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Effects of extraction solvents of dietary plants on lipid lowering activity

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The present study aims to investigate the effects of five dietary plants including *Azadirachta indica* A. Juss. var. siamensis Valeton (flowers), *Bombax ceiba* Linn (pollen), *Citrus hystrix* DC (leaves), *Polygonum odoratum* Lour (leaves), and *Solanum torvum* Sw (fruits) on activity of pancreatic lipase, micellar cholesterol solubilization and bile acids binding. Two different organic solvents (methanol and ethanol) and distilled water (H_2O) were used for plants extraction. Crude extracts in different solvents can inhibit pancreatic lipase activity especially, the ethanolic extracts of *P. odoratum* which exhibited the strongest activity with IC_{50} value of 6.04 mg/mL. Aqueous extract of *P. odoratum* reduced cholesterol solubility by approximately 86%. Ethanolic extract of *S. torvum* had the highest ability to bind to taurodeoxycholic acid upto 97%. Ethanolic and methanolic extracts of *P. odoratum* bound to taurocholic acid 75% and glycodeoxycholic acid 40%, respectively. These findings suggest that lipid lowering activity of these plants were distinguished by organic solvents and water extraction.

Key words: Dietary plant, cholesterol, pancreatic lipase, micelles solubility, bile acid binding

INTRODUCTION

Hyperlipidemia and obesity are associated with cardiovascular disease (CVD) risk factors (Lavie et al., 2009; Last et al., 2011). Therapeutic lifestyle changes are used for primary hyperlipidemia management (Last et al., 2011). Lower cholesterol level is the line for lipid lowering treatment and cardiovascular disease prevention. Cholesterol absorption involves several processes including bile input, lipids digestion, micellar solubilization, cholesterol uptake into enterocytes, and secretion into lymph (Catapano, 2007).

Suppression of the micellar solubility of cholesterol has suggested a potential for the treatment of hypercholesterolemia by reduction of cholesterol absorption (Kirana et al., 2005). In addition, bile acid binding capacity play important roles in regulating cholesterol levels by binding to bile acids in the intestine resulting in the formation of an insoluble complex and then excretion in the feces (Insull, 2006). Moreover,

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Table 1. List of selected plants and their properties.

Scientific names	Common names	Plant part	Properties
A. indica var. siamensis	Siamese neem tree, Nim, Margosa, Quinine	Flowers; Leaves; Flowers; Leaves; Stem; Fruits	Chemopreventive (Kusamran et al., 1998) Against Malaria, HIV/AIDS and cancer (Anyaehie, 2009); Immunomodulatory, anti-inflammatory, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anticarcinogenic (Subapriya and Nagini, 2005); antioxidant (Sithisarn et al., 2005)
B. ceiba	Red cotton tree, Kapok tree, Cotton tree, Silk cotton tree	Root	Improvement of sexual function (Bhargava et al., 2012)
C. hystrix	Leech lime, Mauritus papeda	Peel	Cardioprotective effect induced by doxorubicin (Putri et al., 2013)
P. odoratum	Vietnamese coriander	Leaves whole	Antioxidant, anticancer and antibacterial (Nanasombat and Teckchuen, 2009); Antityrosinase and antioxidant (Saeio et al., 2011)
S. torvum	Plate brush egg plant	-	Treatment of malaria (Asase et al., 2010); Decreased blood pressure (Mohan et al., 2009)

several pharmacological agents have been used for lipid-lowering management including HMG-CoA reductase inhibitors (statins), cholesterol absorption inhibitor (ezetimibe), fibric acid derivatives, bile acid sequestrants, and nicotinic acid. These drugs have different mechanisms of action of lipid lowering.

Single or combination drugs were selectively used to reduce the doses or the adverse effects of drugs (Sampalis et al., 2007; Schmitz and Drobnik, 2003). Additionally, natural products as plant based dietary supplements are alternative choices to reduce blood cholesterol levels and a potential for treatment of hyperlipidemia. It has been reported that hydroalcoholic extract of *Capsicum annuum* L. flowers showed hypolipidemic effect by inhibition of pancreatic lipase acitivity (Marrelli et al., 2016). Ellagic acid-rich extract of pomegranates peel decreased plasma total cholesterol and triglyceride level accompanied by enhancing excretion of fecal bile acid (Liu et al., 2015). Protein and peptides of cowpea showed inhibited the enzyme HMG-CoA reductase activity and reduce cholesterol micellar solubilisation (Marques et al., 2015).

The traditional plants have been used as natural medicines for treatment of many diseases since ancient time. In particular, various medicinal plants, *Azadirachta indica* A. Juss. var. siamensis Valeton (*A. indica var. siamensis*), *Bombax ceiba* Linn (*B. ceiba*), *Citrus hystrix* DC (*C. hystrix*), *P. odoratum* Lour. (*P. odoratum*), and *Solanum torvum* Sw (*S. torvum*), are reported to have many biological activities such as anti-inflammatory, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anticarcinogenic (Table 1). However, there is little evidence to support lipid lowering of these five dietary plants. Therefore this study aimed to investigate the effects of extraction solvents (aqueous, ethanol and methanol) of dietary plants on lipid lowering activity. Pancreatic lipase activity, micellar cholesterol solubilization and bile acids binding were used to evaluate the potential of each extract as lipid lowering agents.

MATERIALS AND METHODS

Chemicals

Folin-Ciocalteu reagent, sodium bicarbonate, 1,2 di-O-lauryl-rac-glycero-3 glutaric acid 6′-methylresorufin ester, Taurocholic acid sodium salt hydrate, glycodeoxycholic acid, taurodeoxycholic acid, hydrazine hydrate solution, ß-nicotinamide adenine dinucleotide (NAD), 3α-hydroxysteroid dehydrogenase, and cholestyramine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). All other chemical reagents used in this study were of analytical grade.

Plant materials

Fresh plant materials (pollen of *Bombax ceiba* Linn, fruits of *Solanum torvum* Sw, flowers of *Azadirachta indica* A. Juss. var. siamensis Valeton, leaves of *Citrus hystrix* DC, and leaves of *P. odoratum* Lour.) were collected from Yong's garden Tumbon Meaka and Buajean's garden Tumbon Bantum, Mueang District, Phayao Province, Thailand. All plant specimens were identified anddeposited in a herbarium of the Faculty of Biology, Naresuan University, Phitsanulok, Thailand.

Preparation of plant extracts

The harvest plants were divided into 3 parts for extracting in two different organic solvents (methanol and ethanol) and distilled water (H_2O). For aqueous extraction, all plant materials were rinsed and 100 g of fresh plants were blended in 300 mL of distilled water and then filtered through cloth filter. The filtrate was lyophilized and the dry crude extract was stored at -20°C prior subsequent experiments. For ethanol or methanol extraction, all plant materials were rinsed and dried in the hot air oven at 50°C. 100 g of dried plant materials were ground and macerated with ethanol or methanol 300 mL for 3 days. Plants were re-extracted by the same process. The ethanol or methanol solution was subsequently filtered through filter paper. The solvents were removed using rotary evaporator. Crude ethanolic and methanolic extracts were stored at -20°C until use.

Determination of total phenolic content

The total phenolic content of plant extracts were determined using Folin-Ciocalteu method and modified according to previous study (Abu Bakar et al., 2009). Briefly, plant extract 2 mg/mL was mixed with 2 µL of the Folin-Ciocalteu reagent (diluted reagent 1:10 with de-ionized water) and added 80 µL of (15g/L) sodium carbonate solution. After 30 min at room temperature, absorbance at 750 nm was read on a spectrophotometer. Gallic acid was used as a standard phenolic compound. The concentration of phenolics was read (mg/mL) from a standard calibration curve; then the total phenolic content in extract was expressed as mg gallic acid equivalent in 1 g of dried extract (mg GAE/ g dry weight).

Determination of pancreatic lipase activity

To determine whether extraction solvents of dietary plants have a differential effect on pancreatic lipase activity, pancreatic lipase activity was measured using the method of Aubry et al. (2012). Varied concentration of extracts was prepared with reaction buffer pH 8.0 (0.8 M Tris-HCl, 150 mM NaCl, and 1.3 mM $CaCl_2$). 500 µL of each extracts solution were centrifuged at 10,000 rpm for 1 min and their supernatants were collected. 25 µL of each supernatant was mixed with reaction buffer (40 µL) and 50 U/mL pancreatic lipase (25 µL) in 96 well plate (black-side and clear-bottom). Finally, the reaction was started by adding 10 µL of 400 µM substrate (1, 2 di-O-lauryl-rac-glycero-3 glutaric acid 6'-methylresorufin ester) and run at 37°C in the dark for 60 min. Amount of fluorescent methylresorufin was measured at Ex 535 nm and Em 595 nm. Orlistat was used as a positive control.

Cholesterol micelles preparation

The micelle preparation was modified from Yamanashi et al. (2007). Briefly, sodium taurocholate was prepared in methanol, while cholesterol and phosphatidylcholine were dissolved in chloroform. The mixed lipid solutions were dried under N_2 gas and stored at -20°C until use.

Micellar cholesterol solubility assay

To determine the effect of extraction solvents of dietary plants on cholesterol solubility, the solubility of cholesterol was adapted from Kirana et al. (2005). Micelles solution was prepared under the conditions as mentioned earlier. The lipid film was hydrated in PBS and sonicated for 1 h before use. The final concentrations of cholesterol micelle composed of 10 mM cholesterol, 1 mM sodium taurocholate and 0.6 mM phosphatidylcholine. Plant extract (1 mg/mL) was mixed to the micelle solution for 3 h at 37°C. After incubation, mixed solutions were then filtered through a 0.22 µm membrane and determined cholesterol content by a cholesterol assay kit. Cholesterol in the filtrate can be defined as micellar cholesterol solubility.

Determination of bile acid binding

To investigate the effect of extraction solvents of dietary plants on bile acid binding activity, the bile acid binding assay was a modification of that by Yoshie-Stark and Wäsche (2004) as previously reported by Adisakwattana et al. (2012). Three bile acids were used in this experiment including taurocholic acid, glycodeoxycholic acid, and taurodeoxycholic acid. In brief, the extracts were dissolved in water (the aqueous extract) or DMSO (the ethanolic and methanolic extract). 200 µL of each extract (1 mg/mL) was incubated with each bile acid (200 µL) at concentration 2 mM in 100 mM phosphate buffered saline (PBS), pH 7.0, at 37°C for 2 h. The mixtures were centrifuged at 10,000 rpm for 10 min and filtrated through 0.22 µm membrane filter to separate the bound from the free bile acids.

The bile acid concentration was determined using the 5th generation randox total bile acids method (Porter et al., 2003). The filtrated-free bile acid (20 µL) was mixed with reaction mixture of 170 µL containing 0.133 mol/L tris buffer (pH 9.5), 1 mol/L hydrazine hydrate, and 7.7 mmol/L NAD. Finally, the 1 unit/mL 3α-hydroxysteroid dehydrogenase (10 µL) was added and incubated at 30°C for 90 min. Two reactions were combined in this kinetic enzyme cycling method. In the first reaction, bile acids were oxidized by 3-α hydroxysteroid dehydrogenase with the subsequent reduction of Thio-NAD to Thio-NADH. In the second reaction, the oxidized bile acids were reduced by the same enzyme with the subsequent oxidation of NADH to NAD. The rate of formation of Thio-NADH was determined by measuring the absorbance at 405 nm.

Statistical analysis

The results were expressed as the mean ± SEM. The data were analyzed by one-way analysis of variance (ANOVA) followed by Scheffe's test. P-values less than 0.05 were considered to be statistically significant.

RESULTS

Extraction yield of plant extracts

The extraction yield of plant extracts in different solvents is shown in Table 2. The solvent, aqueous, ethanol and methanol were used for plant extraction. The methanolic

Table 2. Extraction yield of plant extracts in different solvents.

Plant extracts	% yield		
	Aqueous extracts	Methanolic extracts	Ethanolic extracts
A. indica var. siamensis	4.87	5.13	5.21
B. ceiba	4.67	3.65	1.52
C. hystrix	10.34	15.6	2.33
P. odoratum	3.31	8.93	6.08
S. torvum	4.87	5.76	1.09

Table 3. Total phenolic content of extracts in different solvents.

Plant extracts	Total phenolic content (mg GAE/ g dry weight)		
	Aqueous extracts	Methanolic extracts	Ethanolic extracts
A. indica var. siamensis	12.12±0.76	2.44±0.015	12.78±0.40
B. ceiba	6.12±0.11	1.98±0.06	6.135±0.28
C. hystrix	3.41±0.88	2.76±0.21	9.70±0.31
P. odoratum	10.44±0.48	8.64±0.14	11.88±0.48
S. torvum	3.57± 0.28	1.87±0.0.6	8.72± 0.47

[1]Values are expressed as mean±SEM, N=3.

Table 4. Effect of plant extracts in different solvents on pancreatic lipase activity.

Plant extracts	Pancreatic lipase inhibition (IC_{50}) mg/mL		
	Aqueous extracts	Methanolic extracts	Ethanolic extracts
A. indica var. siamensis	11.75±1.42	24.72±1.54	14.55±1.45
B. ceiba	191.87±1.73	20.99±1.56	18.37±1.39
C. hystrix	11.17±1.46	8.48±1.72	19.54±5.09
P. odoratum	9.15±1.29	19.28±1.70	6.04±1.67
S. torvum	73.96±1.62	40.09±1.57	12.42±1.42
Orlistat	1.97±0.59**	-	-

[1]Values are expressed as mean±SEM, N=3, mg/mL, ** ng/mL.

extract of *C. hystrix* showed the highest extraction yields (15.6%). The percentage of methanol extraction yields of *S. torvum, A. indica var. siamensis*, *C. hystrix*, and *P. odoratum* were higher than aqueous and ethanolic extracts, exception aqueous extraction of *B. ceiba*, showed more the percentage yield than other solvents extraction.

Total phenolic content of plant extracts

The total phenolic contents of plants extracts in different solvents expressed in terms of mg GAE/ g dry weight (Table 3). The total phenolic contents were calculated based on the standard curve of gallic acid. The results showed that the extraction solvents have a vary total phenolic contents of plant extracts.

Ethanolic extracts were a high in phenolics. The ethanolic and aqueous extracts of *A. indica var. siamensis* found highest in total phenolic contents 12.78±0.40 and 12.12±0.76 mg GAE/ g dry weight, respectively. Methanolic extracts of *P. odoratum* showed phenol higher than other methanolic extracts.

Pancreatic lipase inhibitory activity of plant extracts

Pancreatic lipase activity of extracts was investigated using 1, 2 di-O-lauryl-rac-glycero-3 glutaric acid 6'-methylresorufin ester. The inhibitory activities of pancreatic lipase represent as the inhibitory concentration 50% (IC_{50}) values. Crude extracts in different solvents can inhibit pancreatic lipase activity as reported in Table 4. The ethanolic extracts of *P. odoratum*

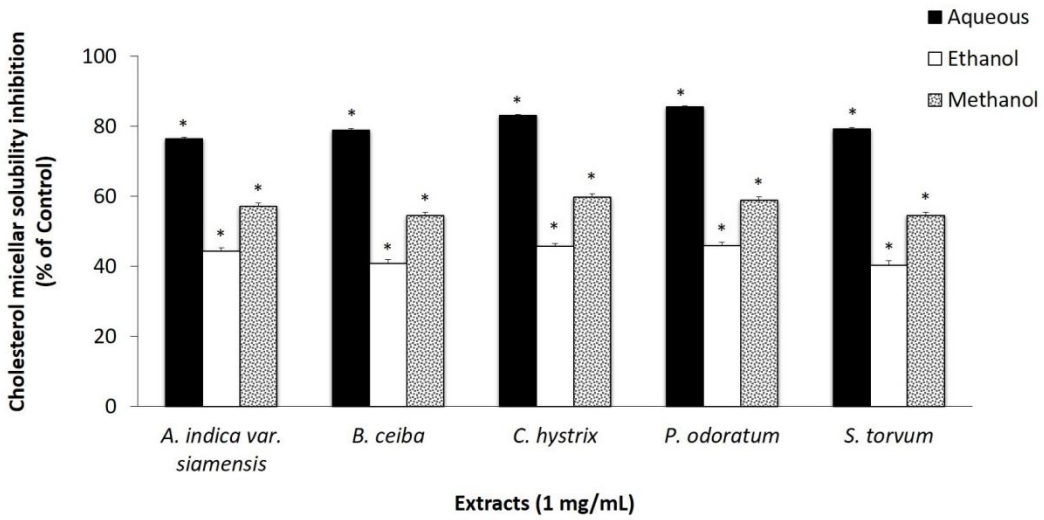

Figure 1. Cholesterol micellar solubility inhibition of plant extracts in different solvents.

exhibited the strongest activity with an IC_{50} value of 6.04± 1.67 mg/mL. The aqueous and ethanolic extracts of *P. odoratum* were more potent than other plants extracted by the same type of solvent. *C. hystrix* methanolic extracts strongly inhibited lipase activity 8.48±1.72 mg/mL when compare with other methanolic extracts. Orlistat, a positive control, inhibited the activity with IC_{50} 1.97±0.59 ng/mL (3.97±1.19 nM).

Effect of cholesterol micellar solubility of plant extracts

The solubility of cholesterol in micelles in the present of plant extracts (1 mg/mL) in different solvents was shown in Figure 1. The result represents the percentage of inhibition of cholesterol solubility in micelles. All aqueous extracts showed potent inhibition of cholesterol solubility 76 to 86%, whereas methanolic and ethanolic extracts reduced cholesterol solubility of 54 to 59 and 40 to 46%, respectively. Aqueous extract of *P. odoratum* Lour. showed highest ability to reduce cholesterol solubility by approximately 86%.

Bile acid binding ability of plant extracts

Taurocholic, taurodeoxycholic, and glycodeoxycholic acids were used as bile acids in this study. Bile acid binding activity of dietary plant extracts in different solvents is shown in Table 5. Interestingly, the *S. torvum* ethanolic extracts revealed strong binding with taurodeoxycholate 97%. Following, the *P. odoratum* of methanolic extracts and ethanolic extract bound to taurodeoxycholate 75%, and taurocholic acid 57%, respectively. The aqueous extracts showed greater bound to glycodeoxycholate than other bile acids, especially *B. ceiba* while the methanolic extracts were more effective bound to taurodeoxycholate than that of the other bile acids, specifically *P. odoratum*.

DISCUSSION

High blood cholesterol and obesity are a major health problem and a risk factor for metabolic syndrome and CVD. The lower blood cholesterol level is the goals of lipid lowering treatment and preventing cardiovascular disease. Many plants such as *Cucurbita moschata*, *Hibiscus sabdariffa* L., *Moringa oleifera* Lam., and *Morus alba L.* show properties in the management of hyperlipidemia and obesity (Chen et al., 2005; Choi et al., 2007; Chumark et al., 2008; El-Beshbishy et al., 2006; Hirunpanich et al., 2006; Xie et al., 2007; Young and Hui, 1999). *B. ceiba*, *S. torvum*, *A. indica var. siamensis*, *C. hystrix*, and *P. odoratum* revealed the biological activity and medicinal values (Anyaehie., 2009; Asase et al., 2010; Bhargavax et al., 2012; Kusamran et al., 1998; Mohan et al., 2009; Nanasombat and Teckchuen 2009; Putri et al., 2013; Saeio et al., 2011; Sithisarn et al., 2005; Subapriya and Nagini 2005). However, there is little evidence to support lipid lowering of these plants. This study investigated the possible mechanism of flowers *of A. indica var. siamensis*, pollen of *B. ceiba*, leaves of *C. hystrix*, leaves of *P. odoratum, and* fruits of *S. torvum*, and on lipid lowering activities.

The inhibition of pancreatic lipase enzymes is expected to limit fat absorption, resulting in delayed triglyceride digestion. Pancreatic lipase plays a role in the breakdown triacylglycerol, it also plays a key role in the absorption of cholesterol. Moreover, it helps to form the lipid emulsion for cholesterol transportation (Young et al., 1999). It was

Table 5. Effect of plant extracts in different solvents on bile acid binding**.**

Plant extracts	Bile acid binding (%)								
	Taurocholic acid			Taurodeoxycholate			Glycodeoxycholate		
	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol	Aqueous	Methanol	Ethanol
A. indica var. siamensis	7.4±0.6*	46.3±0.9*	37.2±1.9*	8.2±2.4	51.4±1.6***	42.1±2.2**	22.0±2.4	17.7±3.1*	24.8±0.8***
B. ceiba	11.3±0.5***	12.6±1.5*	23.6±2.3	19.1±3.8	47.4±1.7***	12.7±3.2	31.0±3.3*	18.7±2.9*	24.4±0.8***
C. hystrix	21.1±0.0***	23.2±0.2***	-8.0±0.9	13.7±2.1*	45.3±0.3***	-13.1±0.0	11.6±0.1	33.2±3.5**	16.8±0.7***
P. odoratum	-0.7±0.3	27.2±1.3***	57.0±1.3	25.5±2.9*	75.6±0.8***	21.0±3.0	14.5±2.7	40.2±2.2*	39.2±0.6***
S. torvum	4.2±0.5*	8.7±1.7	21.5±2.3*	-1.0±3.4	56.6±1.4***	97.3±0.1***	12.5±3.5	31.9±2.5*	31.3±0.7***
Cholestyramine	17.6±1.2*	-	-	25.2±0.8***	-	-	67.1±3.5***	-	-

[1]Values are expressed as mean±SEM, N=3, Water as control of the aqueous extracts. 1% DMSO as control of the methanolic and ethanolic extracts. Plant extracts and cholestyramine were tested 1 mg/mL. ***P<0.001, **P<0.01, *P<0.05.

revealed that transgenic mice lack pancreatic lipase enzyme response to the reduction of cholesterol absorption (Huggins et al., 2003). This observation provided that crude extracts in different solvents can inhibit pancreatic lipase activity. The ethanolic leaves of *P. odoratum* extracts exhibited the strongest activity. In addition, the methanolic extracts of *C. hystrix* and the aqueous extracts of *P. odoratum* were also potent to inhibit the lipase activity, respectively. The study by Han et al. (2001) and Nakai et al. (2005) found that polyphenols and saponins could inhibit pancreatic lipase activity (Han et al., 2001; Nakai et al., 2005). The study results found polyphenolic contents in all plant extracts. Aqueous and methanolic extract of *A. indica var. siamensis* and ethanolic extract of *P. odoratum* showed the highest of polyphenol in each solvents. It is possible that the inhibition of pancreatic lipase may be due to the action of their polyphenolic contents.

The inhibition of fat solubilization in the micelle (Brufau et al., 2008) and/or the changing size of the micelle affected to the absorption of cholesterol (Ikeda et al., 1992; Nagaoka et al., 1999; Raederstorff et al., 2003) in the intestinal lumen. Reduction of cholesterol absorption is also a target site for the treatment of hyperlipidemia. This result demonstrated that plant extracts in different solvents can inhibit cholesterol micelle solubility. The extracts with aqueous, ethanol and methanol at concentrations 1 mg/mL could inhibit the solubility of cholesterol micelle by 70 to 80, 40 to 60, 27 to 48%, respectively.

The study findings indicated that the aqueous extract was more effective to reduce the solubility of cholesterol micelle than methanol and ethanol. The aqueous extract of *P. odoratum* in particular, could reduce cholesterol solubility by upto 86%. Ngamukote et al. (2011) showed that the major polyphenol (gallic acid, catechin, and epicatechin) of grape seeds reduced the solubility of cholesterol micelle (Ngamukote et al., 2011).

The binding of bile acids have been demonstrated as one possible mechanism of lowering plasma cholesterol levels. Bile acids, an acidic steroids, are synthesized from cholesterol in the liver. They are conjugated with glycine or taurine and secreted into duodenum and then reabsorbed at ileum to metabolize in the liver (Kahlon and Smith, 2007).

Cholestyramine, a bile acid sequestrant, depletes the endogenous bile acid pool and increases bile acid synthesis from cholesterol, leading to a decreased plasma cholesterol level (Insull, 2006). The present study indicates that there are differences in bile acid binding between various dietary plants tested. The methanolic and ethanolic of dietary plant extracts were the activity trend to bind with bile acid than the aqueous extracts. The studies suggested that the dietary plant extracts had bile acid binding activities, especially *P. odoratum* and *S. torvum*.

Conclusion

The study findings demonstrate that the five dietary plants extracts with different solvents extraction could inhibit pancreatic lipase activity and reduce cholesterol solubility and bind with bile acids in distinguish activities. In particular, the leaves of *P. odoratum* exhibit potential as the inhibitor of pancreatic lipase and cholesterol micelles solubility. The fruits of *S. torvum* also

show high binding ability to taurodeoxycholic acid. Taken altogether, this study provides the evidence for the potential these dietary plants usage and possible development into natural supplement for lipid lowering product. However, more preclinical and perhaps the clinical studies might be needed.

Conflict of Interests

The authors have not declared any conflict of interests.

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