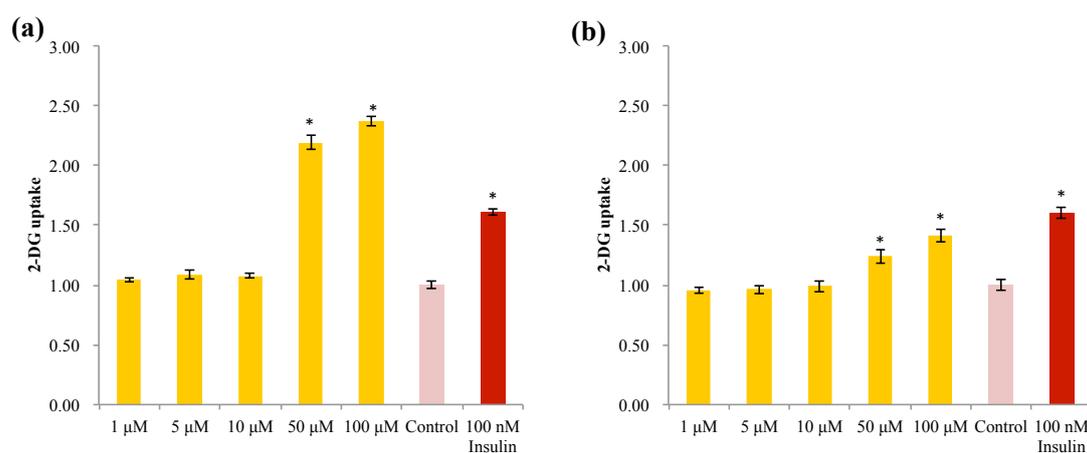


Figure 52. Glucose uptake activity of brazilin (**6**, a) and sappanchalcone (**7**, b) in skeletal muscle cells. (* $P < 0.01$)

4.4 Discussion

4.4.1 Intestinal maltase inhibitors of *C. sappan* wood

In this study, the constituents in *C. sappan* wood that are able to inhibit intestinal maltase were isolated and identified using enzyme assays and chromatographic techniques. The inhibitory activity was found in the ethyl acetate soluble fraction of the initial 50% methanol extract. Fractionation and purification was done using silica gel chromatography and preparative HPLC to yield four active compounds: brazilin (**6**), sappanchalcone (**7**), protosappanin B (**8**), and protosappanin C (**9**). The structures of the isolated compounds are shown in Figure 53. Brazilin (**6**) is a well-known homoisoflavone constituent of *C. sappan* (Namikoshi et al., 1987), and sappanchalcone (**7**) is considered to be its biogenetic precursor (Nagai et al., 1984). Protosappanins B (**8**) (Nagai and Nagumo, 1986) and C (**9**) (Nagai and Nagumo, 1987) have also been identified.

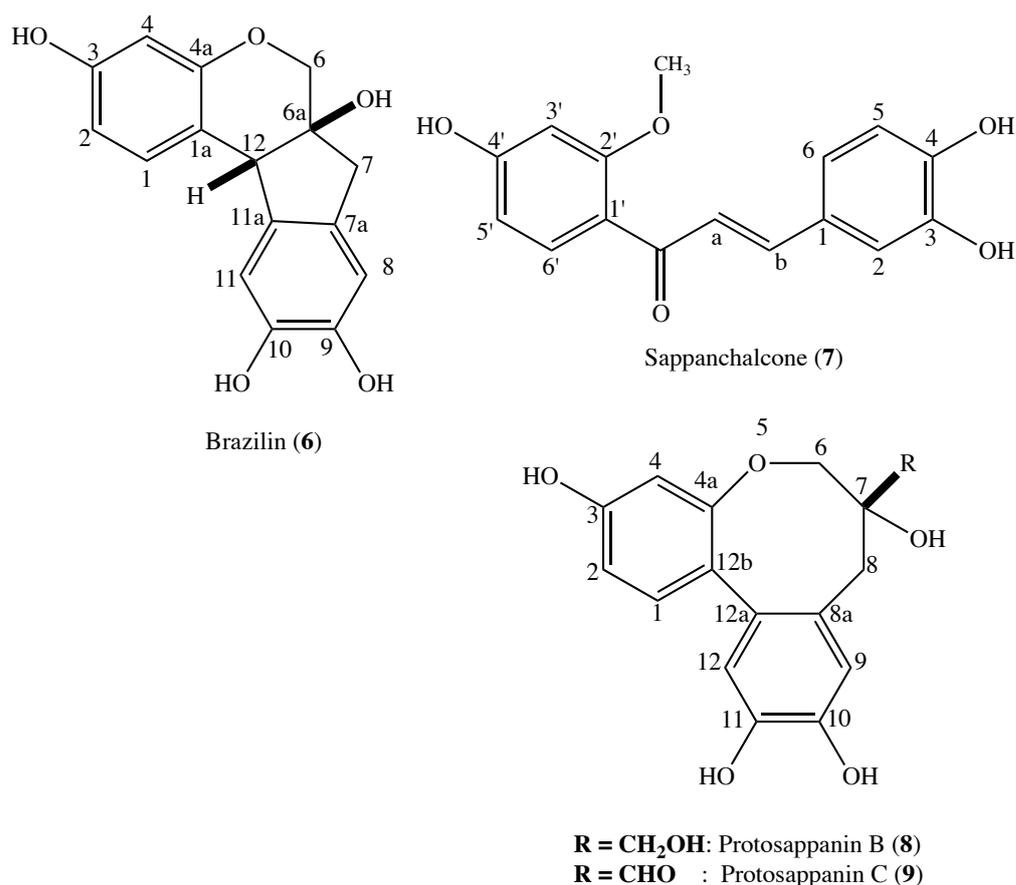


Figure 53. Intestinal maltase inhibitors of *C. sappan* wood.

Protosappanins B and C contain a dibenz[*b,d*]oxocin ring. Protosappanin B is known to exist as an equilibrium mixture of conformers (Nagai and Nagumo, 1986), although Fu et al. (2008) presumably misassigned them as epimers. Nagai and Nagumo (1986) reported that protons at C-1 and C-12 in protosappanin B, two *ortho* positions of their biphenyl system, are not bulky enough to restrict rotation around the C-C single bond between the two benzene rings, and thus the biphenyl system could more or less exist as an equilibrated mixture of rotamers. This is well consistent with the ^1H NMR data of CSE 3-4 and 3-5 (Section 4.3.2.4). In addition, protosappanin C has an aldehyde group on the C-7 position in place of the hydroxymethyl group in protosappanin B. Nagai and Nagumo (1987) reported protosappanin C would exist as

a mixture of aldehyde, hydrate and hemiacetal form in alcoholic solvent. It is well known that an aldehyde and hydrate or hemiacetal are easily convertible to each other in the presence of water or alcohol. The complex inseparable nature of CSE 2-4-3, 2-4-4 and 2-4-5 was thus arising from the equilibrated mixtures shown in Figure 55.

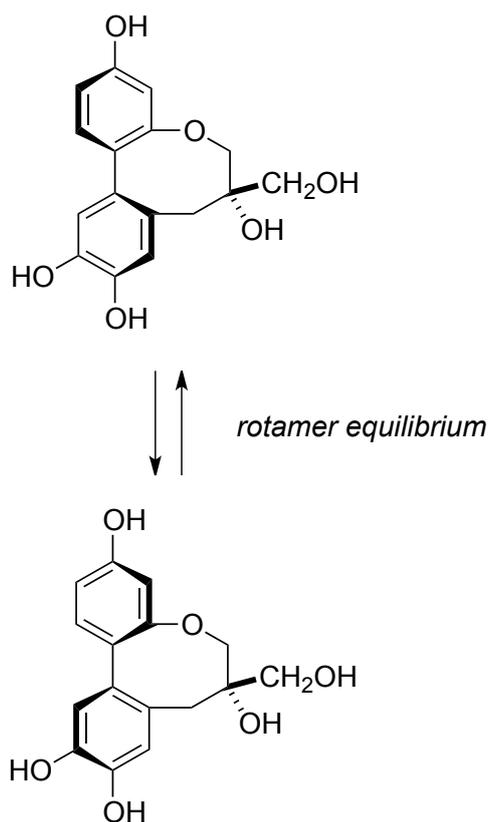


Figure 54. Conformation of protosappanin B.

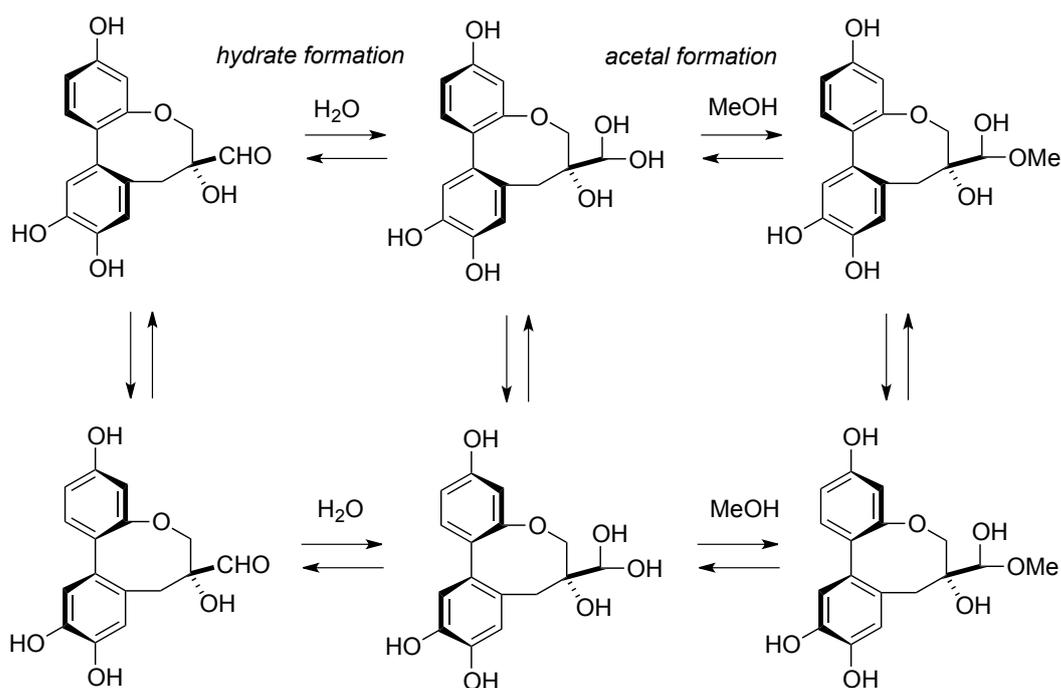


Figure 55. Equilibration of protosappanin C (refer to Nagai and Nagumo, 1987).

4.4.2 Carbohydrate digestive enzymes inhibition of *C. sappan* isolates

In this study, the antidiabetic potential of the widely used traditional medicine, *C. sappan* wood was investigated, since this valuable herb has not previously been investigated for antidiabetic activity relating to inhibition of carbohydrate digestive enzymes, in particular, intestinal maltase. Additionally, to clarify the wider applicability of the isolated compounds to other glucosidases, their inhibitory activity against intestinal sucrase and α -amylase were investigated.

Compounds **7** and **8** showed relatively higher maltase inhibitory activity than **6** and **9**. In contrast, **7** and **8** did not show significant inhibitory activity against intestinal sucrase and pancreatic α -amylase (Table 15). This indicates that **7** and **8** are more likely to have a higher affinity to MGAM than SI complexes. This is the first report of **7** and **8** as intestinal maltase inhibitors obtained from *C. sappan* wood, although many homoisoflavonoid-related biological activities have been identified in this plant (Lin et al., 2014). Therefore, the use of sappanchalcone (**7**) and

protosappanin B (**8**) as intestinal maltase inhibitors could be investigated further in the future.

Compound **9** has the same dibenz[*b,d*]oxocin structure as **8** and yet did not show significant inhibitory activity below a concentration of 5 mM. The presence of a hydroxymethyl group on the C-7 position of **8** might result in a higher maltase inhibitory activity than the aldehyde group in **9**.

Though **6** showed the lowest inhibitory activity against intestinal maltase, it showed moderate inhibition against intestinal sucrose. The results were indicative of the fact that the structure of **6** does not specifically have a strong affinity for carbohydrate digestive enzymes.

4.4.3 Glucose uptake enhancement of *C. sappan* isolates

Four isolated compounds were evaluated for their enhancement of glucose utilization ability through glucose uptake assay using skeletal muscle cells. From the results, compounds **6** and **7** showed significant glucose uptake enhancement, whereas compounds **8** and **9** did not show any activity. Compound **6** has been reported for the hypoglycemic effects through peripheral glucose utilization in isolated soleus muscles from streptozotocin-induced diabetic rats and stimulation of the glucose transport in 3T3-L1 cells. The result of this study concurred with those reported by Moon et al (1990). This is the first finding for compound **7** as a glucose uptake enhancer in skeletal muscle cells. This indicates that homoisoflavone structure in compound **6** and the chalcone structure in **7** are likely preferable in stimulating glucose uptake rather than dibenz[*b,d*]oxocin skeleton contained in compounds **8** and **9**. Therefore the use of **6** as well as **7** as alternative hypoglycemic agent from *C. sappan* wood could be investigated further in future.

4.5 Conclusions

Considering the yield and inhibitory activity of isolates in the extract, the intestinal maltase-inhibiting property of *C. sappan* wood is most likely owing to the presence of protosappanin B (**8**). Although the enzyme inhibitory activities of the isolated compounds were assayed *in vitro*, the results from this work should be relevant to the treatment of humans.

Overall, brazilin and sappanchalcone showed antihyperglycemic action both in carbohydrate digestive enzymes inhibition and glucose uptake enhancement in skeletal muscle cells. This study is the first to report on the use of active compounds from the potentially antidiabetic Indonesian herb, *C. sappan* wood to lower blood glucose through the inhibition of digestive enzymes (α -glucosidase and α -amylase) and glucose uptake enhancement *in vitro*. The inhibitory activities of the isolates are relatively low compared to commercially available glucosidase inhibitors, such as acarbose and glucose uptake enhancer, insulin. However, considering that *C. sappan* wood is a non-endangered plant that has been widely used throughout history, this research may be useful for the development of functional foods, alternative medicines, and complementary therapies for diabetes prevention and management.

Chapter 5. OVERALL CONCLUSIONS, RECOMMENDATIONS AND FUTURE PERSPECTIVES

5.1 Overall conclusions

A promising approach for treating diabetes mellitus (DM) is to decrease postprandial hyperglycemia by suppressing carbohydrate digestion using α -glucosidase inhibitors. This work has demonstrated an effective finding of the potential of Indonesian indigenous plants as an alternative for antihyperglycemic sources. In Chapter 2, aqueous methanol extracts of twenty-eight Indonesian herbs were screened for searching active compounds against maltase. Among them, 7 herbs showed high maltase inhibitory activity (above 60%). These are *Syzygium polyanthum* (64%), *Blumea balsamifera* (77%), *Melaleuca leucadendra* (62%), *Abrus precatorius* (63%), *Starchytapheta jamaicensis* (61%), *Pluchea indica* (79%), and *Caesalpinia sappan* (63%). *P. indica* leaves and *C. sappan* wood were chosen for further investigation to identify the responsible active principles exerting antihyperglycemic effect through intestinal α -glucosidase inhibition, in particular intestinal maltase. The finding for active principles was done by *in vitro* assay-guided isolation.

The finding of intestinal maltase inhibitors from *P. indica* leaves was discussed in Chapter 3. Enzyme assay-guided fractionation by chromatography yielded five active caffeoylquinic acid derivatives (**1-5**). Their structures were elucidated by mass spectrometry and NMR analysis and completed by comparison with reference data – 3,5-di-*O*-caffeoylquinic acid (**1**, 0.01% of weight plant, IC_{50} = 1.16 mM), 4,5-di-*O*-caffeoylquinic acid methyl ester (**2**, 0.08%, 0.21 mM), 3,4,5-tri-*O*-caffeoylquinic acid methyl ester (**3**, 0.04%, 0.002 mM), 3,4,5-tri-*O*-caffeoylquinic acid (**4**, 0.02%, 0.013 mM), and 1,3,4,5-tetra-*O*-caffeoylquinic acid (**5**, 0.02%, 0.011

mM) were isolated. Comparison of the activities of each isolate suggested that both methyl esterification of quinic acid and the number of caffeate groups in the molecule were important to the inhibitory activity (Arsiningtyas et al., 2014).

The findings of antihyperglycemic principles from *C. sappan* wood were discussed in Chapter 4. The ethyl acetate-soluble layer from aqueous methanol extracts of *C. sappan* wood was fractionated by successive silica gel column chromatography with chloroform–methanol and hexane–ethyl acetate solvent systems and by HPLC (ODS) to yield four phenolic compounds, brazilin (**6**, 0.03% of weight plant), sappanchalcone (**7**, 0.01%), protosappanin B (**8**, 0.5%) and protosappanin C (**9**, 0.12%), as intestinal maltase inhibitors. The intestinal maltase inhibitory activity was in the order of **8** ($IC_{50} = 0.81$ mM), **7** (0.96 mM), **9** (2.59 mM), and **6** (3.83 mM). Compounds **7** and **8** showed a higher maltase-inhibitory activity than **6** and **9**. The isolated compounds were also investigated for their intestinal sucrase and α -amylase inhibitory activity. Although **6** showed moderate inhibition of intestinal sucrase ($IC_{50} = 1.12$ mM), compounds **7**, **8**, and **9** did not significantly inhibit sucrase. Compound **7** showed weak PPA inhibitory activity, whereas **6**, **8**, and **9** showed no inhibition. Interestingly, compound **6** and **7** showed significant enhancement on glucose uptake in L6 muscle cell lines. Hence, *C. sappan* wood showed antihyperglycemic activity through the inhibition of carbohydrate digestive enzymes (α -glucosidase, and α -amylase) and stimulation of muscle glucose uptake.

This study provides basic information for further examination the suitability of *P. indica* and *C. sappan* as a functional food, alternative medicines and complementary therapies for diabetes prevention and management.

5.2 Recommendations

This research reveals the potency of Indonesian medicinal plants as α -glucosidase inhibitors. *C. sappan* and *P. indica* are well known medicinal plants in Indonesia. These findings revealed the active principles against α -glucosidase inhibition. The isolated compounds should be investigated for their selectivity in binding the individual subunits of maltase-glucoamylase and sucrase-isomaltase, for the potential of modulating on glucose release could be optimized. Further investigation related to human safety, such as *in vivo*-experiment, should be done for developing the plant use as a functional food, alternative and complementary medicine for diabetes treatment and management.

5.3 Future perspectives

DM has become a serious problem of modern society due to severe long-term health complications associated with it. Natural compounds contained in indigenous plants may be feasible alternatives for the diabetes treatment or reinforcements to currently used treatments. It is a positive aspect, since indigenous plants are vast and can be large amounts/whole plants, therefore the intended medical use can be achieved.

This study revealed the natural constituents from some potential Indonesian indigenous plants. The use of indigenous plants, as traditional medicine or food sources, will almost continue to grow in world. The continuous revealing of bioactive principles from indigenous plants may be utilized to develop drugs and applied in the mainstream public health systems as a way of coping DM.

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