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Full Length Research Paper

Impact of Colombian yellow fruits and tropical fruits drinks consumption on the antioxidant status of healthy women

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Reactive oxygen species are responsible for causing different types of damage in the body which are associated with the onset of chronic non-communicable diseases. A strategy to counteract these effects is by a diet rich in antioxidants compounds found in fruits. The aim of this study was to determine the antioxidant capacity in plasma and the content of total phenolic compounds in healthy women who consumed a drink prepared with Nativanox® Colombian tropical fruits or a drink prepared with Nativanox® Colombian yellow fruits during a short period of time. Nineteen healthy women received a daily 200 mL drink with Nativanox® Colombian tropical fruits or a drink with Nativanox® Colombian yellow fruits for 14 days. Before and after the intervention period blood and plasma were obtained to analyze C-reactive protein levels, lipid profile, total phenolic content and antioxidant status through FRAP, ABTS and EROS methods. Regular consumption of a drink with Nativanox® Colombian tropical fruits had a positive impact on the lipid profile and the antioxidant capacity on plasma of healthy women. The consumption of a drink with Nativanox® Colombian yellow fruits diminished diastolic blood pressure. These results showed that a Nativanox® Colombian yellow and tropical fruit contains bioactive compounds that can improve oxidative status in plasma and contribute to reduce the risk of cardiovascular disease.

Key words: Antioxidant, polyphenols, tropical fruits, yellow fruits, lipid profile.

INTRODUCTION

Epidemiological studies have found that eating foods rich in antioxidants, such as fruits and vegetables, has been associated with a low risk of oxidative stress associated

with non communicable chronic diseases such as cancer and cardiovascular disease (Limón-Pacheco and Gonsebatt, 2009). The oxidative damage theory suggests

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that mitochondrion produce reactive oxygen species (ROS) or free radicals from electron transporter chain (Harman, 1956). Free radicals are also produced by enzymatic reactions such as NADPH oxidase reactions of phagocytes to destroy invading microbes or xanthine oxidase. In addition, external sources such as pollution, cigarette smoke and sunlight produce ROS which are incorporated into organisms and cells. Excessive production of ROS leads to damage of lipids, proteins, carbohydrates and DNA (Dean et al., 1997). The membranes exposed to free radicals lose their ability to transport nutrients properly; lipoproteins become oxidized forms; and DNA damage has the potential to accumulate consecutive mutations, which can lead to carcinogenesis (Ames et al., 1993). Therefore, the oxidative damage theory strictly recalls the concept that antioxidant molecules are capable to slow oxidative process and very important for homeostasis normal body metabolism.

An antioxidant with biological function is defined like a substance present at low concentrations able to decrease or prevents oxidation of a substrate. Such substances may have direct action by the neutralization of free radicals and non-radical reactive species or indirect, through induction of enzyme systems such as glutathione reductase, catalase and superoxide dismutase (Halliwell and Whiteman, 2004; Hicks et al., 2006). Among the antioxidants of dietary origin, that is, present in fruits and vegetables are carotenoids and polyphenols, the phenolic acids and flavonoids comprise 60% of the phenolic compounds obtained from diet (Ramos, 2007). Polyphenols are secondary plant metabolites that contribute to the organoleptic qualities, color and defense against pathogens attacks. The chemical structure of phenols having one or more aromatic rings with one or more hydroxyl groups which confer activity radical scavenging inactivating directly ROS or by binding to pro-oxidant metal ions through their groups OH (Rice-Evans et al., 1997). In recent years, it has been shown that a diet rich in polyphenols can improve health and reduce the incidence of cardiovascular disease (Shroeter et al., 2006). These effects are primarily due to its antioxidant properties that can usually justify their vasodilatory and vasoprotective actions and their antithrombotic, antilipemics, antiarterosclerotics, anti-inflammatory and anti-apoptotic (Potenza et al., 2007) shares. In addition, some studies have shown that these compounds can also inhibit angiotensin converting enzyme (ACE) and inhibition of this enzyme would justify its vasodilating and cardio-protective effects (Ojeda et al., 2010; Andriambeloson et al., 1997). One of the most studied properties of polyphenols is the ability to improve the lipid profile (Aviram and Rosenblat, 1994), thus, can prevent the development and occurrence of atherosclerosis, a disease characterized by progressive clogging of the arteries as a result of lipid accumulation in the arterial wall. These compounds are capable of attenuating the

onset and progression of this disease due to their ability to mitigate LDL oxidation by increasing the concentration of HDL-cholesterol and inhibit the proliferation of vascular smooth muscle (Osakabe et al., 2001).

Phenolic compounds are the largest non-energy plant substances group, so that products derived from fruit can be consumed to improve health. Furthermore, the combined food can have more benefits to consume the active components alone, because the set of nutrients can have a synergistic effect (Betoret et al., 2009). Andean berries and yellow Colombian native fruits with trade name Nativanox® prepared by the company Tecnas SA, rich in flavonoids, anthocyanins, tannins, polyphenols and carotenoids were determined ORAC value (Oxygen Radical Absorbance capacity) whose values were 55,000 $\mu\text{mol Trolox}/100\text{ g}$ for Nativanox® tropical fruits, 40,000 $\mu\text{mol Trolox}/100\text{ g}$ for Nativanox® Andean berries and 40,000 $\mu\text{mol Trolox}/100\text{ g}$ for fruits Nativanox® yellow (Peñaloza and Rojano, 2014). These results suggested that the consumption of a drink rich in bioactive compounds such as polyphenols, with high antioxidant capacity determined by ORAC can improve antioxidant status after consumption regularly for a short-time in healthy women.

According to data reported by the National Survey of Nutritional Status of Colombia (ENSIN) in 2010, 33.2 and 71.9%, a low percentage of Colombians consume daily fruits and vegetables, respectively. Only 1 in 5 Colombians consume whole foods, and just 25% of the population eats fruits and vegetables daily. Therefore, as a strategy to encourage the consumption of fruits and vegetables, has been proposed based on drinks fruit as an option accepted by the population (Wootton-Beard and Ryan, 2011). Fruits or fruit juices are good sources of antioxidants such as polyphenols. These could fulfill the role of mediating biological processes, resulting in the prevention of non-communicable chronic diseases such as dyslipidemia, hypertension, atherosclerosis and cancer (Morton et al., 2000). The aim of this study was to determine the impact on the antioxidant status of healthy women who consumed a drink prepared with Nativanox® colombian tropical fruits or consumed a drink prepared with Nativanox® colombian yellow fruits, obtaining a significant antioxidant activity for 2 weeks.

MATERIALS AND METHODS

Characterization of Nativanox

The bioactives of Nativanox were characterized by carotenoids, tannins, flavonoids, polyphenols, scavenging capacity hydroxyls $\bullet\text{OH}$ radicals and reactive oxygen species (ROS).

Determination of carotenoids

A representative portion of the sample was taken in a test tube. 4 ml of acetone was added and stirred in a vortexer for 2 min. The

Table 1. Bioactive compounds contained in Nativanox®.

Nativanox®	Flavonoids (mg catechin/mg dry weight)	Anthocyanins (mg cyanidin-3-glycosid/kg dry weight)	Tanins (mg catechin (mg dry weight)	Carotenoids (mg β -carotene/100 g sample)	Total polyphenols (mg gallic acid/100 g dry weight)	DPPH (TEAC μ mol Trolox/100 g sample)	Recommended dose/day by USDA-FDA/OMS
Colombian tropical fruits	1203-1500	0	8000-8492.40	0	4500-5012.8	18000 ^a 19700	5-8 g
Colombian yellow fruits	530	0	4000-6000	6.12	2100-2500	8000 ^a 9000	8-12 g

mixture was centrifuged at 4000 rpm/10 min and the supernatant was collected in another test tube. The absorbance of the solution was determined at 449 nm.

Determination of tannins

The analysis was performed according to the methods of the pharmacopoeia and the AOAC method after some modifications. 25 ml of the Nativanox extract were measured in a 1 L conical flask, and 25 ml of indigo solution and 750 ml of deionized water were added. Titration was performed with an aqueous solution of potassium permanganate (KMnO₄) 0.1 N until the blue solution turned green. The target of the test was made with 25 ml of indigo carmine solution in 750 ml of deionized water. The samples were analyzed in triplicate.

Determination of flavonoids

Determining flavonoids was performed following the method described by Debnath et al. (2011). A standard curve using catechin was constructed. Results were expressed as mg catechin/100 g Nativanox®. Readings were taken at 510 nm.

Evaluation of scavenging capacity hydroxyls •OH radicals

The hydroxyl radical scavenging activity was determined by fluorescence (Yang and Guo, 2001). The reaction was carried out in phosphate buffer (pH 7.4). 300 μ l of sodium

terephthalate, 420 μ l of buffer, 100 μ l of Nativanox®, 90 μ l EDTA solution and 90 μ l of iron solution (Fe⁺²) were mixed. The mixture was allowed to stand for 6 min with constant aeration and at room temperature (26°C), and then the fluorescence intensity was measured. Results are expressed as mg of DMSO/100 g of Nativanox®, by constructing a standard curve using different concentrations of DMSO.

Evaluation of the total capacity to trap reactive oxygen species (ROS) of Nativanox®

Capacity of Nativanox® to trap reactive oxygen species (ROS) was evaluated. ROS are generated by the azo compound, 2,2'-diazobis (2-amidinopropane) dihydrochloride) (AAPH), which in aqueous medium free radicals produced at a constant speed (Martín-Romero, 2008). The reaction was carried out in phosphate buffer (pH 7.4). 50 ml of a solution of AAPH, 50 ml of an ethanolic solution of dichlorofluorescein diacetate, 2850 ml of buffer and 50 μ l of Nativanox® were mixed. Immediately, fluorescence intensity was emitted during the first 10 min and compared to the intensity emitted in the absence of the sample which was read. Results are expressed as values TEAC (μ mol Trolox/100 g Nativanox®), by constructing a standard curve using different concentrations of Trolox.

Preparation of drinks

A powder enriched in polyphenolic compounds was obtained, packaged in foil pouch protected from light, with trade name Nativanox® produced by the company TECNAS S.A. Passion fruit (*Passiflora edulis*), Anana

(*Ananas comosus*) smooth cayenne variety, Granadilla (*Passiflora ligularis*), Common Guava (*Psidium guajava* L.) and Feijoa (*Acca sellowiana*) were used. The edible portion of each fruit was obtained and homogenized in a vegetable processor Black & Decker model FP1550S and homogenized in Ultraturrax T-50 Basic IKA-WERK containing the bioactive compounds in Table 1. The drink was prepared from the powdered extract. Following the recommendations of USDA and FDA for ORAC units/d indicated for preventing degenerative diseases, 5 g of Nativanox® colombian tropical fruits or 8.2 g of Nativanox® colombian yellow fruits representing 5,000 ORAC which were diluted in 200 mL of a Colombian blend mineral water and stored at 4°C until consumption were used. These preparations were made daily and consumed 1 h after dilution.

Study design

Double-blind trial with an intervention period of 14 days (2 weeks) was used. Twenty healthy women were recruited and given 200 ml/day a drink with tropical fruits (n = 9) or a drink with yellow fruits (n = 11). During the study, the diet or lifestyle of the participants did not change though they were instructed to avoid the consumption of fruits or similar elements contained in the juices. Each subject's weight and height were measured for calculating the body mass index (BMI). The subjects' diets were assessed before and at the end of the intervention using 72 h recall. Women received the drink packed in plastic bottles sterilized for consumption weekend, they were asked refrigerated, protected from direct light and heat. Fasting venous blood samples were collected before and after 2 weeks of intervention from each subject. Blood was collected in

heparinized tubes and centrifuged to obtain plasma for antioxidants analysis and non-heparinized tubes to obtain serum for lipid profile, glucose and C reactive protein analysis.

Subjects

Twenty healthy women (aged 18 to 60 years) from the University of Antioquia at Eastern Region were recruited in the study. The study protocol was approved by the Human Ethics Committee of Dentistry Faculty from the University of Antioquia University, and informed consent was obtained from each subject. All the subjects were in good health, with a regular medical history, and none of the subjects were on any medication, smoking, heavy physical exercise or took vitamin/mineral, antioxidant or herbal supplements.

Anthropometrics and blood pressure

Before (initial time, day 1) and after the intervention (final time, day 14), weight, size, body mass index (BMI), systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using Tanita BC-1500 Ironman Radio Wireless, measuring tape Seca 201 and digital sphygmomanometer Welch Allyn, respectively.

Total phenols in blood plasma

The total phenolic content was determined according to the adapted Folin-Ciocalteu method (Singleton, 1999). Plasma deproteinized by perchloric acid (50 μ l) were mixed with 125 μ l of Folin-Ciocalteu reagent and 400 μ l of sodium carbonate solution (7.1% p/v), and the resulting solution was brought to a final volume of 1000 μ l. The mixture was stirred and stored at room temperature for 30 min in the dark. The absorbance was measured at 760 nm against a control sample. Aqueous solutions of gallic acid were used to build a calibration curve. The results were expressed as gallic acid equivalents (GAE)/ml.

FRAP assay (Ferric Reducing/Antioxidant Power) in blood plasma

The antioxidant capacity of wine was estimated according to the procedure described by Benzie and Strain (1996), with some modifications. This method is based on the increase of absorbance due to the formation of 2, 4, 6-tripyridil-s-triazine (TPTZ)-Fe (II) in the presence of reducing agents. A volume of 50 μ l of deproteinized plasma was mixed with 950 μ l FRAP reagent previously dissolved in acetate buffer (pH 3.6). The absorbance increase was measured at 590 nm. The FRAP values were expressed as ascorbic acid equivalent antioxidant capacity (AEAC: mg ascorbic acid per ml) using an ascorbic acid standard curve.

ABTS assay in blood plasma

ABTS radical was produced through an oxidative reaction of ABTS using potassium persulphate. The capacity of plasma samples to scavenge ABTS radical was evaluated by reducing the absorbance after 30 min at 732 nm. Results were presented as TEAC values/L solution using a Trolox standard curve (Van der Berg et al., 1999).

Evaluation of ROS scavenging capacity in blood plasma

The method described by Rojano (2008) was used. 2,7-dichlorodihydrofluorescein (DCFH) reacts with ROS produced by 2,2'-

diazobis (2-amidinopropane dihydrochloruro) (AAPH) in an aqueous medium and forms the compound 2,7-dichlorofluorescein (DCF) fluorescence. 50 μ l of 0.3 M AAPH solution were mixed with 50 μ l of 2,7-dichlorofluorescein diacetate ethanolic solution 2.4 mM, 2850 μ l of 75 mM phosphate buffer, pH 7.4 and 50 μ l of the sample were evaluated. The intensity of fluorescence emitted during the first 10 min was analyzed and compared to the intensity emitted in the absence of the sample (λ excitation: 326 nm, one λ emission: 432 nm and 10 nm slit). Results were expressed as % relative value.

Statistical analysis

Data are presented as mean \pm standard deviation (SD) for normally distributed data or median and p25- p75 for data with non-normal distribution. For statistical differences, Student's t-test for repeated samples and the Wilcoxon test were used. To determine the correlation between antioxidant activity values, blood biomarkers and the total content of polyphenolic compounds, the Pearson correlation coefficient was calculated. ANOVA with repeated measures was used to evaluate interaction between time and type of drink, this type of ANOVA was chosen because participants of the study were measured at two times to see the changes to the intervention with Nativanox® Colombian yellow fruits or Nativanox® Colombian tropical fruits. A post-test analysis was performed for significant differences. The level of significance was $p < 0.05$. All analyses were performed using SPSS version 22.

RESULTS

After two weeks (14 days) of consumption of a drink prepared with Nativanox® Colombian tropical fruits and other prepared with Nativanox® Colombian yellow fruits, no significant changes were observed in the anthropometric variables weight and BMI, but significant decrease in diastolic blood pressure in the group of yellow fruits was observed (Table 2).

An improvement in lipid profile parameters was evidenced in both groups, but only a statistical significance was reached ($p < 0.05$) in the group of Nativanox® Colombian tropical fruits for total cholesterol and LDL-cholesterol (Table 3).

The antioxidant capacity measured in plasma using ABTS and ROS methods, and the total concentration of phenols in both groups showed no significant difference between day 0 and day 14 of intervention. However, in the group of participants who received the drink prepared with Nativanox® Colombian tropical fruits, a significant difference in reducing activity of plasma determined by FRAP assay was observed (Table 4).

There was an inverse Pearson correlation between the total phenolic compounds content and triglyceride, VLDL-cholesterol, total cholesterol, FRAP and ROS values in the group Nativanox® Colombian tropical fruits. On the contrary, the group that received drink prepared with Nativanox® Colombian yellow fruits did not show significant Pearson correlation (Table 5).

A significant interaction time vs. intervention was found for cholesterol-LDL and FRAP values ($p < 0.05$) (Table 6), indicating a different in time variation in every group, being significant for the group that received the drink

Table 2. Effect of treatment on antropometrics and blood pressure values.

Parameter	Yellow fruits			Tropical fruits		
	Initial time (day 0)	Final time (day 14)	P	Initial time (day 0)	Final time (day 14)	P
Weight (kg)	57.5 (57-60)*	57.6 (56.6-60.2)*	0.2	58.07±10.3	58.02±9.91	0.84
BMI (kg/m ²)	23.9 (23.1-24.4)*	23.4±2.8	0.3	22.38±2.43	22.35±2.32	0.62
SBP (mmHg)	110 (100-110)*	100 (100-112)*	0.14	110 (110-110)*	109.37±4.42	0.098
DBP (mmHg)	73.63±8.96	70.09±7.77	0.022**	70.5±7.61	68.5±9.22	0.45

Mean ± SD, T-student for repeated samples, *Me (Rq), Wilcoxon, **Statistical significance p<0.05. SAP: Systolic arterial pressure; DAP: diastolic arterial pressure.

Table 3. Lipid profile, reactive C protein and blood glucose.

Parameter	Yellow fruits			Tropical fruits		
	Initial time (day 0)	Final time (day 14)	p	Initial time (day 0)	Final time (day 14)	p
C reactive protein	0.09 (0.02-0.24)*	0.11 (0.04-0.17)*	0.75	0.13 (0.37-0.65)	0.26±0.27	0.61
Total cholesterol (mg/dl)	167 (142-183)*	159 (145-180)*	0.16	180.62±29.03	165±30.61	0.023**
Tricilglycerides (mg/dl)	99 (52-212)*	92 (49-162)*	0.05	96.25±51.71	66 (66-167.7)	0.88
Cholesterol-HDL (mg/dl)	50.54±10.21	52.83±9.04	0.171	48.18±9.23	50.23±8.48	0.07
Cholesterol-LDL (mg/dl)	91.3 (82.4-11.2)*	85.1 (76-105.2)*	0.79	113.18±23.95	96.15±24.34	0.012**
Cholesterol-VLDL (mg/dl)	19.8 (10.4-42.4)*	18.4 (9.8-32.4)*	0.05	19.25±10.34	13.2 (9.4-33.5)	0.89
Glucose (mg/dl)	74.81±7.93	74.63±7.71	0.91	72.37±4.53	74±8.08	0.54

Values are mean ± SD, T-test for repeated samples. *Me (Rq), Wilcoxon. **Statistically significant p<0.05.

prepared with Nativanox® Colombian tropical fruits. Additionally, the values of DBP, total cholesterol, HDL-cholesterol and LDL-cholesterol were significantly different between the two groups, likewise significant changes of FRAP values after the intervention period were evidenced (Table 6).

DISCUSSION

The results of several studies show the beneficial effect of polyphenols on the cardiovascular system, through mechanisms that improve endothelial function, platelet aggregation and anti-inflammatory function (Vita, 2005). In this study, a positive effect of consumption drink prepared with Nativanox® Colombian yellow fruits on the diastolic blood pressure after ingesting daily for 14 days was observed, suggesting a health benefit of this product on the vascular homeostasis. Similar findings after consumption of a diet rich in berries showed a high antioxidant content (Erlund et al., 2008) or after consumption during 4 weeks of rich tomato lycopene (Engelhard et al., 2006).

Taking account of the bioactive compounds present in Nativanox® Colombian yellow fruits (Table 1), this preparation has an important content of tannins and

carotenoids. Tannins are present in pomegranate juice (Ignarro et al., 2006; Stowe, 2011) and Sumac berries (*Rhus coriaria*) which are able to reduce diastolic pressure and the vascular smooth muscle cell (VSMC) migration by 62% (Zargham and Zargham, 2008). It has been shown that hydrolysable tannins are able to increase nitric oxide (NO) a vasoactive molecule endothelial-derived whose alteration is involved in the occurrence of atherosclerosis (Moncada and Higgs, 2006). In relation to the carotenoids present, only in Nativanox® Colombian yellow fruits, β -carotene is widely distributed in fruits in vegetables and is considered beneficial to endothelial functions and vascular health because of the ability to increase NO that leads to a downregulation of the expression of NF-kB-dependent adhesion molecules in endothelial cells involved in the proinflammatory response, by which (Aizawa et al., 2003; Gammone et al., 2015). Thus, it will be interesting to investigate the type of tannins (hydrolysable or condensed) and carotenoids present in Nativanox® Colombian yellow fruits and to determine its effects on nitric oxide metabolites after consumption.

In other hand, the findings in this study regarding lipid profile show that consumption of Nativanox® Colombian tropical fruits rich in polyphenols such as flavonoids and tannins, decreased the concentration of total cholesterol

Table 4. Antioxidants in plasma.

Parameter	Yellow fruits			Tropical fruits		
	Initial time (day 0)	Final time (day 14)	p	Initial time (day 0)	Final time (day 14)	p
Total phenols (GAE/ml)	1334.7 ± 157.33	1454.13 ± 123.3	0.05	1345.17 (1345.1 - 1411.1)	118716.75 ± 33342.0	0.67
FRAP (mg ascorbic acid/L solution)	76.71 ± 12.76	77.77 (61.86 - 80.2)*	0.85	75.28 ± 7.8	85.76 (79.6 - 91.7)	0.017**
ABTS TEAC (µmol Trolox/L solution)	4518.98 ± 211.9	4733 ± 377.2	0.07	4428.14 (4272.1 - 4606.7)	4305.43 (2354 - 4305.4)	0.78
ROS (% relative value)	0.0 (0.0-0.0)*	0.0 (0.0-0.0)*	0.59	0.0 (0.0 - 0.0)	0.0 (0.0-52.72)	0.18

Values are mean ± SD, T-test for repeated samples. *Me (Rq), Wilcoxon, **Statistically significant at p<0.05.

Table 5. Pearson's correlation coefficient.

Parameter	Yellow fruits		Tropical fruits	
	(r)	p	(r)	p
DBP (mmHg)	-0.37	0.26	0	0.9
SBP(mmHg)	-0.21	0.62	0.62	0.78
C reactive protein	-0.015	0.96	-0.35	0.38
Total cholesterol (mg/dl)	0.438	0.17	-0.59	0.12
Triglycerides (mg/dl)	0.348	0.29	-0.71	0.04*
Cholesterol-HDL (mg/dl)	0.208	0.62	0.208	0.62
Cholesterol-LDL (mg/dl)	-0.519	0.10	-0.44	0.27
Colesterol VLDL (mg/dl)	0.348	0.29	-0.71	0.04*
Glucose (mg/dl)	0.565	0.7	-0.92	0.48
FRAP (mg ascorbic acid/L solution)	-0.132	0.69	-0.84	0.008*
ABTS TEAC (µmol Trolox/L solution)	0.532	0.92	0.43	0.28
ROS (% relative value)	-0.191	0.57	-0.86	0.005*

*Statistically significant at p<0.05.

and LDL-cholesterol. A study shows similar findings of the relationship between cocoa catechins and epicatechins with the LDL-cholesterol concentration (Baba et al., 2007). Beneficial effects have been observed in humans after intake of chocolate (Baba et al., 2007; Mursu et al., 2004), grape juice (Freedman et al., 2001)

and black tea (Duffy et al., 2001) with high content of phenolic compounds. Epidemiological studies suggest that the rate of cardiovascular events is reduced by about 1% for every 1% decrease in LDL (Brown et al., 2006), which is important in the prevention and management of these diseases. A possible mechanism involved in the results

observed here on LDL-cholesterol levels is the evidenced obtained with phenolics of cocoa that decrease levels of LDL-cholesterol by reducing protein and mRNA expression of ApoB protein and mRNA which is contained in LDL-cholesterol lipoprotein, whereas cocoa polyphenols increase apolipoprotein (Apo) A1 and mRNA expression

Table 6. ANOVA repeated measures anthropometric, blood and plasma markers.

Parameter	p ²		
	Treatment	Time	Interaction
Weight (kg)	0.283	0.823	0.426
BMI (kg/m ²)	0.307	0.457	0.427
SBP (mmHg)	0.61	0.837	0.504
PBD (mmHg)	0.049*	0.530	0.562
C reactive protein	0.721	0.689	0.782
Total cholesterol (mg/dl)	0.005*	0.817	0.071
Triglycerides (mg/dl)	0.220	0.334	0.355
Cholesterol-HDL (mg/dl)	0.045*	0.565	0.906
Cholesterol-LDL (mg/dl)	0.004*	0.482	0.016*
Cholesterol-VLDL (mg/dl)	0.220	0.334	0.355
Glucose (mg/dL)	0.623	0.624	0.54
Total phenols (GAE/ml)	0.408	0.687	0.074
FRAP (mg ascorbic acid/L solution)	0.286	0.041*	0.044*
ABTS TEAC (μmol Trolox/L solution)	0.731	0.052	0.145
ROS (% relative value)	0.233	0.241	0.205

*Statistically significant at $p < 0.05$.

present in HDL-cholesterol lipoprotein (Andújar et al., 2012).

With regard to total phenol content in the drink prepared with Nativanox® Colombian tropical fruits and triglycerides and VLDL-cholesterol, a negative correlation was found, indicating that the higher concentration of total phenols lower levels of these parameters. These effects on triglycerides and VLDL has been observed in people following diets naturally rich in polyphenols (2776 to 2903 mg) for 8 weeks (Annuzzi et al., 2014) that significantly reduced fasting triglyceride concentrations (2-factor ANOVA) in plasma ($P = 0.023$) and large very-low-density lipoproteins (VLDLs) ($P = 0.016$) and postprandial triglyceride total area under the curve in plasma ($P = 0.041$) and large VLDLs ($P = 0.004$) (Anuzzi et al., 2014).

In the case of hydroxyl radicals, they are generated by the Fe^{2+} -EDTA/ H_2O_2 system. The mechanism is carried out in three stages: first, the oxidation of the pair Fe^{2+} -EDTA occurs with molecular oxygen to form Fe^{3+} -EDTA and superoxide radical. In the second, the superoxide radical in the presence of hydrogen is dismutated to H_2O_2 ; and in the last stage, the $\text{Fe}(\text{II})$ -EDTA catalyzes the decomposition of H_2O_2 to $\bullet\text{OH}$ (Yang and Guo, 2001). After being generated, hydroxyl radicals react with terephthalic acid to form a highly fluorescent product monohydric, 2-hydroxitereftalato acid. The capacity of Nativanox® to catch hydroxyl radicals decreases the amount of product 2-hydroxitereftalato, which can be evidenced on the decreased fluorescence intensity. Values for Nativanox® of 36147 mg DMSO/100 g dry weight have a considerable value as a bioactive expression.

Similarly, some bioactive of Nativanox have a similar

behavior like anthocyanins. This actives compounds might provide a lot of effects such as the reactive oxygen species scavenger capacity, chelate metals, stimulating the expression of enzymes, reducing the formation of oxidative DNA adduct, reducing lipid peroxidation inhibiting toxins and environmental mutagenesis carcinogens, and reducing cell proliferation by modulating the signal transduction pathways (Wang and Jiao, 2000; Wang and Stoner, 2008). Nativanox contains bioactive with a high capacity to trap peroxy free radicals ($\text{ROO}\bullet$), hydroxyl ($\text{OH}\bullet$), and generally reactive oxygen and nitrogen species (ROS and RNS). An intake of 200 ml Nativanox drinks provides about 4,000 IU ORAC, enough to maintain a good oxidative balance.

The study of antioxidant status in plasma of participants after 14 days of intake 200 ml of a drink prepared with Nativanox® colombian tropical fruits led to a significant increase in the reducing capacity of plasma determined by FRAP method, a trend also reported by Pedersen et al. (2000) in a group of 9 healthy women who consumed a cranberry juice for 1 week. This effect was attributed to the phenolic content and vitamin C of juice.

The correlation between the total phenol content in the beverage prepared with Nativanox® Colombian tropical fruits and FRAP value was inversely significant, suggesting that this antioxidant activity in plasma could be attributed to other polyphenolic compounds presents in drink such as tannins, carotenoids and vitamin C.

CONCLUSION

Results of this study show favorable changes in diastolic

blood pressure, total cholesterol and LDL-cholesterol as well as improvement in antioxidant status in plasma measured by the FRAP method, and an inverse relationship between total phenolic content and triglycerides and VLDL-cholesterol levels after consumption of a drink containing Nativanox® Colombian tropical fruits and other drink with Nativanox® Colombian yellow fruits for a short period of time in healthy women. These findings are important because they can explain the protective role against cardiovascular risk of a diet that includes fruits, and propose the development of food matrices containing active ingredients mixtures of yellow and tropical fruits for the prevention of non-communicable chronic diseases associated with oxidative stress. It is also demonstrated that the combination of fruits has a synergistic effect on the antioxidant capacity, which opens the door to the development of nutraceuticals or functional foods from the Nativanox® Colombian yellow or tropical fruits. Additional studies are needed to identify the bioactive compounds and the mechanisms responsible for these observed effects and their effect on cardiovascular risk population.

Conflict of interests

The authors state that they have no conflict of interest.

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Full Length Research Paper

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* Valetton (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 (H₂O) 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 IC₅₀ 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
<i>A. indica var. siamensis</i>	Siamese neem tree, Nim, Margosa, Quinine	Flowers; Leaves; Flowers; Leaves; Stem; Fruits	Chemopreventive (Kusamran et al., 1998) Against Malaria, HIV/AIDS and cancer (Anyaehe, 2009); Immunomodulatory, anti-inflammatory, antihyperglycaemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anticarcinogenic (Subapriya and Nagini, 2005); antioxidant (Sithisarn et al., 2005)
<i>B. ceiba</i>	Red cotton tree, Kapok tree, Cotton tree, Silk cotton tree	Root	Improvement of sexual function (Bhargava et al., 2012)
<i>C. hystrix</i>	Leech lime, Mauritius papeda	Peel	Cardioprotective effect induced by doxorubicin (Putri et al., 2013)
<i>P. odoratum</i>	Vietnamese coriander	Leaves whole	Antioxidant, anticancer and antibacterial (Nanasombat and Teckchuen, 2009); Antityrosinase and antioxidant (Saeio et al., 2011)
<i>S. torvum</i>	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 activity (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* Valetton (*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, β -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* Valetton, leaves of *Citrus hystrix* DC, and leaves of *P. odoratum* Lour.) were collected from Yong's garden Tumbon Meaka and Buajejan's garden Tumbon Bantum, Mueang District, Phayao Province, Thailand. All plant specimens were identified and deposited 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 (H₂O). 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 μ L of the Folin-Ciocalteu reagent (diluted reagent 1:10 with de-ionized water) and added 80 μ L 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 CaCl₂). 500 μ L of each extracts solution were centrifuged at 10,000 rpm for 1 min and their supernatants were collected. 25 μ L of each supernatant was mixed with reaction buffer (40 μ L) and 50 U/mL pancreatic lipase (25 μ L) in 96 well plate (black-side and clear-bottom). Finally, the reaction was started by adding 10 μ L of 400 μ M 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 N₂ 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 μ m 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 μ L of each extract (1 mg/mL) was incubated with each bile acid (200 μ L) 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 μ m membrane filter to separate the bound from the free bile acids.

The bile acid concentration was determined using the 5th generation radox total bile acids method (Porter et al., 2003). The filtrated-free bile acid (20 μ L) was mixed with reaction mixture of 170 μ L 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 3 α -hydroxysteroid dehydrogenase (10 μ L) 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 \pm 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	Ethanollic extracts
<i>A. indica var. siamensis</i>	4.87	5.13	5.21
<i>B. ceiba</i>	4.67	3.65	1.52
<i>C. hystrix</i>	10.34	15.6	2.33
<i>P. odoratum</i>	3.31	8.93	6.08
<i>S. torvum</i>	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	Ethanollic extracts
<i>A. indica var. siamensis</i>	12.12±0.76	2.44±0.015	12.78±0.40
<i>B. ceiba</i>	6.12±0.11	1.98±0.06	6.135±0.28
<i>C. hystrix</i>	3.41±0.88	2.76±0.21	9.70±0.31
<i>P. odoratum</i>	10.44±0.48	8.64±0.14	11.88±0.48
<i>S. torvum</i>	3.57± 0.28	1.87±0.0.6	8.72± 0.47

¹Values 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	Ethanollic extracts
<i>A. indica var. siamensis</i>	11.75±1.42	24.72±1.54	14.55±1.45
<i>B. ceiba</i>	191.87±1.73	20.99±1.56	18.37±1.39
<i>C. hystrix</i>	11.17±1.46	8.48±1.72	19.54±5.09
<i>P. odoratum</i>	9.15±1.29	19.28±1.70	6.04±1.67
<i>S. torvum</i>	73.96±1.62	40.09±1.57	12.42±1.42
Orlistat	1.97±0.59**	-	-

¹Values 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 ethanollic 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.

Ethanollic extracts were a high in phenolics. The ethanollic 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. Methanollic extracts of *P. odoratum* showed phenol higher than other methanollic 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% (IC₅₀) values. Crude extracts in different solvents can inhibit pancreatic lipase activity as reported in Table 4. The ethanollic extracts of *P. odoratum*

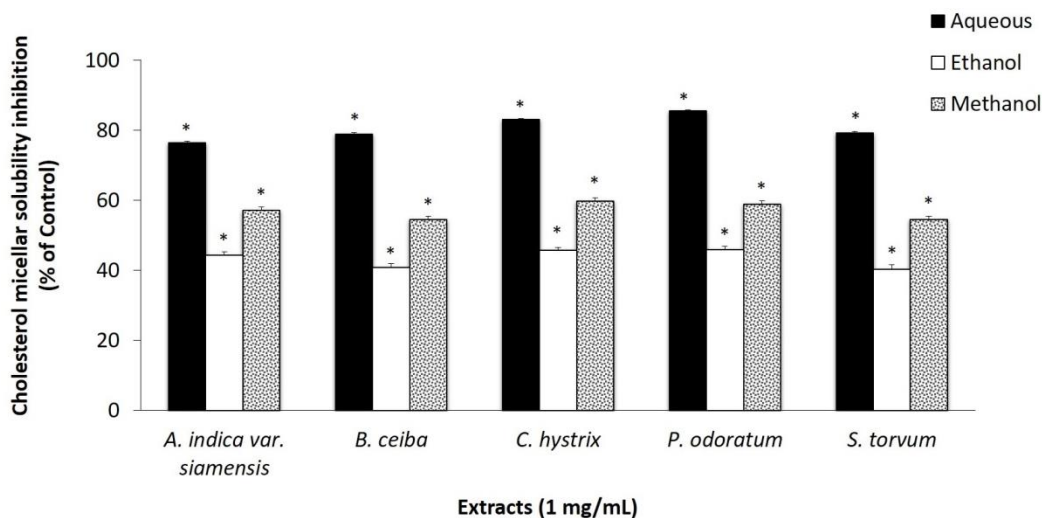


Figure 1. Cholesterol micellar solubility inhibition of plant extracts in different solvents.

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 (Anyaehe, 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
<i>A. indica var. siamensis</i>	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***
<i>B. ceiba</i>	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***
<i>C. hystrix</i>	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***
<i>P. odoratum</i>	-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***
<i>S. torvum</i>	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***	-	-

¹Values 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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Full Length Research Paper

Antibacterial, antifungal and *in vitro* cytotoxic activities of three extracts isolated from mint

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A number of reports have been focusing on chemical compositions and functional properties of essential oils isolated from mint. However, there is little data available on the biological activities of non-volatile constituents. In this study, the antibacterial (against 2 gram-positive bacterial strains: *Staphylococcus aureus* and *Bacillus cereus*; 2 gram-negative bacterial strains: *Escherichia coli* and *Pseudomonas aeruginosa*), antifungal (against *Candida albicans*, *Colletotrichum gloeosporioides* and *Penicillium polonicum*) and cytotoxic (against Human Embryonic Kidney 293 cell line) activities of three non-volatile extracts (Extracts 1 to 3) from the leaves and stems of mint were evaluated. Extract 1 showed significant antibacterial activity against gram-positive pathogens *S. aureus* and *B. cereus*, with an inhibition zone of 12.60 and 12.08 mm, respectively. The minimal inhibitory concentration (MIC) of extract 1 against *S. aureus* was 0.94 mg/ml. On the other hand, it exhibited low cytotoxic activity against normal Human Embryonic Kidney 293 cell line (CI₅₀ nearly 30 mg/ml), which suggested that Extract 1 could be a potential and safe antibacterial agent. Nevertheless, the tested gram-negative bacteria and pathogenic fungi were not susceptible towards Extracts 2 and 3.

Key words: Mint, antibacterial activity, antifungal activity, cytotoxicity.

INTRODUCTION

Antibiotic resistance (ABR) has become a global threat to public health systems due to the misuse and abuse of antibiotics (Ferri et al., 2015). Development of novel antibiotics is one of the effective measurements to address this ABR challenge (Saleem et al., 2010). Medicinal plants are potential sources of antibiotics due to the immense varieties of functionally relevant

secondary metabolites (Ngo et al., 2013; Saleem et al., 2010). Systematic screening of these natural products may result in discovery of novel effective antibiotics.

Plants from the genus *Mentha* are usually aromatic, stimulant and carminative, and are extensively used in the pharmaceutical, food, beverages, cosmetics, and allied industries (Johnson et al., 2011). As a traditional

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medicine, it is often used for treating nerve center, breath and digestive diseases (Thompson and Ernst, 2002). This genus includes 25 to 30 species that grow in the temperate regions of Eurasia, Australia and South Africa, with at least 7 species endemic to Australia ((Lawrence et al. 2006; Tang et al., 2016). Considerable chemical diversities and biological activities are observed in the mint's essential oil (Ahmed et al., 2015; Mimica-Dukic and Bozin, 2008; Petretto et al., 2014; Snoussi et al., 2015). Petretto has analyzed chemical constituents of essential oil from *Mentha suaveolens* species by gas chromatography-mass spectrometry (GC-MS). It shows that essential oil's major compounds are oxygenated monoterpene compounds (82.5%) and have a strong antimicrobial activity against all yeast strains (Petretto et al., 2014). The study by Snoussi et al. (2015) on *Mentha spicata* essential oil on *Vibrio* species biofilm inhibition and eradication reinforces its applicable possibility in the pharmaceutical or food industry as a natural antibiotic and seafood preservative against *Vibrio* contamination. Antioxidant and antibacterial activity of essential oil of *Mentha longifolia* and *Mentha pulegium* enable it to be good for cardiovascular and throat health (Ahmed et al., 2015).

While a number of reports have been focusing on chemical compositions and functional properties of essential oils isolated from mint, there is little data available on the biological activities of non-volatile constituents (such as flavonoids and polyphenolic compounds), which are reported to have broad-spectrum biological and antimicrobial activities and could be good substitutes for existing antibiotic drugs. In this study, the antibacterial, antifungal and cytotoxic activities of three non-volatile extracts from the leaves and stems of mint (the overground parts of *Mentha australis* R. Br.) were evaluated with the aim of finding out natural products as antibiotics.

MATERIALS AND METHODS

Extracts preparation

Three extracts (Extracts 1 to 3) were isolated from leaves and stems of Australian native mint by incubation with 60% ethanol at 80°C for 1 h. Extracts 1 and 2 were purified by macroporous adsorption resin of AB-8 with 4 bed volume (BV) 20 and 30% ethanol, respectively. Finally, the yield of flavonoid extract was 22 mg/g of dried material. With the corresponding reference standards of rutin and rosmarinic acid, the content of the total flavonoids and polyphenol were determined by ultraviolet and visible spectrophotometry. The content of flavonoids in Extract 1 was 90.35% and that in Extract 2 was 79.65%. Extract 3 was purified with macroporous adsorption resin of HPD-400 and the content of polyphenol was 35.47%.

Antibacterial activity

Bacterial strains and culture medium

Both gram-positive and gram-negative rods (*Staphylococcus*

aureus ATCC 6538, *Bacillus cereus* ATCC 11778, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853) were selected as microorganisms according to their pathogenic origin. The Beef-Protein medium was chosen for all bacteria culture.

Fungi strains and culture medium

Candida albicans ATCC 10231, *Colletotrichum gloeosporioides* and *Penicillium polonicum* has been chosen to evaluate antifungal activity. *C. gloeosporioides* and *P. polonicum* were isolated in our laboratory and identified by ITS sequence analysis. All fungi strains have been cultured in Sabouraud Dextrose Agar medium.

Disc diffusion method

The disc diffusion method was applied to determine the antimicrobial activity against four bacteria and three fungal pathogens. Bacteria were cultivated at 37°C for 24 h, fungi at 28°C for 5 days and these pathogens were adjusted to approximately 10⁶ CFU/ml. Sterile paper discs (6 mm diameter) impregnated with tested extracts were diluted in sterile water for 12 h and then placed onto the surface of inoculated plates. Ampicillin (25 µg/ml) was used as positive control against *S. aureus* while streptomycin (25 µg/ml) against other three bacteria strains. Water-soluble amphotericin B (50 µg/ml) was used as positive control against all fungal strains. The plates were left for 15 min at room temperature and then incubated at 37°C for 24 h (for bacteria) or 28°C for 5 days (for fungi). Then the antibacterial activity was evaluated by measuring the inhibition zone around the discs with caliper. Each test was carried out in triplicate.

Micro-Well dilution assay

MIC values were determined by a micro-well dilution method. A high-throughput 96-well micro plate assay procedure was used according to the method of (Bala et al., 2011). The inocula of the bacteria and fungi were adjusted to approximately 10⁶ CFU/ml. Within each sterile 96-well micro plate, the first two rows contained 200 µl medium as sterility check. Test samples were loaded in the next rows with respective concentrations of 30.00, 15.00, 7.50, 3.75, 1.88, 0.94, and 0.47 mg/ml, all comprising 100 µl culture and 100 µl extract. Streptomycin, ampicillin and water-soluble amphotericin B with corresponding concentration were used as positive controls. The same volume of microbiological culture and pure medium were used as negative controls. The test was replicated three times in each run of the experiments. The plates were covered with sterile plate seal and then incubated for 24 h/48 h. The MIC was defined as the lowest concentration at which the extract could be able to inhibit any visible bacterial or fungal growth.

Determination of MBC (MFC)

From the determination of MBC, 0.1 ml liquid medium without bacterial growth had been gotten and coated uniformly onto the surface of inoculated plates. And then they were incubated for more than 24 h at 37°C/28°C and checked whether microorganism grew or not. The minimum mass concentration without microorganism growth was MBC value for bacteria and the minimal fungicidal concentration (MFC) for fungi.

Cytotoxic activity

Cell culture

The normal Human Embryonic Kidney 293 cell line (293FT) were

Table 1. The inhibition zone diameter (mm) of isolated extracts of different concentrations against tested bacteria.

Sample	Concentration (mg/ml)	Inhibition diameter (mm±SD)			
		<i>Staphylococcus aureus</i> (+)	<i>Bacillus cereus</i> (+)	<i>Escherichia coli</i> (-)	<i>Pseudomonas aeruginosa</i> (-)
Extract 1	10	10.05±0.07	9.20±0.01	8.01±0.02	7.54±0.02
	20	11.58±0.49	10.50±0.03	8.75±0.05	8.65±0.01
	30	12.60±0.28	12.08±0.01	9.50±0.01	8.70±0.01
Extract 2	10	7.12±0.03	7.58±0.04	7.70±0.02	NI
	20	7.36±0.01	9.20±0.01	7.75±0.03	NI
	30	7.52±0.01	10.52±0.04	7.90±0.01	7.43±0.03
Extract 3	10	NI	NI	NI	NI
	20	NI	NI	NI	NI
	30	NI	7.44±0.01	NI	NI
Streptomycin ^a	1	-	18.00±0.15	17.40±0.27	14.56±0.04
Ampicillin ^a	1	14.50±0.33	-	-	-
ddH ₂ O ^b		NI	NI	NI	NI

NI, No inhibition observed; (+), gram-positive; (-), gram-negative; a, positive control; b, blank control.

kindly provided by PhD Yanyang Wu from Tsinghua University and seeded at the density of 2×10^5 cells/60 mm culture dish (BD Discovery Labware, Bedford, Massachusetts) (Jain et al., 2011). Cells were incubated at 37°C in the culture medium of DMEM basic (Gibco Cat. C11995500BT) +10% Foetal Bovine Serum (BI Cat. 04-001-1ACS) as culture medium in a humidified atmosphere of 95% air and 5% CO₂. At the onset of the experiments, the cells were at an exponential and asynchronous phase of growth.

Evaluation of cytotoxicity

Cytotoxicity was evaluated with the cell counting kit-8 (CCK8, Dojindo) method. 100 µl cell suspensions were inoculated in the 96-well culture plate and incubated at 37°C. After treatment with various concentrations of extracts, cells were re-incubated for an additional 48 h at 37°C. Then the medium was discarded and the cells re-incubated for 2 h after 10 µl of CCK8 solution added. The CCK8 solution was then removed and 200 µl insoluble formazan crystals were added. The optical density (OD) was measured at 540 nm by using an enzyme-linked immunosorbent assay plate reader. Data were obtained from triplicate wells.

The cytotoxicity index (CI%) was calculated according to the following equation: $CI\% = (C-T)/C \times 100$, where T and C respectively represent the mean optical density of the treated group and vehicle control group (Selim et al., 2015). CI50 was defined as the concentration (µg/ml) of the substrate that causes 50% death of cells.

HPLC analyses of flavonoids compounds

The dry samples (100 µg) were redissolved in 100 µl of methanol and were later injected into an Agilent 1260 infinity series HPLC with an Agilent Zorbax SB-C18 column (250×4.6 mm, 5 µm) using acetonitrile and 0.5% acetic acid as the mobile phase (flow rate, 1 ml/min), and the detector was set at 254 nm. Chromatographic peaks were identified by comparing the retention times and spectra against the standards of diosmin (97%, Sigma).

Statistical analysis

Statistical analysis was performed by using analysis of variance (ANOVA). The Statistical Package for Social Sciences (SPSS) 18.0 software package (SPSS Inc., Chicago) was used to perform statistical analysis and *P* value of ≤ 0.01 was considered to be significant.

RESULTS AND DISCUSSION

Antibacterial activity

The antibacterial activities of three non-volatile extracts with different concentrations were evaluated by using disc diffusion method against gram-positive (*S. aureus* ATCC 6538 and *B. cereus* ATCC 11778) and gram-negative (*E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853) bacterial strains. Two standard antibiotics (streptomycin and ampicillin) were used as positive controls in this assay. The antibacterial activity, which presented as a clear inhibition zone against these four bacterial species, is summarized in Table 1. At the same time, we have tested minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) to evaluate the antibacterial activities of three extracts as shown in Table 2.

Extract 1 was the most active one against bacteria among these three extracts (Tables 1 and 2). Especially, it showed significant antibacterial activities against gram-positive bacteria *S. aureus* and *B. cereus*, with inhibition zone of 12.60 and 12.08 mm and MIC of 0.94 and 7.50 mg/ml, respectively. Its inhibition effect was closely related with the dose (Table 1). The decreasing growth of

Table 2. MIC and MBC for antibacterial activities of three extracts.

Sample		<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>
		(+)	(+)	(-)	(-)
Extract 1	MIC	0.94	7.50	7.50	15.00
	MBC	1.88	7.50	7.50	15.00
Extract 2	MIC	7.50	15.00	15.00	15.00
	MBC	15.00	15.00	15.00	15.00
Extract 3	MIC	7.50	15.00	15.00	15.00
	MBC	15.00	15.00	30.00	15.00
Streptomycin ^a	MBC	-	<1.00	<1.00	<1.00
Ampicillin ^a	MBC	<1.00	-	-	-

Note: MIC, minimum inhibitory concentration (mg/mL); MBC, minimum bactericidal concentration (mg/mL); -, not been tested.

Table 3. The inhibition zone diameter (mm) of isolated extracts of different concentrations against tested fungi.

Sample	Concentration (mg/ml)	Inhibition diameter (mm±SD)		
		<i>Candida albicans</i>	<i>Colletotrichum gloeosporioides</i>	<i>Penicillium polonicum</i>
Extract 1	10	7.53±0.33	NI	NI
	20	7.21±0.23	NI	NI
	30	8.02±0.32	NI	NI
Extract 2	10	7.53±0.34	NI	NI
	20	8.04±0.27	NI	NI
	30	8.86±0.35	NI	NI
Extract 3	10	7.50±0.41	NI	NI
	20	7.52±0.30	NI	NI
	30	7.72±0.45	NI	NI
Amphotericin B	1	15.70±0.65	14.52±0.69	15.44±0.55
ddH ₂ O		NI	NI	NI

NI, No inhibition observed.

the bacteria was observed with the increasing dose of compounds used. The highest antibacterial activity was observed against *S. aureus* by 30 mg/ml Extract 1, with an inhibition zone of 12.60 mm.

Compared with the activities against gram-positive bacteria, the results of inhibition zone and MIC and MBC all showed that it had lower activity against gram-negative bacteria, even by Extract 1 (Tables 1 and 2). This finding agreed with previous reports describing the effectiveness of antimicrobial agents. This may be attributed to the porous nature of the outer peptide-glycan layer of cell wall of gram-positive bacteria that extracts could pass through the cell wall easier (Nostro et al., 2000).

The concentration of extracts with no bacteria growing on the agar disc after 48 h incubation was considered as

MBC. Except those against *S. aureus*, the MIC and MBC of three extracts against bacteria were at the same concentration. It suggested that the inhibited effects of these three compounds are long lived. With the prolonged incubation time, *S. aureus* was only susceptible at higher concentration. That maybe suggests that *S. aureus* could overcome the antibacterial effect of these three extracts.

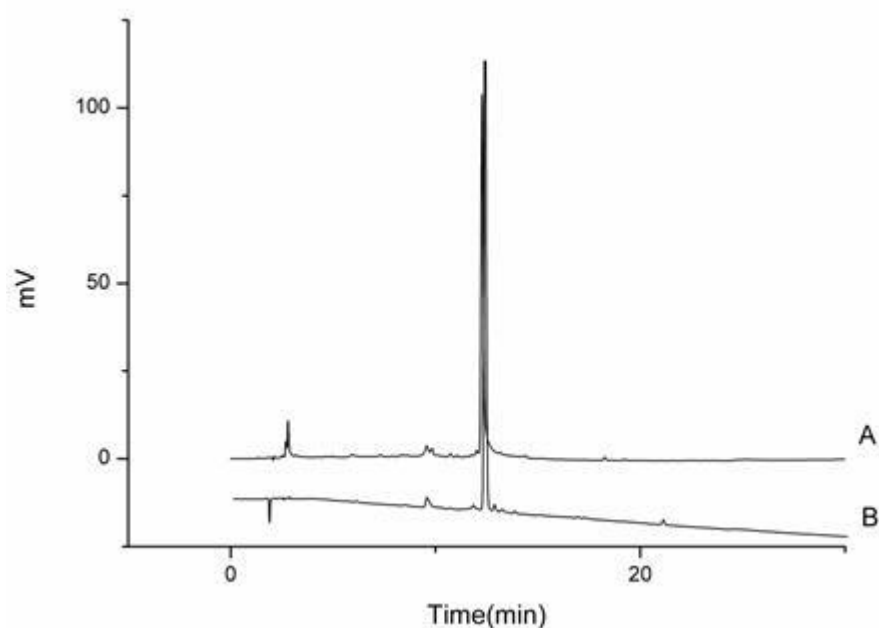
Antifungal activity

The activities of three extracts against fungi (*C. albicans*, *C. gloeosporioides* and *P. polonicum*) are shown in Tables 3 and 4. The results of inhibition zone did not follow the pattern observed in the MIC or MFC

Table 4. MIC and MFC for antifungal activities of three extracts.

Sample		<i>Candida albicans</i>	<i>Colletotrichum gloeosporioides</i>	<i>Penicillium polonicum</i>
Extract 1	MIC	>30.00	7.50	15.00
	MFC	-	7.50	15.00
Extract 2	MIC	>30.00	7.50	>30.00
	MFC	-	15.00	-
Extract 3	MIC	>30.00	>30.00	>30.00
	MFC	-	-	-
Streptomycin ^a	MFC	-	-	-
Ampicillin ^a	MFC	-	-	-
Amphotericin B	MFC	<1.00	<1.00	<1.00

MIC, Minimum inhibitory concentration (mg/mL); MFC, minimal fungicidal concentration (mg/mL); -, not been tested.

**Figure 1.** HPLC analysis of Extract 1. A, the standard of diosmin; B, Extract 1.

determination. The biggest inhibition zone was shown as 8.86 mm by 30 mg/ml Extract 2 against *C. albicans*, while in the MIC and MFC results, Extract 1 showed more active, with the MIC/MFC at 7.5 and 15.0 mg/ml against *C. gloeosporioides* and *P. polonicum*, respectively. That may be ascribed to (1) the spores of *C. gloeosporioides* and *P. polonicum* were easy to auto-infect when cultured on the solid medium; (2) the extracts could be thoroughly exposed in the liquid medium. Consequently, the inhibited effect against *C. gloeosporioides* and *P.*

polonicum could not be accurately measured on the agar discs.

Though we have purified and obtained the diosmin, which was reported to inhibit the growth of *Bacillus subtilis* and the human pathogenic fungus *Trichophyton rubrum*, from Extracts 1 and 2 by HSCCC, and verified by HPLC with the reference standard (Figure 1), the antimicrobial activities of the extracts are difficult to correlate with diosmin due to their complexities and variabilities. Some reports considered that there may be

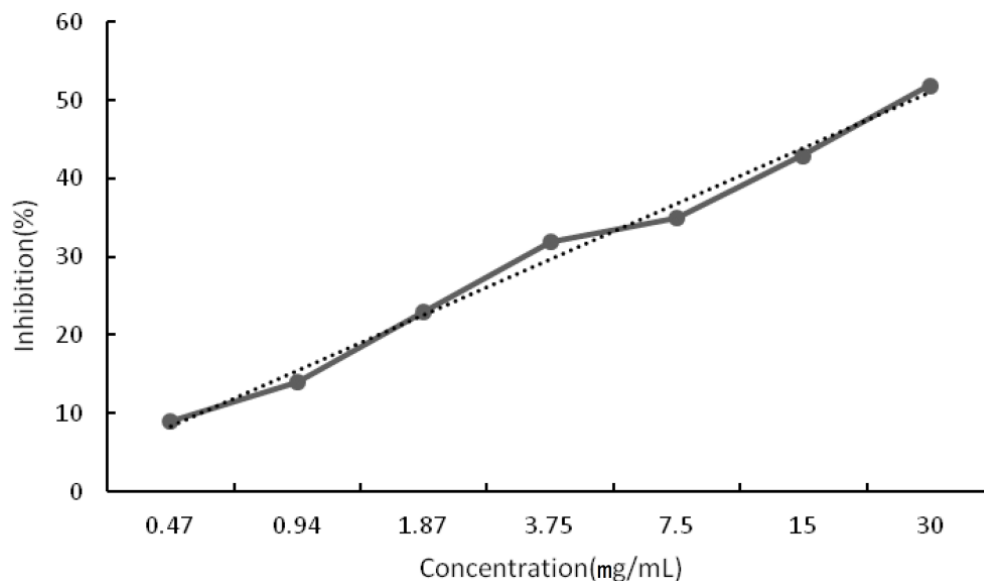


Figure 2. Cytotoxic activity of different concentrations of Extract 1 on Human Embryonic Kidney 293 cell line (HEK-293FT).

some relation between the most abundant components of compounds and the antimicrobial activity (Oliveira et al., 2014). In this study, the content of flavonoids in Extract 1 was the highest (90.35%) in these three extracts. That may be why the antimicrobial effect of Extract 1 was the most significant.

CCK8 cytotoxic activity

A preliminary *in vitro* cytotoxicity assay against a normal cell line (Human Embryonic Kidney 293 cell line (HEK-293FT)) was performed by using the Cell Counting Kit-8 (CCK8).

The cytotoxicity of Extract 1 was determined at various concentrations, and expressed in half maximal cytotoxicity index (CI50, mg/ml) (Figure 2). The results showed Extract 1 exhibited low cytotoxic activity against human normal cell line with CI50 values approximately 30 mg/ml.

CONCLUSION

This study is the first report on the antibacterial, antifungal and cytotoxic activities of three non-volatile extracts from the leaves and stems of mint. Extract 1 showed antibacterial activity against various pathogens with the most significant activity against *S. aureus*. It exhibited low cytotoxicity against cell line 293FT with the CI50 approximately 30 mg/ml. Therefore, Extract 1 could be suggested as a potential and safe antibacterial agent. However, further research is necessary to establish the pharmacological mechanism of tested extracts.

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Conflict of interests

The authors have not declared any conflict of interests.

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