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Research Article

Phytochemical Screening and Study of Cholesterol Esterase Inhibitory Activity of Extracts from Stem of *Dipterocarpus intricatus* Dyer

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ABSTRACT

Dipterocarpus intricatus Dyer is a medicinal plant used for relieving muscle soreness (bark) and curing wounds (resin). This study investigates chemical composition and cholesterol esterase inhibitory activity of *D.intricatus* stem harvested from Phrasaeng District, Surat Thani Province, Thailand. Using chromatographic method and spectroscopic analysis, two known compounds, dipterocarpolic acid (1) and bergenin (2), were isolated and identified as a triterpene and a coumarin, respectively. Cholesterol esterase inhibition activity of dipterocarpolic acid (1) and bergenin (2) at concentration of 1.0 mg/mL was 35.22±1.44%, and 42.01±1.65%, respectively, whereas methanolic extract showed activity of 31.70±1.23% at 5.0 mg/mL, compared to orlistat (99.55±0.53%). Therefore, *D. intricatus* extracts are another alternative for further studies in herbal medicine and natural product development.

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Dipterocarpus intricatus Dyer; cholesterol esterase; phytochemical screening; spectroscopic methods

1. Introduction

Phytochemical constituents are great sources of therapeutic drugs. These natural bioactive compounds, often referred as secondary metabolites, are formed during plant's normal metabolic processes. Commonly these chemicals include flavonoids, anthraquinones, alkaloids, terpenoids, tannins, saponins, steroids and glycosides. Many secondary metabolites from plants are widely used as anti-inflammatory, antioxidants, anti-diabetic, anti-pancreatic cholesterol esterase and antibacterial agents. Traditionally, plant crude extracts or decoctions are commonly used to treat infections. It is important for the phytochemical constituents to be determined and tested for their biological activities in order to support their medicinal use. (Vasanthi *et al*., 2014).

Hypercholesterolemia is a genetic disorder, defined as high levels of cholesterol in the blood circulation (Ghule *et al*., 2009; Cho *et al*., 2006; Sergent

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et al., 2012). Low-density lipoproteins (LDL) are the primary carrier of cholesterol in the blood to liver cells and throughout the body. High LDL-cholesterol is associated with an increased risk of cardiovascular diseases (Handa *et al*., 2013; Al-Shaer *et al*., 2004; Ngamukote *et al*., 2011).

Pancreatic cholesterol esterase is the only important enzyme synthesized in the pancreas that catalyzes the hydrolysis of cholesterol esters to free cholesterol. The inhibition of pancreatic cholesterol esterase may provide a method for treating hypercholesterolemia and atherosclerosis by limiting the absorption of free cholesterol derived from cholesterol esters. (Wei *et al*., 2014). Simvastatin, is used for decades to lower LDL cholesterol by inhibiting HMG-CoA reductase required for the biosynthesis of cholesterol (Endo, 2008). More recently, simvastatin has been reported to inhibit cholesterol esterase *in vitro* and *in vivo* (Chiou *et al*., 2006). However, simvastatin can cause a condition that results in the breakdown of muscle tissue, potentially leading to renal failure (Endo, 2008). Therefore, the search for a natural, safe and highly effective cholesterol esterase inhibitor produced from botanical materials may be beneficial.

Among Dipterocarpaceaeous plants, *Dipterocarpus* has been known as the third largest genus with 75 species distributed in tropical regions of Asia, particularly in Southeast Asia (Ashton, 2014). Many species in this genus have been used as folk medicine for treatments of rheumatism, dysmenorrhea (Wibowo *et al*., 2012; Pauline, 2000), gonorrhea, urinary gleets, ulcer, ringworm, and skin diseases (Aslam *et al*., 2015). *Dipterocarpus intricatus* Dyer is a deciduous species, 15 - 25 meters tall, whose habitat is dry deciduous dipterocarp forest. In Thailand, parts of *Dipterocarpus intricatus* have been traditionally used as treatments for several diseases. Liquid gum is ground and used to treat gonorrhea and skin diseases. The barks are used to treat rheumatism. Several groups of active compounds have been isolated and identified from Dipterocarpaceaeous plants including sesquiterpenes, triterpenes and coumarin derivatives as well as their bioactivities isolated using different solvents (Wibowo *et al*., 2012; Aslam *et al*., 2015). Extracts of *Dipterocarpus* plants show many bioactivities. For example, extract from the flowers of *D. intricatus* exhibits antibacterial, antioxidant and cytotoxic activity (Le *et al*., 2021). In addition, Bergenin, an isocoumarin derivative in woody parts of the Dipterocarpaceae family, was reported to significantly reduced the levels of triglycerides (TG), total cholesterol (TC), and low-density lipoprotein cholesterol (LDL-c) while simultaneously increasing high-density lipoprotein cholesterol (HDL-c) levels in zebrafish model (Zhang *et al*., 2023). Oral administration of bergenin at 10 mg/kg reduced blood glucose level after 60 and 90 minutes in rat oral glucose tolerance test. In the same study, administration of 10 mg/kg bergenin reduced blood glucose levels and improved lipid profiles in streptozotocin-nicotinamide induced diabetic rats after 14 days. Kumar *et al*. (2012), it is reported that bergenin reduced the lipid profile of diabetic rats, though not total cholesterol and triglycerides. Therefore, the study on chemical profiles of *D. intricatus* and their bioactivity is required to investigate its potential for use and development into food, nutraceutical, and pharmaceutical products. This study is the first to report anti-pancreatic cholesterol esterase activity of methanolic extract of *D.intricatus* and two known compounds, a triterpene: dipterocarpolic acid (1), and a coumarins: bergenin (2) isolated from *D.intricatus* stems.

2. Materials and Methods

2.1 Extract preparation

Stem of *Dipterocarpus intricatus* Dyer were collected from Phrasaeng District, Surat Thani Province, Thailand in November 2022. It was cut and dried in a hot air oven at 50ºC before being ground. Air-dried ground stem of *D. intricatus* (1.2 kg) was macerated with 10 L methanol for 5 days at room temperature twice. Crude methanolic extracts were combine and filtered. It was concentrated using a rotary evaporator under reduced pressure to give crude extract (71.6 g). Yield of crude extract from dry weight was 6.0%.

2.2 Preliminary screening of phytochemical test

The qualitative phytochemical screening tests were performed to identify the main groups of chemical constituents (alkaloids, flavonoids, anthraquinones, coumarin, saponins, tannins, phlobatannins, terpenoids, steroids and cardiac glycosides) present in the extracts using the color reactions described elsewhere (Tukiran, 2013; Bhatt and Dhyani, 2012; Mouafi *et al*., 2014 and Vittaya and Chalad, 2016) as follows:

2.2.1 Test for alkaloids

0.2 g of the extract was boiled with 1.0 mL of 10% sulfuric acid for 5 minutes in a water bath, filtered, and allowed to cool. The filtrate was added 5 drops of Dragendroff's reagent was added to filtrate and shaken gently. The formation of a reddish brown precipitate (with Dragendroff's reagent) was taken as positive for a test for alkaloids.

2.2.2 Test for alkaloids

Development of a yellow or orange color appearance after addition of few magnesium turning followed by dropwise addition of conc. hydrochloric acid to 0.2 g of the extract to 1.0 mL of distilled 50% Ethanol indicates the presence of flavonoids.

2.2.3 Test for anthraquinone

0.2 g of the extract was boiled with 1.0 mL of 10% sulfuric acid for 5 minutes in a water bath, filtered, and allowed to cool. The filtrate was added 0.5 mL of 10% NH_3. The formation of a rose pink color in the ammonia layer indicated the presence of anthraquinone.

2.2.4 Test for coumarin

Development of intense yellow color after addition of 1.0 mL of 50% EtOH followed by 1.0 mL of 6 M NaOH to 0.2 g of the extract indicates the presence of coumarin.

2.2.5 Test for saponins

0.2 g of the extract was heated with 5 mL of water for 5 minutes in a water bath. After filtration, 5 mL of distilled water was added and shaken well. The formation of froth showed the presence of saponin.

2.2.6 Test for tannins

0.2 g of the extract was boiled with 1.0 mL of water for 5 minutes in a water bath, filtered, and allowed to cool. The filtrate was added 5 drops of 1% $FeCl_3$ and shaken. Then, it was observed for brownish green or a blue black coloration.

2.2.7 Test for phlobatannins

0.2 g of the extract was boiled with 1.0 mL of water for 5 minutes in a water bath, filtered, and allowed to cool. The filtrate was added 5 drops of 1% H_2SO_4 and shaken. The solution was heated for 5 minutes in a water bath, it was observed for brownish green or a blue black coloration.

2.2.8 Test for terpenoids

Development of a reddish brown coloration at the interface after addition of 1.0 mL of chloroform followed by 0.5 mL of concentrated sulfuric acid to 0.2 g of the extract indicates the presence of terpenoids.

2.2.9 Test for steroids

Development of a dark green or dark blue color after addition of 1.0 mL of chloroform followed by further addition of 0.5 mL of glacial acetic acid followed by further addition of 3 drop of concentrated sulfuric acid to 0.2 mg of extract indicate the presence of steroids.

2.2.10 Test for cardiac glycosides

Development of a brown ring at the interface after addition of 2 mL of glacial acetic acid that contained 5 drops of 1% of ferric chloride solution followed by further addition of 0.5 mL of concentrated sulfuric acid to 0.2 mg of extract dissolved with 1.0 mL of chloroform indicate the presence of cardiac glycosides.

2.3 Chemical isolation and structure elucidation

The crude extract (50.6 g) was dissolved in methanol and then partitioned by using n-hexane. The methanol fraction was concentrated by using rotary evaporator. The crude methanol extract (2.43 g) was further purified by Quick Column Chromatography on silica gel using hexane as eluent and increasing polarity with EtOAc and methanol, successively giving nine fractions (M1-M9). Fraction M3 (112.9 mg) was purified by column chromatography (CC) on silica gel using EtOAc: hexane (3:7) as eluent to give compound 1 (25.4 mg, 0.050% of crude extract: R_f = 0.36, EtOAc: hexane, 3:7). Fraction M7 (354.4 mg) was subjected to CC on silica gel using EtOAc: acetone: methanol (4:5:1) to yield compound 2 (39.7 mg, 0.078% of crude extract: R_f = 0.72, EtOAc: acetone: methanol, 4:5:1). The compound was structurally elucidated by using 400 MHz Bruker FTNMR Ultra ShieldTM spectrometers recorded in deuterochloroform and deuteromethanol, respectively and were recorded as δ value in ppm downfield from TMS (internal standard δ 0.00 ppm).

2.4 Identifcation of compounds 1 and 2

Compound **1** colorless viscous oil; IR v_{max} (KBr) cm^{-1}: 3415 (OH), 1686 (C=O); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.11 (1H, t, J =6.8 Hz, H-24), 2.50 (1H, ddd, J = 8.4, 9.6, 15.6 Hz, H-2) 2.43 (1H, ddd, J = 4.4, 7.2, 14.4 Hz, H-2), 2.17 (2H, m, H-23), 2.00 (1H, dd, J = 7.2, 14.4 Hz, H-1), 1.87 (1H, m, H-12), 1.77 (1H, m, H-17), 1.75 (1H, m, H-16), 1.73 (1H, m, H-13), 1.67 (3H, m, H-27), 1.59 (3H, m, H-26), 1.57 (1H, m, H-6), 1.55 (1H, m, H-5), 1.53 (1H, m, H-11), 1.50 (1H, m, H-16), 1.47 (1H, m, H-5), 1.47 (1H, m, H-22), 1.46 (1H, m, H-22), 1.45 (1H, m, H-1), 1.45 (1H, m, H-15), 1.40 (1H, m, H-9), 1.38 (2H, m, H-7), 1.36 (1H, m, H-6), 1.36 (1H, m, H-11), 1.32(1H, m, H-12), 1.13(1H, m, H-15), 1.08 (3H, m, H-29), 1.04 (3H, m, H-28), 0.99 (3H, m, H-18), 0.94 (3H, m, H-19), 0.86 (3H, m, H-30); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 218.9 (C=O, C-3), 180.8 (COOH, C-21), 132.7 (C, C-25), 123.4 (CH, C-24), 77.8 (C, C-20), 55.6 (CH, C-5), 50.1 (CH_2, C-7), 50.0 (C, C-14), 48.0 (CH, C-9), 47.6 (CH, C-17), 40.6 (C, C-4), 40.3 (CH, C-13), 40.2 (C, C-8), 38.4 (CH_2, C-22), 36.8 (CH_2, C-1), 34.6 (C, C-10), 34.1 (CH_2, C-2), 30.5 (CH_2, C-15), 29.7 (CH_2, C-12), 26.5 (CH_3, C-28), 25.7 (CH_3, C-27), 23.9 (CH_2, C-16), 22.4 (CH_2, C-11), 22.1 (CH_2, C-23), 21.0 (CH_3, C-29), 19.5 (CH_2, C-6), 17.6 (CH_3, C-26), 16.1 (CH_3, C-30), 15.9 (CH_3, C-19), 15.3 (CH_3, C-18).

Compound 2 colorless viscous oil; IR v_{max} (KBr) cm^{-1}: 3381 (OH),1699 (C=O); ^{1}H NMR (CD_3OD, 400 MHz) δ 7.08 (1H, s, H-2′), 4.97 (1H, d, J = 10.4, Hz, H-1), 4.06 (1H, dd, J = 10.4, 9.6 Hz, H-2), 3.90 (3H, s, OMe), 3.79 (1H, t, J = 9.2 Hz, H-3), 3.72 (1H, m, H-6a), 3.67 (1H, m, H-5), 3.43 (1H, t, J = 9.2 Hz, H-4), 3.01 (1H, m, H-6b); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 164.4 (C, C-7), 151.0 (C, C-3′), 148.1 (C, C-5′), 141.0 (C, C-4′), 118.0 (C, C-1′),115.9 (C, C-6′), 109.7 (CH, C-2′), 81.7 (CH, C-5), 80.0 (CH, C-2), 74.2 (CH, C-3), 72.9 (CH, C-1), 70.5 (CH, C-4), 61.3 (CH_2, C-6), 59.5 (OCH_3).

2.5 Pancreatic cholesterol esterase inhibitory activity

A modification of the method detailed by Gururaja *et al*. (2015) was used. Fractions and isolated compounds were prepared in ethanol as a stock solution (2 mg/mL) and diluted to 0.2 mg/mL with deionized water. Twofold serial dilutions were performed for IC_{50} determination. The final proportion of ethanol in the reaction was 2%. Pancreatic cholesterol esterase solution (2 µg/mL) was dissolved in 100 mM sodium phosphate buffer (pH 7.0). Various concentrations of the tested substances (100 µl) were pre-incubated with 50 µl of pancreatic cholesterol esterase solution at 37 °C for 15 min. p-nitrophenyl butyrate (p-NPB), a substrate, was used to assay enzyme activity. The substrate solution was combined with a sodium phosphate buffer (0.1 M, pH 7.0) containing 1 volume each of 5.16 mM taurocholic acid sodium salt, 0.2 mM p-NPB, and 100 mM NaCl. After pre-incubation, the reaction was initiated by adding 50 µl of substrate solution to the mixtures, which were then continuously incubated at 37 °C for 15 min. The reaction was stopped with the addition of 100 µl of ethanol. After incubation, the absorbance of the mixtures was measured at 405 nm. Orlistat was used as positive controls.

Table 1 Qualitative phytochemical analysis in the extract of stem of *D. intricatus*

Phytoconstituents	Crude methanolic extract
Alkaloids	-
Flavonoids	+
Anthraquinones	-
Coumarins	-
Saponins	-
Tannins	+
Phlobatannins	-
Terpenoids	+
Steroids	-
Cardiac glycosides	-

Note: + means present and - means absent

3. Results and Discussion

3.1 Phytochemical screening

The qualitative phytochemical screening of crude methanolic extract of stem of *D. intricatus* revealed the presence of some secondary metabolites or bioactive compounds such as flavonoids, tannins, and terpenoids as shown in Table 1. These phytochemical compounds are known to have medicinal and pharmacological importance (Adebayo and Ishola, 2009), giving reason for their study as potential pharmaceuticals. In this work, the stem contained flavonoid, tannin, and terpenoid compounds, which are one of the most unique groups of plant metabolites (Singh *et al*., 2007)

3.2 Structure elucidation

The methanolic extract of the *D. intricatus* stem was purified using silica gel column chromatography, yielding a terpenoid dipterocarpolic acid (1), and a coumarin: bergenin (2).

Compound 1 was obtained as a colorless viscous oil. The IR spectrum showed absorption band of a hydroxyl group at 3415 cm^{-1} and a carbonyl group at 1686 cm^{-1}. The ^{13}C and ^{1}H NMR spectra data showed characteristic of the 3-oxodammarane skeleton. The ^{13}C NMR spectral data (Figure 1) recorded in $CDCl_3$ showed 31 signals for 31 carbons. Analysis of DEPT 90° and DEPT 135° spectra of this compound suggested the presence of seven methyl [δ 26.5 (C-28), 25.7 (C-27), 21.0 (C-29), 17.6 (C-26), 16.1 (C-30), 15.9 (C-19) and 15.3 (C-18)], two carbonyl [δ 218.9 (C-3), 180.8 (C-21)], two olefinic [δ 132.7 (C-25), 123.4 (C-24)] and a oxyquaternary carbons [δ 77.8 (C-20)]. The ^{1}H NMR spectral data (Figure 1) showed seven methyl singlet signals at δ 1.08, 1.04, 0.99, 0.94 and 0.86 including two vinylic methyl at δ 1.67 and 1.59, an olefinic methine proton were shown at δ 5.11 (1H, t, J =6.8 Hz, H-24) and a typical dammarane δ_H -17 proton at δ 1.77 (m). The position of the hydroxyl group at C-20 was determined through an HMBC experiment in which the methine proton at δ 1.77 (H-17) showed correlations with C-12 (δ 29.7), C-14 (δ 50.0), C-15 (δ 30.5), C-20 (δ 77.8), C-21 (δ 180.8) and C-22 (δ 38.4). The position of an olefin methine proton at C-24 was determined from HMBC correlation of H-24 (δ 5.11) with C-22 (δ 38.4), C-26 (δ 17.6) and C-27 (δ 25.7). These data confirmed the side chain consisted of a carboxylic acid, a hydroxyl group as well as one double bond between quaternary carbon and methine carbon. Thus, on the basis of its spectroscopic data and comparison of the ^{1}H and ^{13}C NMR spectral data with the previously reported data of dipterocarpolic acid (Smirnovaa *et al*., 2012), compound 1 was assigned as dipterocarpolic acid.

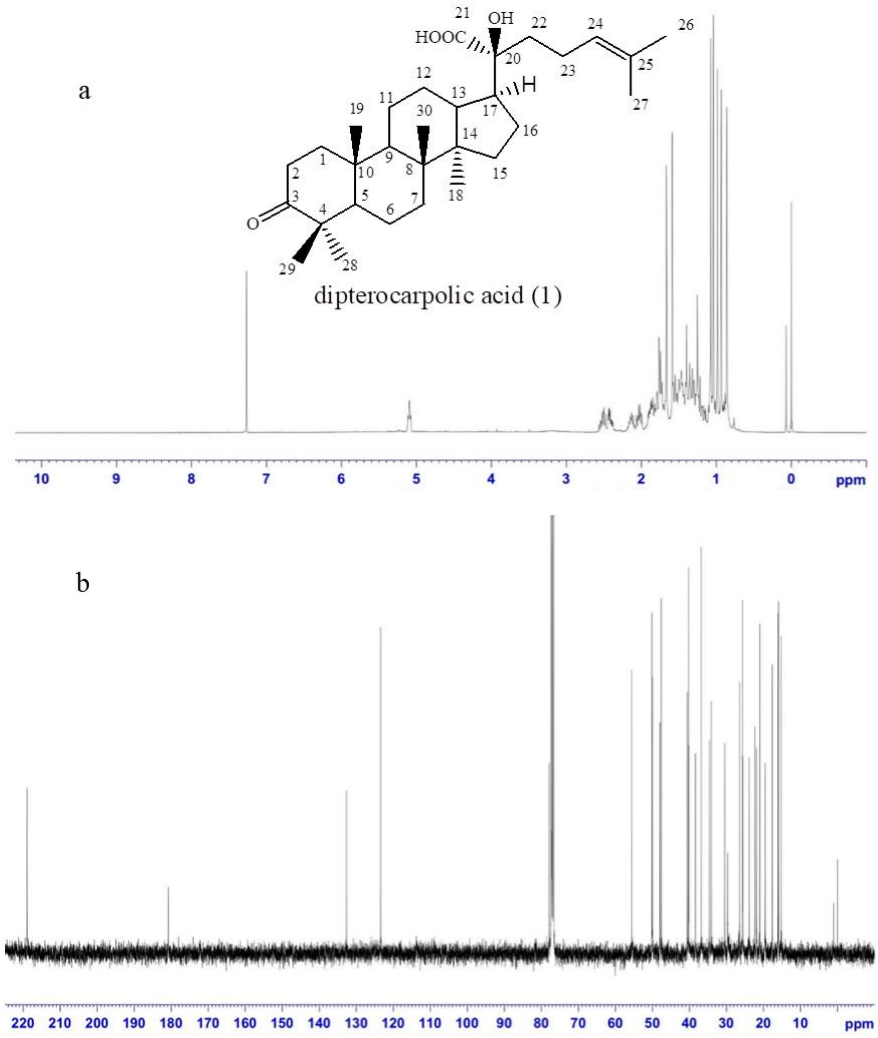

Figure 1 The ^{1}H (400 MHz, a) and ^{13}C NMR (100 MHz, b) spectrum of dipterocarpolic acid (1) (in $CDCl_3$)

Compound 2 was obtained as a colorless viscous oil. The IR spectrum showed absorption band of a hydroxyl group at 3381 cm^{-1} and a carbonyl group at 1699 cm^{-1}. The ^{13}C NMR spectral data (Figure 2) indicated the presence of 14 carbons including six aromatic, five oxymetine, one oxymethylene, one methoxyl and one carbonyl carbons. The ^{1}H NMR spectral data (Figure 2) displayed the presence of characteristic signal of sugar moiety. The anomeric proton at δ 4.97 (d, *J* = 10.4 Hz, H-1), was inferred to β-configuration of sugar moiety based on the value of the coupling constant. Other proton signal of sugar moiety were resonances at δ 3.01 (m, H_b-6), 3.43 (t, *J* = 9.2 Hz, H-4), 3.67 (m, H-5), 3.79 (t, *J* = 9.2 Hz, H-3), 3.72 (m, H_a-6) and 4.06 (dd, *J* = 10.4, 9.2 Hz, H-2) and the large vicinal coupling constants ($J_{ax,ax}$ = 10.4 , 9.2 Hz), confirming the β-*C*-glucoside ring . The proton signal displayed the presence of a one proton singlet at δ 7.08 which was assigned as H-2′. From the HMBC experiments, the aromatic proton at δ 7.08 (H-2′) showed correlations with the carbons at δ 72.9 (C-1), 115.9 (C-6′), 118.0 (C-1′), 141.0 (C-4′), 151.0 (C-3′) and 164.4 (C-7), the oxymethine proton at δ 4.06 (H-2) with the carbons at δ 72.9 (C-1), 74.2 (C-3), 115.9 (C-6′) and 164.4 (C-7), and the anomeric proton at δ 4.97 (H-1) with the carbons at δ 74.2 (C-3), 80.0 (C-2), 81.7 (C-5), 115.9 (C-6′), 118.0 (C-1′), 141.0 (C-4′) and 148.1 (C-5′). These data suggested an aryl β-*C*-glucoside and an aryl δ-lactone ring. Therefore, compound 2 was identified as bergenin (Wang *et al.*, 2005)

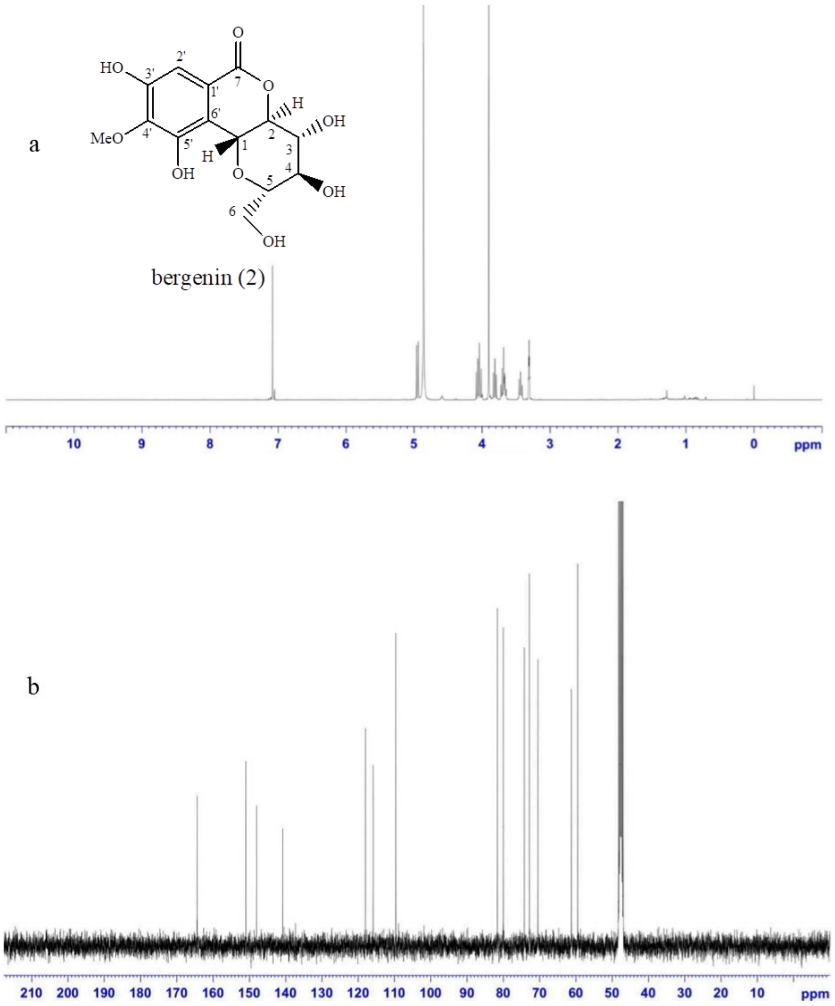

Figure 2 The ^{1}H (400 MHz, a) and ^{13}C NMR (100 MHz, b) spectrum of bergenin (2) (in CD_3OD)

All isolated from the *D. intricatus* stem were evaluated for their pancreatic cholesterol esterase inhibitory activity. The percentage inhibition of this radical was concentration-dependent. Cholesterol esterase inhibition of 1.0 mg/mL of dipterocarpolic acid (1), bergenin (2) and methanolic extract (5.0 mg/mL) were 35.22±1.44%, 42.01±1.65% and 31.70±1.23% respectively. The activities of isolated compounds and methanolic extract were weaker compared with orlistat (99.55±0.53%) in Figure 3. Bergenin was reported to reduce the lipid profile of diabetic rats. Significant reduction in total lipid levels was also observed in hyperlipidaemic rats given oral berginin for 14 (Jahromi *et al.*, 1992; Kumar *et al.*, 2012). To our knowledge, this study is the first to report *D. intricatus* phytochemical profile and pancreatic cholesterol esterase inhibitory activity of the extract, along with dipterocarpolic acid and bergenin isolated from *D. intricatus* stems.

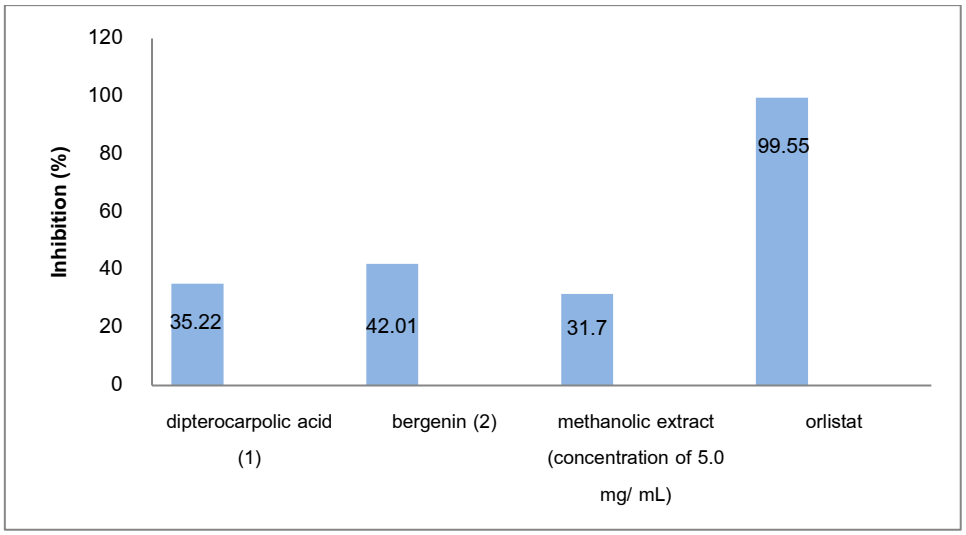

Figure 3 Percentage inhibitory pancreatic cholesterol esterase activity of dipterocarpolic acid (1), bergenin (2), methanolic extract, and orlistat at concentration of 1.0 mg/mL. Values represent mean ± SD (n = 3), $p < 0.05$.

4. Conclusion

The results of this study revealed that different medically important phytochemicals were present in extracts from *D. intricatus* stem. The result revealed that terpenoid dipterocarpolic acid (1), and coumarins: bergenin (2) exhibited moderate activity pancreatic cholesterol esterase inhibitory. This study has helped in establishing scientific evidences in the rationality of traditional use of plants for curing different human diseases. It has also assisted in exploring the medicinal values and creating a database of medicinal plants available in Thailand.

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