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Full Length Research Paper

Investigation of biological activities of *Jasminum matthewii*

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The crude methanol extract of leaves of *Jasminum matthewii* as well as its hexane, carbon tetrachloride, chloroform and aqueous soluble partitionates were subjected to screening for antioxidant, cytotoxic, thrombolytic, membrane stabilizing, antimicrobial, analgesic, anti-diarrheal and central nervous system depressant activity assays. The antioxidant potential was evaluated in terms of total phenolic content and free radical scavenging activity using butylated hydroxytolune (BHT) and ascorbic acid as standards. Among the test samples of *J. matthewii*, the highest free radical scavenging activity was demonstrated by the aqueous soluble fraction (IC_{50}= 41.55±0.51 µg/ml), whereas in case of brine shrimp lethality bioassay, the hexane soluble fraction revealed the highest cytotoxic activity with LC_{50} value 0.19±0.32 µg/ml. In thrombolytic activity assay, the aqueous soluble fraction of *J. matthewii* extractives showed 62.29±0.29% of clot lysis, whereas standard streptokinase demonstrated 66.77% clot lysis. Among the test samples, the crude methanol extract inhibited 70.15±0.39% haemolysis of red blood cells induced by hypotonic solution. In case of heat-induced condition, the aqueous soluble fraction demonstrated 25.25±0.31% inhibition of haemolysis of red blood cells. None of the test samples revealed any zone of inhibition in disc diffusion assay. In peripheral analgesic activity assay, the crude methanol extract of *J. matthewii* demonstrated 51.02% inhibition of writhing at a dose of 400 mg/kg body weight dose compared to 74.49% inhibition by standard diclofenac sodium. In anti-diarrheal activity assay, the methanolic crude extract reduced diarrheal feces by 89.00±0.15% at 400 mg/kg dose. *J. matthewii* extractives potentiated phenobarbitone sodium-induced sleeping time in a dose dependent manner.

Key words: *Jasminum matthewii*, free radical scavenging activity, brine shrimp lethality, thrombolysis, membrane stabilization, hypotonic solution, zone of inhibition, writhing.

INTRODUCTION

Ethnobotanical and traditional uses of natural compounds of plant origin received much attention in recent years. According to the estimates of the WHO, more than 80% of people in developing countries depend on traditional medicine for their primary health needs (Kabir et al., 2015). The practice of herbal medicine is common in rural

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areas where western medicines are too expensive or not available (Adamu et al., 2004). Herbal preparations are generally believed to be safe for human use. Humans have frequently used plants to treat common infectious diseases and some of these traditional medicines are still part of the habitual treatment of various maladies. A recent survey shows that more than 60% of cancer patients use vitamins or herbs as therapy (Madhuri and Pandey, 2008; Sivalokanathan et al., 2005). People's reliability on drugs from plant sources is continuously increasing. It is therefore essential for systematic evaluation of plants used in traditional medicine for various ailments. Hence, there is need to screen medicinal plants for promising biological activity (Chowdhury et al., 2009). Drugs derived from unmodified natural products or drugs semi-synthetically obtained from natural sources corresponded to 78% of the new drugs approved by the Food and Drug Administration (FDA) between 1983 and 1994 (Cragg et al., 1997).

Jasmine (taxonomic name *Jasminum*) is a genus of shrubs and vines in the olive family, Oleaceae. Jasmines are native to tropical and subtropical regions of Eurasia, Australasia and Oceania, although only one of the 200 species is native to Europe (Schmidt et al., 2002). Their center of diversity is in South Asia and Southeast Asia (Panda, 2005). Jasmines are widely cultivated for the characteristic fragrance of their flowers. *Jasminum matthewii* P.S.Green is an ornamental plant of *Jasminum* genus in the Oleaceae family. The plant is widely distributed in India. Many species of this genus possess significant medicinal properties and have been used as traditional medicines for years. For example, *Jasminum grandiflorum* is documented to possess beneficial effects as odontalgic, thermogenic, aphrodisiac, antiseptic, emollient, anthelmintic, deobstruant, suppurative, tonic, in fixing loose teeth, ulcerative stomatitis, leprosy, skin diseases, ottorrhoea, otalgia, wounds, corns and aromatherapy (Warrier et al., 2004). The leaf and flower extract of *Jasminum officinale* Linn has blood purifying property and is traditionally used in cough and fever. Again the root and flower extract of *Jasminum humile* Linn is used as astringent and tonic (Haq et al., 2011). To the best of our knowledge from the literatures, the biological activities of *J. matthewii* were not explored extensively.

As part of our ongoing effort to investigate the medicinal plants of Bangladesh and assemble their activities for further systematic evaluation (Sharmin et al., 2013; Sarker et al., 2014), the crude methanol extract of leaves of *J. matthewii* growing in Bangladesh as well as its organic and aqueous soluble fractions were subjected to the study of antioxidant potential in terms of total phenolic content and free radical scavenging property; cytotoxic, thrombolytic, membrane stabilizing, antimicrobial, peripheral analgesic, anti-diarrheal activities and phenobarbitone sodium-induced sleeping time test for the first time and we, here in, report the results of our preliminary investigations.

MATERIALS AND METHODS

Plant

The leaves of *J. matthewii* were collected from Dhaka, Bangladesh, in May 2015. A voucher specimen (DUSH - 4569) for this plant has been maintained in Dhaka University Salar Khan Herbarium for future reference.

After collection of the plant materials, they were cleaned and sun dried. The powdered leaves (300 g) were macerated in 1.5 L of methanol for 7 days. Using fresh cotton bed and finally with Whatman filter paper number 1, the macerated plant material was filtered. The filtrate was then concentrated using a rotary evaporator at reduced temperature and pressure. 5 g of the concentrated methanol extract was fractionated by modified Kupchan (VanWagenen et al., 1993) partition protocol and the resultant partitionates were evaporated to dryness with rotary evaporator to yield hexane (HXSF, 1.0 g), carbon tetrachloride (CTCSF, 1.0 g), chloroform (CSF, 1.5 g) and aqueous (AQSF, 1.0 g) soluble materials. The residues were then refrigerated until further use.

Animal

Healthy Swiss-albino mice of either sex, aged 5 to 6 weeks were used for investigation on animal model to evaluate analgesic, antidiarrhoeal and sleep inducing properties. The Animal Resources Branch of the International Centre for Diarrhoeal Diseases and Research, Bangladesh (ICDDR, B) supplied the animals. After purchase, the animals were reserved under standard environmental condition and fed with ICDDR, B formulated rodent food and water. They were housed in isolation in cages and were kept at steady room temperature (25.0±3.0°C), humidity 35 to 60% and 12 h light and 12 h dark cycle to get them adapted with the new surroundings of the laboratory, before being employed in any experimentation (Hawk et al., 1954).

Total phenolic content

Using the method developed by Harbertson and Spayd (2006), the total phenolic content of the extractives was determined. Folin-Ciocalteau reagent was used in the test procedure.

DPPH free radical scavenging assay

According to the method developed by Brand-Williams et al. (1995), in DPPH free radical scavenging activity assay, butylated hydroxytoluene (BHT) and ascorbic acid were used as standards. The stable 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical was utilized to assess antioxidant activity of the test samples.

Brine shrimp lethality bioassay

This single day *in vivo* assay was designed by Meyer et al. (1982). This method is useful for the estimation of toxic properties of different plant extractives prepared using dimethylsulfoxide (DMSO) against *Artemia salina* in a single day *in vivo* assay. In this assay, vincristine sulphate was used as positive control.

Thrombolytic activity

Following the method developed by Prasad et al. (2006), the

Table 1. Total phenolic content, free radical scavenging activity and cytotoxic activity of *J. matthewii*.

Sample/Standard	Total phenolic content (mg of GAE/g of dried extract)	Free radical scavenging activity IC_{50} (µg/ml)	Brine shrimp lethality bioassay LC_{50} (µg/ml)
ME	8.25±0.12	49.34±0.45	0.33±0.44
HXSF	3.32±0.44	83.20±0.39	0.19±0.32
CTCSF	2.63±0.11	58.54±0.44	0.31±0.25
CSF	1.56±0.34	123.21±0.80	0.43±0.53
AQSF	44.13±0.53	41.55±0.51	0.32±0.11
VS	-	-	0.45±0.04
BHT	-	27.50±0.54	-
Ascorbic acid	-	5.80±0.21	-

ME: Methanolic crude extract; HXSF: hexane soluble fraction; CTCSF: carbon tetrachloride soluble fraction; CSF: chloroform soluble fraction; AQSF: aqueous soluble fraction; VS: vincristine sulfate; BHT: butylated hydroxytolune

thombolytic action of the plant extractives was determined. Streptokinase was used as positive control in this assay.

Membrane stabilizing activity

The membrane stabilizing potency of the extractives was determined by using the method developed by Omale et al. (2008). The test samples were evaluated by assessing their ability to inhibit hypotonic solution and heat induced haemolysis of human erythrocytes.

Antimicrobial screening

Disc diffusion method (Bayer et al., 1966) was used to determine the antimicrobial activity of the extractives.

Peripheral analgesic activity

Peripheral analgesic activity of the extractives was evaluated by determining their ability to inhibit acetic acid-induced abdominal writhing in mice (Kaushik et al., 2012).

Anti-diarrheal activity

Following the method of castor oil induced diarrhea in mice (Shoba and Thomas, 2001), the plant extractives were tested for having antidiarrheal potential.

Phenobarbitone induced sleeping time

Phenobarbitone induced sleeping time test was carried out according to the method of Williamson et al. (1996).

Statistical analysis

For all bioassays, three replicates of each sample were used for statistical analysis and the values are reported as mean ± standard deviation (SD).

RESULTS

The intend of the study was to estimate different organic and aqueous soluble materials of the crude methanol extract of *J. matthewii* for antioxidant, cytotoxic, thrombolytic, membrane stabilizing, antimicrobial, peripheral analgesic, anti-diarrheal activities and phenobarbitone sodium-induced sleeping time test.

Different extractives of *J. matthewii* demonstrated free radical scavenging potential with IC_{50} values ranging from 41.55 to 123.21 µg/ml. The highest free radical scavenging activity was demonstrated by the aqueous soluble fraction (IC_{50}= 41.55±0.51 µg/ml) which could be correlated to its phenolic content 44.13±0.53 mg of gallic acid equivalent (GAE)/g of extractives (Table 1).

In case of brine shrimp lethality bioassay, all the fractions demonstrated significant cytotoxic potential against *A. salina* with LC_{50} values ranging from 0.19 to 0.43 µg/ml. The hexane soluble fraction revealed maximum cytotoxic activity with LC_{50} value 0.19±0.32 µg/ml. All the other test subjects also revealed noteworthy cytotoxic potentials as compared to LC_{50} value 0.45 µg/ml for Vincristine sulphate (Table 1).

The extractives of *J. matthewii* demonstrated moderate to significant potential to promote thrombolysis. The aqueous soluble fraction showed 62.29±0.29% of clot lysis whereas streptokinase, used as standard in the assay, demonstrated 66.77% clot lysis (Table 2).

J. matthewii extractives significantly inhibited the haemolysis of red blood cell (RBC) induced by hypotonic solution and heat at concentration 1.0 mg/ml which is comparable to the standard acetyl salicylic acid (0.10 mg/ml). The crude methanol extract inhibited 70.15±0.39% haemolysis of RBCs induced by hypotonic solution, whereas under heat-induced condition, the aqueous soluble fraction was proven to inhibit 25.25±0.31% haemolysis of RBCs as compared to 71.90 and 42.12% by acetyl salicylic acid, respectively (Table 3).

In disc diffusion assay, none of the *J. matthewii* test samples demonstrated any zone of inhibition. Therefore, the plant may not posses any antimicrobial potential.

At a dose of 400 mg/kg body weight, *J. matthewii* leaf

Table 2. Thrombolytic activity of *J. matthewii*.

Sample/Standard	% of lysis of RBCs
ME	26.03±0.47
HXSF	24.62±0.39
CTCSF	49.09±0.22
CSF	27.59±0.53
AQSF	62.29±0.29
Water	3.79±0.55
Streptokinase	66.77±0.36

ME: Methanolic crude extract; HXSF: hexane soluble fraction; CTCSF: carbon tetrachloride soluble fraction; CSF: chloroform soluble fraction; AQSF: aqueous soluble fraction.

Table 3. Effect of different extractives of leaf of J. matthewii on heat and hypotonic solution-induced haemolysis of erythrocyte membrane.

Sample/Standard	% Inhibition of haemolysis	
	Heat induced	Hypotonic solution induced
Hypotonic medium	-	-
ME	1.60±0.19	70.15±0.39
HXSF	1.80±0.82	69.81±0.45
CTCSF	12.86±0.24	58.12±0.17
CSF	10.65±0.43	53.55±0.11
AQSF	25.25±0.31	69.36±0.29
ASA	42.12±0.38	71.90±0.78

ME: Methanolic crude extract; HXSF: hexane soluble fraction; CTCSF: carbon tetrachloride soluble fraction; CSF: chloroform soluble fraction; AQSF: aqueous soluble fraction; ASA: acetyl salicylic acid.

Table 4. Effect of the crude methanol extract of *J. matthewii* on acetic acid-induced writhing in mice.

Groups (n = 5)	Dose (mg/kg)	Number of writhing*	Inhibition of writhing (%)
Control	10 ml/kg	19.6	-
Diclofenac sodium	50	5.00±0.68	74.49
ME	400	9.60±0.12	51.02
ME	200	15.0±0.26	23.47

ME: Methanolic crude extract. *Values are Mean ± SEM.

extractives exposed significant analgesic activity. The mean number of writhing was significantly lower in mice when compared with the negative control but higher than that of the standard diclofenac sodium used in the assay. At 400 mg/kg body weight dose, the crude methanol extract demonstrated 51.02% inhibition of writhing whereas the standard diclofenac sodium was found to produce 74.49% inhibition of acetic acid induced writhing in the test animals (Table 4).

The crude methanol extract of *J. matthewii* showed highly significant antidiarrhoeal property in castor oil induced diarrhea in mice. The methanolic crude extract at 400 mg/kg doses reduced diarrheal feces by 89.00±0.15% whereas the same extract at 200 mg/kg dose showed 82.00±0.39% reduction of diarrheal feces. Both of these assessments were found to be more significant when compared with the standard loperamide (71.42%) (Table 5).

J. matthewii extract was found to potentiate the phenobarbitone sodium-induced sleeping time in a dose dependent manner. The time of onset of sleep was 15.8 min in control group, whereas in experimental group it

Table 5. Effect of methanolic crude extract of *J. matthewii* on castor oil (1 ml/mice) induced diarrhea in mice.

Groups (n=5)	Treatment	Dose (mg/kg)	Number of diarrheal faeces	% Reduction of diarrhea
I	Control (saline)	10 ml/kg	16.8±0.48	-
II	Standard (loperamide)	50	4.8±0.76	71.42±0.42
III	Methanolic extract	200	2.20±0.24	82.00±0.39
IV	Methanolic extract	400	1.80±0.62	89.00±0.15

Values are expressed as Mean ± SD from the experiments.

Table 6. Effect of the crude methanol extract of *J. matthewii* on phenobarbitone sodium–induced sleep.

Groups (n = 5)	Treatment	Dose (mg/kg)	Time of onset of sleep (min)	Total sleeping time (min)
I	Control	10 ml/kg	15.8 ± 1.19	118.6 ± 2.81
II	Methanolic extract	200	29.4± 2.20	126.2± 2.85
III	Methanolic extract	400	12.6± 1.76	159.2± 3.21

Values are expressed as Mean ± SD from the experiments.

was 29.4 and 12.6 min at doses of 200 and 400 mg/kg body weight, respectively. The total sleeping time was about 126.2 and 159.2 min at 200 and 400 mg/kg, respectively, while it was 118.6 min in the control group (Table 6).

DISCUSSION

The potential of the plant under investigation *J. matthewii* for different biological activities have been explored for the first time. Although, many other plants under the same genus have been used from generation to generation for medicinal purposes, such folkloric uses of *J. matthewii* have not been reported yet. Therefore, the findings in our investigation can only be correlated with those of the plants of the same genus. Besides, plants belonging to Oleaceae family are rich sources of various pharmacologically active substances. However, the identity of many of these phytoconstituents and their mechanisms of action are not still clear (Rahman et al., 2014). These species including *J. matthewii* may contain very important secondary plant metabolites that may contribute to their biological activities.

J. matthewii extractives demonstrated mild to moderate free radical scavenging and highly significant cytotoxic potential in brine shrimp lethality bioassay. According to literature study, the essential oil and the crude methanol extract from *Jasminum sambac,* another species of the same genus, have *in vitro* antioxidant activities (Abdoul-Latif et al., 2010). *J. sambac* also fashioned very prominent cytotoxic activity in brine shrimp lethality bioassay. Several Jasminium species have been reported to be used in cancers (Rahman et al., 2011). Plants belonging to Oleaceae family contain very important compounds like alkaloids, flavonoids, tannins, etc. (Rahman et al., 2014) that were reported to have cytotoxicity in different cell lines (Bun et al., 2009; Matsuo et al., 2005; Jiang et al., 2008). These compounds may contribute to the antioxidant and cytotoxic potentials of the plant under exploration.

The extractives of *J. matthewii* showed significant thrombolytic potential in our investigation. This may be a key finding that may have imperative implications in cardiovascular diseases (Hussain et al., 2014), because blood clot formation is considered to be a serious event in which endothelial cell surfaces or blood vessels are clogged by the deposition of fibrin, platelets and tissue factor (Furie and Furie, 2008). In addition, this finding may indicate the possibility of developing novel thrombolytic agents from the flowers of the plant. The presence of phytochemicals like tannins and alkaloids have been reported for plants under the genus *Jasminum* and these compounds are the probable reason for demonstrating the thrombolytic activity.

J. matthewii extractives significantly inhibited haemolysis of RBC induced by hypotonic solution and heat. Human red blood cell membranes resemble lysosomal membrane components (Mounnissamy et al., 2008). Therefore, the inhibition of hypotonic solution and heat induced haemolysis of red blood cell can be considered as a measure of the mechanism of anti-inflammatory effect of the plant extract. Membrane stabilization results from prevention of leakage of serum proteins and fluids into the tissues during phases of augmented permeability caused by inflammatory mediators (Chaitanya et al., 2011). Phytochemical screening of other plant extracts of the same genus *Jasminum* came to the findings that these plants contain flavonoids which may have reportable anti-inflammatory

property. The anti-inflammatory activity is probably due to the inhibitory effect on enzymes involved in the production of the chemical mediators of inflammation and metabolism of arachidonic acid (Oweyele et al., 2005).

Although, *J. matthewii* extractives showed no antimicrobial activity, many other species under the same genus like leaves of *Jasminum multiflorum* or *J. sambac* have been reported to have significant antimicrobial activity (Abdoul-Latif et al., 2010; Ankita et al., 2014). This justifies our attempt to assess *J. matthewii* extractives whether they possess any antimicrobial activity or not.

J. matthewii extractives showed considerable analgesic and anti-diarrheal properties. Such results can be correlated with similar findings with plant of the same genus, *Jasminum amplexicaule*. By tradition, this plant has been commonly used in ailments like dysentery, diarrhea and bellyache in China. The crude methanol extract of *J. amplexicaule* and different fractions of this extract were studied for anti-diarrheal and analgesic activities by Jia et al. (2008). The anti-diarrheal potential of the plant was tested using castor oil-induced, magnesium sulphate-induced diarrhoea models, anti-enteropooling assay and gastrointestinal motility models in mice. By means of hot-plate, writhing and formalin models in mice, the analgesic activities were studied. Jia et al. (2008) found that at the doses of 100, 200 and 400 mg/kg, the crude methanol extract of *J. amplexicaule* showed significant and dose-dependent anti-diarrheal and analgesic activities in these models. These results supported its traditional use in diarrhoea and pain. The leaf extract of another species under the same genus, *J. sambac* also demonstrated significant writhing inhibition in acetic acid-induced writhing in mice (Rahman et al., 2011). Other studies also showed that *J. sambac* root has analgesic effect (Bhoumik et al., 2013). Such findings support the traditional use of its root in ancient China to treat headaches, insomnia, and pain due to dislocated joints and broken bones (Rahman et al., 2011).

J. matthewii extractives potentiated phenobarbitone sodium-induced sleeping time in a dose dependent manner. Other *Jasminum* species like *J. sambac* extract is traditionally used as sedative (Rahman et al., 2011). The ethanol extract of *J. multiflorum* showed marked CNS depressant action (Pal et al., 2007). Species under the same genus may possess same phytochemicals that may contribute to their similar biological properties.

Conclusion

Jasmines are plants with variety of biological potentials. Although the plant under our investigation has not been explored for bioactivities to that extent, it is clearly evident from the aforementioned findings that the test samples of *J. matthewii* possess different types of bioactivities. The phytoconstituents which are mainly responsible for these biological activities can be isolated, purified and identified by different chromatographic and spectroscopic techniques. Therefore, the plant is a good candidate for the isolation, characterization and evaluation of biological activities of the isolated phytoconstituents to correlate with the findings in our investigation.

Conflict of Interests

The authors have not declared any conflict of interests.

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Alpha-glucosidase inhibitory activity and phytochemical investigation of *Borassus flabellifer* Linn.

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The determination of α-glucosidase inhibitory activity and isolation of active compounds from *Borassus flabellifer* Linn. are the main objectives of this study. The colorimetric method and chromatographic technique were done for bioassay and phytochemistry. The results showed that, at concentration of 2 mg/ml, ethyl acetate extract inhibited α-glucosidase equally as positive standard, acarbose as 80% while water and ethanol extracts showed lower activity. Four compounds were isolated as glucosyl-(6-1)-glycerol, mixture of β–sitosterol and stigmasterol, 5-hydroxymethyl-furfural and tyrosol. In addition, 2 isolated compounds, tyrosol and glucosyl-(6-1)-glycerol showed moderate and mild α-glucosidase inhibitory activities with IC_{50} as 1041.5 ± 205.5 µg/ml and 30% inhibition at 1000 µg/ml, respectively when compared with acarbose (IC_{50} = 125.6 ± 9.3 µg/ml). Since, *B. flabellifer* extract and the isolated compounds, tyrosol and glucosyl-(6-1)-glycerol exhibited α–glucosidase inhibition, this plant can be further subjected to *in vivo* anti-diabetic studies.

Key words:α-Glucosidase inhibitor, *Borassus flabellifer*, tyrosol, glucosyl-(6-1)-glycerol, anti-diabetes.

INTRODUCTION

Diabetes mellitus is one of the most important diseases in group of non-communicative diseases (NCD). In recent reports, the number of diabetic patients would dramatically increase from around 400 million in 2013 to almost 600 million in 2035 (Guariguata et al., 2014). In addition, diabetes could lead to the other chronic diseases such as hypertension and diabetes itself and also has serious complications including loss of vision or can cause life threatening event and hyperglycemic shock (Chan et al., 2014). Inhibition of α-glucosidase is one of anti-diabetes strategies and it has been used clinically. Alpha-glucosidase is involved in a carbohydrate metabolism process via hydrolysis of the α-glycosidic bond among polysaccharide chain and release a single glucose molecule, which would be absorbed into the blood stream. Inhibition of this enzyme can slowdown the carbohydrate metabolism and lead to reduction of blood sugar level in the end (Van de Laar, 2008).

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Borassus flabellifer Linn. has a common English name "Sugar palm". *B. flabellifer* is widely spread through Southern Asia including Bangladesh, India, Laos, Myanmar and Thailand, and it has different local names across this region such as "Tal", "Palmyra", "KoK mak tan", "Htan" and "Thann", respectively. Sugar palm has been used by traditional medicine according to the part of the plant, for example; root part treats aphthous ulcer, fever and helminthes; fresh petiole part is used as antidiarrhea (Siri Ruckhachati Nature Park, 2015).

B. flabellifer or sugar palm has been claimed traditionally to treat many disorders such as anti-inflammatory activity, diuretic, sedative and laxative effects (Paschapur et al., 2009). Recently *B. flabellifer* was reported to have anti-diabetic activity in animal model and in inhibition of glucose transportation in *in vitro* study (Uluwaduge et al., 2005; Uluwaduge et al., 2006). However, it is possible that *B. flabellifer* may have more than one mechanism in reducing blood glucose level. Many phytochemical substances from natural products were found, α-glucosidase inhibition (Yin et al., 2014) which is the one of mechanism for anti-diabetes treatment. So, *B. flabellifer* is fascinating in the investigation of the α-glucosidase inhibitory activity of the extracts and isolated compounds. This study will be the first report on α-glucosidase inhibitory activity and phytochemical study of *B. flabellifer*.

MATERIALS AND METHODS

General experiment procedures

UV spectra were prepared in methanol and obtained from a spectronic Genesys 6 and IR spectra were recorded on Perkin Elmer FT-IR by neat technique on NaCl dish. Moreover, NMR spectra were operated in suitable solvents such as deuterated chloroform, methanol and water on NMR spectrometer, Varian unity or Bruker, 300 MHz or 500 MHz (^{1}H - NMR) and 75 MHz or 125 MHz (^{13}C - NMR), respectively and two dimension NMR including HMQC and HMBC were repeated on the same instruments. Mass spectra were obtained from two methods including electron impact mode from Thermofinnigan MAT 95 XL mass spectrometer and electron spray ionization mode from Alliance – micromass Walters 2690 – LCT. The enzymatic reaction was determined at 405 nm on DTX 880 microplate reader. Column chromatography was undertaken on both silica gel 60 H (Merck) and Sephadex® LH – 20 (GE Healthcare). Thin layer chromatography analysis was performed on silica gel 60 GF_{254} (Merck) using 50% sulphuric acid and anisaldehyde - sulphuric reagent as spraying reagents.

Chemicals and reagents

All solvents for extraction and isolation processes were purchased from Thail Oil Co. Ltd., Thailand. Alpha-glucosidase from *Saccharomyces cerevisieae*, *para*–nitrophenyl–alpha–D–glucopyranosidse and acarbose were obtained from Sigma-Aldrich, Germany.

Plant material

The fruits of sugar palm (*B. flabellifer* Linn.) were collected from Singhanakorn district, Songkhla province, Thailand. The voucher specimen number of this plant was SKP 136/217 02 06 01. It was collected at the Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

Extraction and isolation

The fresh sugar palm's fruits were cleaned. Their peels were removed and the yellowish pulps were cut into small pieces, and dried under hot-air oven at 60°C for 5 days. Next, all dried samples were macerated with 4 different solvents, which started with petroleum ether (Pet. ether), ethyl acetate (EtOAc), ethanol (EtOH) and water, respectively. In this step, samples were soaked in solvent for 3 days and then filtrated. The extract was dried by a rotary evaporator and water bath and the residues were re-macerated twice before moved to next solvents. On water part, the samples were boiling at 70°C for 6 h instead of maceration. After drying, all extracts were kept at 4°C until determination.

The EtOH extract (30 g) was loaded over the silica gel (Merck, 0.063 – 0.2 mm) on the quick column chromatography, which started with mobile phase with chloroform ($CHCl_3$) 100% until it reached 60:40:2 ratio of $CHCl_3$, EtOAc and water. All fractions from the column were grouped into three fractions according to their thin layer chromatography (TLC) patterns. The second fraction (F2, 11.5 g) was further isolated by classical column chromatography. After three columns with same mobile system as before, but in different ratio as 70:30:2, one column of Sephadex® LH – 20 (GE healthcare), compound 1 (47 mg) was found.

The EtOAc extract also was isolated by two methods. Firstly, 30 g of the EtOAc extract was loaded on the normal phase quick column chromatography, which used the same solvents system like previous column. After evaluation by TLC pattern, all fractions were combined into five fractions. The second fraction (F2, 19.5 g) was brought to the other columns to seek the pure components. After silica gel column chromatography, the crystals appeared on the fifth fraction (F25, 4.8 mg). So, the crystals were washed by methanol (MeOH) and further purified by Sephadex® LH – 20 columns, by using $CHCl_3$ and MeOH with 50:50 ratio as the mobile phase. So, compound 2 (2.5 mg) was received from this column. However, the first fraction of the second column (F21, 190 mg) showed possibility to isolate another component. Thus, F21 was further isolated with the normal phase and Sephadex® columns until compound 3 (5.3 mg) was obtained. Secondly, the 3 g of this extract was solved with 50:50 mixtures of water and MeOH and it was partition with Pet. ether, EtOAc and $CHCl_3$, consequently. Moreover, compound 4 (0.8 mg) was found from the EtOAc partition part after two classical columns with 80:20 ratio of $CHCl_3$ and EtOAc and one column of Sephadex® with 50:50 mixtures of $CHCl_3$ and MeOH.

In vitro α-glucosidase inhibitory activity

Alpha-glucosidase inhibition was determined for all extracts, which was performed following the previously report assays (Walker et al., 1995; Kim et al., 2008; You et al., 2011) This protocol would be described briefly subsequently. After hydrolysis reaction, a substrate such as *p*-nitrophenyl-alpha-D-glucopyranoside (*p*NPG) turned to *p*-nitrophenol as a product, which had a yellow color and could be detected at 405 nm. Firstly, 50 µl of 8 mg/ml sample solution were mixed in the wells with both 50 µl of phosphate buffer solution, which had 2 mg/ml of bovine serum albumin and 0.2 mg/ml sodium azide (PBS) and 50 µl 1 unit/ml of α-glucosidase enzyme in PBS. In the control well, 5% of DMSO solution was used. Moreover, 8 mg/ml of acarbose solution replaced the sample solution for positive control well. All wells were incubated at 37°C for 2 min before adding the 4 mM of *p*NPG into the mixed wells.

Next, entire reaction was detected at 405 nm every half minutes for 5 min by micro-plate reader. The velocity of the reaction was performed (Equation 1) for calculation of the percent inhibition (Equation 2).

$$\text{Velocity} = \frac{\Delta \text{ Absorbance}}{\Delta \text{ Time}} \quad (1)$$

$$\text{Inhibition (\%)} = \frac{V_{\text{control}} - V_{\text{sample or positive control}}}{V_{\text{control}}} \times 100 \quad (2)$$

The IC_{50} of all compounds was performed cover 6 concentrations starting at 1000 µg/ml and 3 – folds dilution technique was used. The relationships between concentrations and % inhibition would show as a curve graph in both normal and semi – log scales. Furthermore, the IC_{50} values were determined following equation 3, a non – linear relationship (Copeland, 2005) by SPSS version 22.0 program (SPSS Inc., Chicago, Illinois, USA).

$$\text{Inhibition (\%)} = \frac{100}{1+(IC_{50}/\text{Inhibitor conentration})^{\text{hill slope}}} \quad (3)$$

RESULTS

After extraction series of *B. flabellifer* (dried weight, 11.4 kg), all extracts were weighted and calculated as % yield. As a result, EtOH extract showed the highest % yield (15.5 %, 1.7 kg). Water extract were the second (3.64%, 416 mg). Furthermore, the third and the last % yield were on Pet. ether (0.5 %, 63.3 mg) and EtOAc extracts (0.4%, 54.7 mg), respectively. Next, all the samples were evaluated for α-glucosidase inhibitory activity (Figure 1). EtOAc had the strongest activity at 80.1 ± 3.3% that was an equivalent activity to acarbose as standard drug (80.0 ± 3.2 %). The moderate activity was found in both water and EtOH extracts as 55.5 ± 3.8 and 42.8 ± 4.2%, respectively.

To identify an inhibitors in active extracts, EtOAc and EtOH extracts were isolated by column chromatography techniques. After isolation and purification processes, four fractions were obtained; three pure compounds and one mixture component (Figure 2). Three pure compounds were glucosyl-(6-1)-glycerol (compound 1), 5-hydroxymethyl–furfural (compound 3) and tyrosol (compound 4). In addition, one mixture component was a mixture between β–sitosterol and stigmasterol (compound 2).

Compound 1

Compound 1 appeared as yellowish oil. The UV spectrum in MeOH showed absorption band at λ_{max} 202mn, 242 (shoulder) and 284 nm (shoulder), and the IR peaks exhibited at 3367 (-OH), 2931 (-CH_2), 1419 and 1363 (-CH_2) and 1076 cm^{-1} (-CO). The molecular formula of compound1 was determined as $C_9H_{18}O_8$, which deducted which deducted from ESI–MS pseudo–ion peak at 277.1 m/z [M+Na^+] and NMR spectroscopic data, which would be showed next. The ^{1}H–NMR spectrum showed signals in an anomeric region of the sugar moiety. The signals were found at δ 5.13 (H-1) and 4.55 (H-1'), which indicated α- and β-glucopyranose molecules consequently.

First anomeric signal, δ 5.13 (H-1), showed small *J*-value as 3.66 Hz and doublet in splitting pattern, which indicated a coupling constant between H-1 and H-2 of α-glucopyranose. Second signal at 4.55 (H-1') had also the same splitting pattern (doublet) but larger *J*–value as 8.05 Hz than the previous anomeric signal that was a characteristic coupling constant between H-1'' and H-2'' of β-glucopyranose. After that, the remaining protons on both α and β-isomer were determined based on COSY spectrum and the previous report (13) that were shown in Table 1. Finally, the last four signals at δ 3.75 (2H, d, *J* = 7.82 Hz), 3.78 (1H, m), 3.63 (1H, dd, *J* = 5.88 and 12.44 Hz) and 3.70 (1H, dd, *J* = 6.84 and 13.42 Hz) were identified as protons of glyceryl part that were determined at H-1''' to H-3'''A and B, consequently (14). Next, the ^{13}C-NMR spectrum showed fifteen signals. Two signals at δ 89.49 (C-1) and 93.31 (C-1'') were assigned as anomeric carbons of α-glucopyranose and β-glucopyranose, respectively. The other signals were assigned by comparison with the previous report (13). The last three signals were defined as carbons of glyceryl residue including δ 66.77, 68.85 and 60.62, which were C-1''' to C-3''' (15). Finally, the linkage bonds between glucopyranosyl and glyceryl structures were indicated by HMBC. According to the entire spectroscopic data, mass spectrum and by comparing all spectroscopic data with the previous reports (Bondu et al., 2007; Goffin et al., 2009; Pretsch et al., 2009; Nihira et al., 2014), compound 1 was identified as glucosyl-(6-1)-glycerol. The summary NMR spectrum of compound 1 was shown on Table 1.

Compound 2

Compound 2 is colorless needles. The UV spectrum in MeOH showed absorption band at λ_{max} 202 and 238 nm (shoulder), IR (NaCl, neat) 3363 (-OH), 2933 and 2863 cm^{-1} (-CH_3 and –CH_2), 1679 (C=C), 1457 and 1374 (-CH_3 and –CH_2) and 1051 cm^{-1} (-CO) ,^{1}H – NMR (500 MHz, $CDCl_3$) and ^{13}C – NMR (125 MHz, $CDCl_3$) were in close agreement with the recent published article (Abdel-Wahab et al. 2014). So, compound 2 was established as a mixture of β–sitosterol and stigmasterol.

Compound 3

Compound 3 is yellowish oil, The UV spectrum in MeOH showed absorption band at λ_{max} 202nm, 222

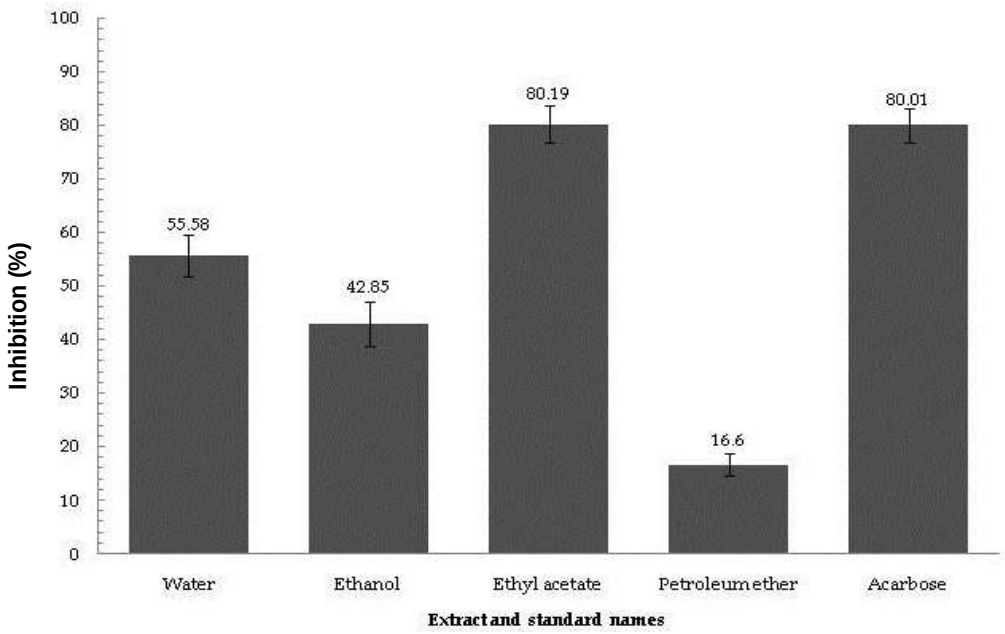

Figure 1. Alpha-glucosidase inhibitory activity of *B. flabellifer* extracts and standard drug, acarbose.

(shoulder) and 280 nm. IR (NaCl, neat) 3366 (-OH), 2924 and 2815 cm^{-1} (–CH_2), 1734 (-C=O), 1672 (C=C), 1419 (-CH_2) and 1188 cm^{-1} (-CO). ^{1}H – NMR (500 MHz, CD_3OD) δ_H 9.53 (H-1, 1H, *s*), δ_H 7.37 (H-3, 1H, *d*, *J* = 3.50 Hz), δ_H 6.57 (H-4, 1H, *d*, *J* = 3.50 HZ) and δ_H 4.60 (H-6, 2H, *s*). ^{13}C–NMR (125 MHz, CD_3OD) δ_C 179.42 (C-1), δ_C 163.23 (C-5), δ_C 153.98 (C-2), δ_C 124 (C-4), δ_C 110 (C-3) and δ_C 57.65 (C-6). EI-MS m/z 125.9 (97), 108.9 (97), 96.9 (70) and 69.0 (41). The spectroscopic data was confirmed with previous report (Khalil et al., 2003). So, compound 3 was 5–hydroxymethyl–furfural.

Compound 4

Compound 4 is a yellowish powder. The UV spectrum in MeOH exhibited λ_{max} at 202nm, 224 and 178 nm. The IR spectrum showed several peaks at 3369 (-OH), 2919 and 2845 (-CH_2 and –CH_3), 1697 (overtone of =CH), 1596 and 1384 cm^{-1} (aromatic C=C), 1121 (-CO) and 828 cm^{-1} (=CH). The molecular compound ($C_8H_{10}O_2$) was deducted from NMR data and EI-MS that showed the molecular ion peak at 138 m/z $[M]^+$, base peak at 107 m/z $[M\text{-}CH_2OH]^+$ and aromatic peak at 77 m/z $[M\text{-}H_2O\text{-}C_2H_5OH]^+$. Moreover, the 1H–NMR spectrum exhibited important signals in aromatic region at δ_H 7.00 (H-2 and 5, 2H, *d*, *J*= 8.54 Hz) and δ_H 6.67 (H-3 and 6, 2H, d, J = 8.66 Hz). This information indicated aromatic structure with para - substitution. In addition, other signals appeared at δ H 3.66 (H-8, 2H, t, J = 7.20 Hz) and □ H 2.69 (H-7, 2H, t, J = 7.14 Hz).

These signals were assigned as an ethyl alcohol, connected with phenyl structure. After matching all information with a recent report, compound 4 could be determined as tyrosol (Guzmán-López et al., 2007).

In *in vitro,* α–glucosidase inhibitory activity and IC_{50} determination, all isolation of compounds took place for identification of inhibitors and the results were reported visually in concentrations response curve (Figure 3A and B).

Firstly, the maximum concentration of all samples started at 1000 µg/ml and 3-folds dilution was used to dilute concentrations of all until reaching last concentration, 4.1 µg/ml. As a result, acarbose (positive control), tyrosol (compound 4) and glucosyl-(6-1)-glycerol (compound 1) could inhibit α–glucosidase enzyme in dose dependent manner.

Starting with standard drug, acarbose demonstrated the highest activity at IC_{50} of 125.6 ± 9.3 µg/ml and hill coefficient of acarbose was 0.6. In addition, tyrosol showed the moderate inhibitory activity with this enzyme at IC_{50} value as 1041.2 ± 203.5 µg/ml and hill coefficient of tyrosol was 0.3, which was lower than acarbose. Lastly, glucosyl-(6-1)-glycerol also could inhibit α–glucosidase enzyme but with mild activity, 29.2% inhibition at the concentration of 1000 µg/ml (Table 2). Fortunately, glucosyl-(6-1)-glycerol had enough amounts for further investigation while the others were not. So, the concentration of glucosyl-(6-1)-glycerol was enhanced from 1000 µg/ml to 4000 µg/ml for IC50 evaluation once again. Same as before, the 3-folds diluted protocol was applied until the lowest concentration became 16.4 µg/ml. Finally, the IC50 value of glucosyl-(6-1)-glycerol was obtained at 4000.0 ± 687.0 µg/ml with hill coefficient at 0.5 values, which closed to acarbose.

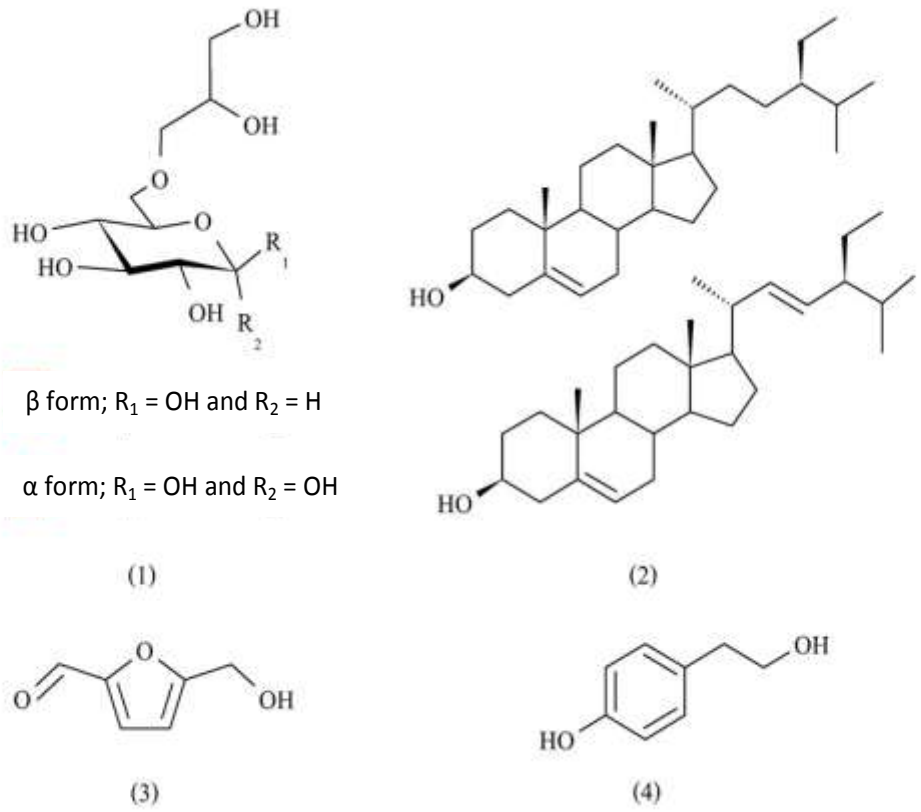

Figure 2. Four isolated compounds from *B. flabellifer*: (1) Glucosyl-(6-1)-glycerol (compound 1); (2) the mixture of β-sitosterol and stigmasterol (compound 2); (3) 5-hydroxymethylfurfural (compound 3) and (4) tyrosol (compound 4).

Table 1. ^{1}H-^{13}C NMR of glucosyl-(6-1)-glycerol (compound 1).

Position	^{13}C-NMR	^{1}H-NMR		
	δ	δ	Pattern	*J* value
1	89.49	5.13	d	3.66
2	68.89	3.44	dd	3.66/ 9.52
3	70.17	3.62	dd	9.52/ 9.76
4	67.07*	3.31	t	9.52
5	68.28*	3.73	ddd	10.00/ 5.37/ 2.40
6A	58.02*	3.58	dd	6.59/ 12.21
6B		3.81	dd	2.20/ 12.20
1'	93.31	4.55	d	8.05
2'	71.55	3.15	dd	8.05/ 9.27
3'	73.35	3.39	t	9.2
4'	67.02*	3.30	dd	9.27/ 9.58
5'	73.17*	3.37	ddd	9.27/ 4.89/ 2.20
6'A	58.17*	3.67	dd	5.37/ 12.21
6'B		3.81	dd	2.20/ 12.20
1''	66.77*	3.75*	d	7.82
2''	68.85*	3.78	m	-
3''	60.62*	3.63	dd	5.88/ 12.44
		3.70	dd	6.84/ 13.42

*Show the ^{1}H-^{13}C correlation on the structure base on HMBC data.

Table 2. The IC_{50} determination of isolated compounds from *B. flabellifer*.

Compound Name	IC_{50} (µg/ml)	Standard error (SE)	Hill coefficient	Maximum inhibition at the highest concentration (%)
Acarbose	125.6	9.3	0.6	81.0
Tyrosol	1041.5	205.5	0.3	51.7
Glucosyl-(6-1)-glycerol	4000.0	687.0	0.5	50.0
	> 1500.0	-	-	29.2
Mixture of steroids	> 1500.0‡	-	-	13.1
5 – Hydroxymethyl - furfural	> 1500.0‡	-	-	8.9

‡Indicate insufficient amount for testing.

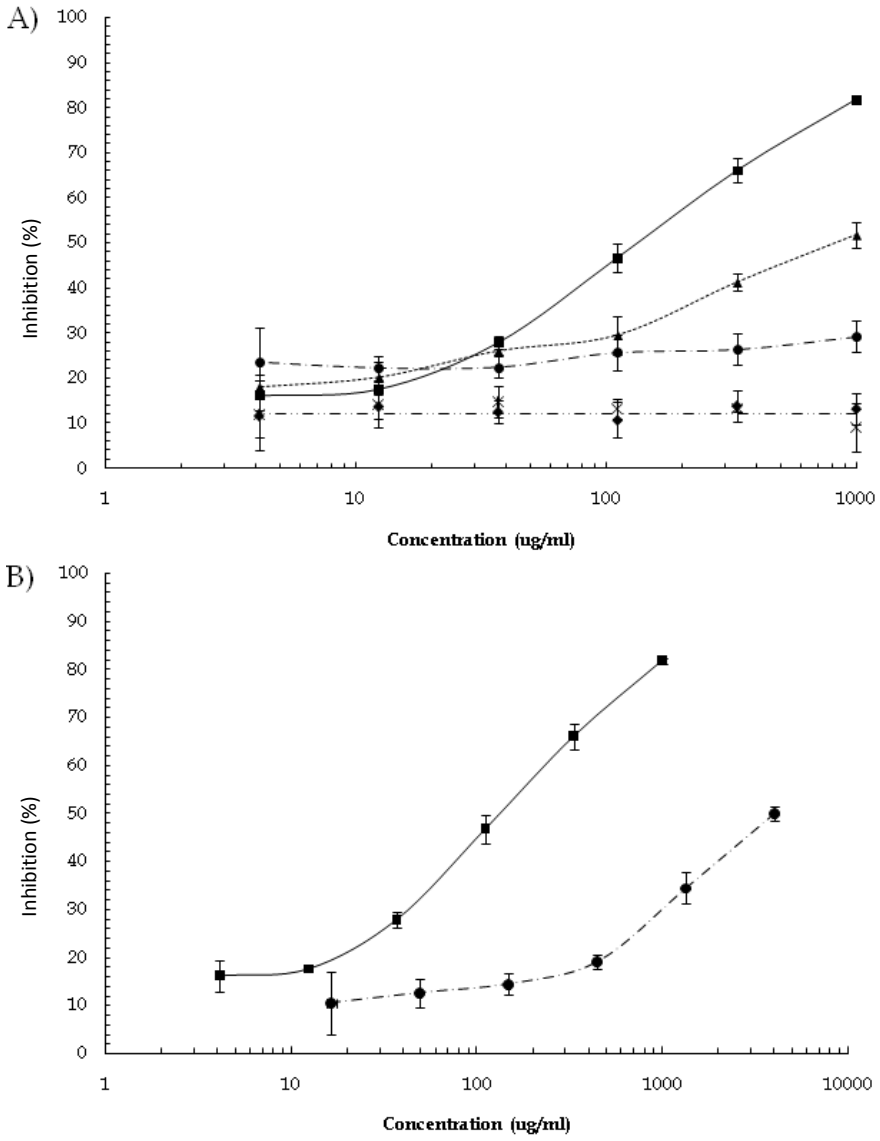

Figure 3. A) The concentration response curve at 1000 µg/ml of isolated compounds, as glucosyl-(6-1)-glycerol (Compound 1; ●), the mixture of β-sitosterol and stigmasterol (Compound 2; ◆), 5–hydroxymethyl–furfural (Compound 3; ∗), tyrosol (Compound 4; ▲), and standard drug (acarbose; ■); B) The semi-log curve of the concentration response curve at 4000 µg/ml of glucosyl-(6-1)-glycerol (Compound 1; ●) as compared to standard drug (acarbose; ■) at 1000 µg/ml.

DISCUSSION

In summary, the EtOAc extract of *B. flabellifer* could inhibit α–glucosidase like acarbose in the screening step at 80% inhibition, while water and EtOH extracts inhibited the same enzyme at lower potency, 55.5 and 42.8% inhibition, respectively (Figure 1). After phytochemical investigation of EtOAc and EtOH extracts, four components were found as glucosyl-(6-1)-glycerol (compound 1), the mixture of β-sitosterol and stigmatosterol (compound 2), 5-hydroxymethyl-furfural (compound 3) and tyrosol (compound 4), which are shown in Figure 2. However, only two compounds could inhibit α–glucosidase. Firstly, tyrosol exhibited the lower IC_{50} value than glucosyl-(6-1)-glycerol (1041.2 ± 203.5 and 4000.0 ± 687.0 µg/ml, accordingly). However, tyrosol had a smaller structure, at 138 g/mol, when compared with the structure of glucosyl-(6-1)-glycerol, which was 254.23 g/mol. Based on this fact, the activity of both compounds was almost close to others in term of molarity as 15.73 mM for tyrosol and 9.60 mM for glucosyl-(6-1)-glycerol, respectively (Figure 3). In detail, hill coefficient values had been used commonly in pharmacology to evaluate the stoichiometric interaction between inhibitor and target enzyme, which could give clue about the behavior of inhibitor (Copeland, 2005). Tyrosol had hill coefficient value lower than acarbose, which was known as a competitive behavior that one molecule of inhibitor could bind at one active site of enzyme. The lower hill value of tyrosol might indicate that tyrosol more than one molecule could interact with an enzyme, which was possibly a behavior of non-competitive inhibitor. Next, glucosyl-(6-1)-glycerol had a hill coefficient value was almost close to that of acarbose. So, it was possible that glucosyl-(6-1)-glycerol could have the same behavior like acarbose. Moreover, the structure of glucosyl-(6-1)-glycerol could also be used to support this finding because its structure was interpreted as a carbohydrate derivative.

In addition, the result of this study showed the correlation with the recent reports. The previous reports showed that *B. flabellifer* could reduce the blood sugar level in animal model (Uluwaduge et al., 2006) and in clinical pilot study (Uluwaduge et al., 2008). Besides, the mechanism that might be response was reported as Na/K ATPase (Uluwaduge et al., 2005), which involved the glucose transportation process from intestinal lumen to blood stream. The steroidal saponins were claimed as a active components (Ariyasena et al., 2010). Moreover, the findings in this study demonstrated the other possible mechanism (α–glucosidase inhibition), which is also involved in carbohydrate metabolism process, and active components (tyrosol as phenolic compound) that could be response in anti–diabetes activity of *B. flabellifer*.

Eventually, it is quite clear that *B. flabellifer* has potency in anti-diabetes activity according to the scientific evidences until now. Moreover, the results of this study also supported that *B. flabellifer* extracts and its components could inhibit α–glucosidase that was one of mechanisms for lowing blood sugar because there are many mechanisms, which can reduce glucose level.

Conclusion

B. flabellifer extract and the isolated compounds, tyrosol and glucosyl-(6-1)-glycerol showed α–glucosidase inhibitory activity. Further, the plant has to be explored for anti-diabetic activities.

Conflict of Interests

The authors do not have any conflict of interest for this work.

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