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ORIGINAL ARTICLE **OPEN ACCESS**

Toxicity and hypoglycemic effect of tannin-containing extract from the mangrove tree *Sonneratia alba* Sm.

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ABSTRACT
The diverse ethno-medicinal value of tropical mangrove trees is widely known, but few studies on their toxicity and anti-diabetic properties have been conducted. Methanolic leaf extracts from the mangrove plant, Sonneratia alba Sm. were investigated for toxicity and hypoglycemic properties. Methanol was used to obtain crude leaf extract, while column chromatography, gradient and isocratic elution and liquid separation were utilized to extract tannin-containing polar fraction. Brine Shrimp Lethality Test was used to analyze toxicity properties of crude and tannin-containing extracts. Hypoglycemic effects of the tannin-containing extract was investigated using streptozotocin (STZ)-induced hyperglycemic IRC-mice, Mus musculus. Following a completely randomized design experiment, we injected the tannin-containing polar fraction intraperitoneally to hyperglycemic mice once daily for 9 days. The crude extract showed mild toxicity, but the fraction recovered from liquid separation was not toxic to brine shrimp larvae. STZ-induced hyperglycemic mice showed a significant drop in blood sugar level by an average of 39.6% 6 hours after leaf extract injection and 56.4% 12 hours after injection, with decreased blood sugar levels comparable to those in control mice. This study revealed the hypoglycemic potential of the non-toxic tannin-containing polar fraction of S. alba leaf extract, and this finding underscores the potential economic value of tropical mangrove trees as source of natural anti-diabetic drug.
Keywords:*hypoglycemia, STZ-induced diabetic mice, brine shrimp toxicity test, mangrove, Sonneratia alba.*

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INTRODUCTION

Mangrove plants perform vital ecological roles for a wide variety of animals and other species as areas for habitat, foraging, roosting, breeding, and other activities [1,2]. Mangroves also buffer coastal zones from strong weather disturbances, protect shorelines, help prevent coastal erosion, and protect vital coral reefs and sea grass beds from the damaging siltation due to the filtering effects of mangrove forests [2,3]. We believe that studies on the potential medicinal values of mangrove plants may further enhance protection and conservation of dwindling mangrove forest resources in many tropical regions.

Reaching a global prevalence of 360 million by 2030, the chronic disorder diabetes mellitus affect 2.8% of the global population attributed mainly to lifestyle, unhealthy food products, and genetics [4,5]. The disorder is often associated with obesity, such that in the USA alone, up to 90% of the obese and type 2 diabetic patients are characterized by hyperinsulinemia, hyperlipidemia, and hyperglycemia [6]. Pharmaceutical companies have introduced a variety of hypoglycemic and/or anti-hyperglycemic drugs to treat diabetes, but most of these are adipogenic or promoting weight gain [6].

Various medicinal plant extracts have been tested to treat diabetes mellitus and showed insulin-like properties [5,7]. For instance, crude polysaccharide from purslane had hypoglycemic effect in mice [8]. Hypoglycemic activity, improvement in lipid profile and regeneration of beta cells of pancreas were reported in alloxan-induced diabetic rats treated with *Cassia occidentalis* extract [9]. Extracts from *Linumusitatisumum*, *Morus alba*, and *Ocimum tenuiflorum* have shown potential porcine pancreatic alpha amylase inhibition leading to a reduction in starch hydrolysis lowering thereby glucose levels [10]. Similarly, root extract of *Circium japonicum* showed inhibition of carbohydrate-hydrolyzing enzyme

alpha-glucosidase activity [11]. Another species, *Lagerstroemia speciosa* L. of Family Lythraceae, has both anti-diabetic and anti-adipogenic apart from its diuretic properties [6,12].

Anti-diabetic medicinal properties of a few mangrove tree species have been reported [13]. For example, *Ceriops decandra*, possesses antidiabetic property comparable with that of the synthetic antidiabetic drug glibenclamide [14], while triterpenoids from *Rhizophora mangle* has been clinically used in the control of diabetes [15]. Recently, Gurudeeban et al. (2012) [16] reported anti-diabetes dipeptidyl peptidase IV inhibitors from extracts of *Rhizophora mucronata*, while Prabhu and Guruvayoorappan (2012) [13] have identified hypoglycemic compounds from *Avicennia marina*. We recently reported the hypoglycemic potential of slightly insoluble polysaccharides from the extract of *S. alba* in mice [17]. However, the toxicity of extracts from mangrove and other medicinal plants is rarely evaluated [18] (Krishnaraju et al., 2005).

Molecular markers are said to be similar among closely related species belonging to the same family or taxon [19]. Since tannins have been identified as the active antidiabetic and antiadipogenic components of *Lagerstroemia speciosa*, we explored the marine member of Family Lythraceae, the mangrove tree species *Sonneratia alba* Sm., which is rich in tannin that may show hypoglycemic property [19,20]. The present study was designed to determine toxicity and hypoglycemic properties of tannin-containing polar fraction from *Sonneratia alba* Sm. leaf extract. Specifically, this study aimed to (1) extract tannin-containing polar fraction from *S. alba* leaves using flash column chromatography with liquid separation, (2) determine the toxicity level of the crude and polar fraction extracts using the Brine Shrimp Lethality Test, and (3) measure blood sugar level in streptozotocin (STZ)-induced hyperglycemic IRC-mice treated and untreated with the tannin-containing polar fraction.

MATERIALS AND METHODS

Sampling and drying of the plant materials

Plant sample of 900 grams of *S. alba* leaves was obtained from Kauswagan, Lanao del Norte, Philippines (Greenwich coordinates: 8o 11' 13'' N, 124o 06' 23''E). Leaves were washed with distilled water to remove the dust on leaf surface. Clean leaves were placed in net bags air-dried for at least two weeks. Air-dried leaves were macerated and pulverized using a blender prior to the extraction process.

Extraction

The entire 900 g of air-dried and pulverized leaf samples of *S. alba* were cut into smaller pieces and soaked with 3.75 L methanol for 7 days. Supernatant liquid was dried *in vacuo* using a rotary evaporator (Buchi R110, Japan) and the residues that comprised the crude extract were then reconstituted to a small volume (ca. 100 mL).

Isolation by flash column chromatography and liquid separation

The reconstituted crude extract was loaded to hp-20 column (9.7 cm x 15 cm). The column was eluted following step-wise gradient with the following concentrations: 50%, 70%, 90% and 100% methanol:water, respectively. Elution volume was two times its column volume. Each fraction was collected separately. Since tannin was identified in Lagerstroemia speciosa leaf extract by Liu et al. (2005) to have anti-diabetic effect, chemical assay for the presence of tannin was done using a modified colorimetric method of [21]. Collected fractions from the stepwise gradient were tested for the presence of tannin by adding 0.1% $FeCl_3$ and observed for brownish green or blue-black coloration.

Majority of the tannins were detected on fractions that were eluted with 50:50 methanol: water (v/v) and so this was then evaporated to dryness and loaded again to silica column with isocratic elution of 60:40 water:methanol (v/v) to obtain more of these molecules at decreased methanol concentration. Fractions that were positive for the presence of tannin were collected and then dried *in vacuo* for further purification.

Liquid separation

Since tannin is polar, the non-polar components from the fraction recovered from isocratic elution were removed through liquid partitioning using methanol:water:ethylacetate (5:25:70, respectively, v/v ratio). The tannin-containing polar fraction recovered through liquid separation was then evaporated to dryness in vacuo and was reconstituted for blood sugar assay.

Reconstitution of fraction

The dried tannin-containing polar fraction from *S. alba* leaf extract was reconstituted at 32 mg per ml of sterile normal saline water with a dosage of 16 mg per 20 grams body weight of mice [17]. This dosage was used as a working solution for the hypoglycemic assay.

Toxicity test

The Brine Shrimp Lethality Test assay was done to investigate the toxicity of the methanol crude extract and the polar fraction recovered through liquid separation. The protocol described by Meyer et al. (1982),

McLaughlin (1991) and Krishnaraju et al. (2005) [18,22,23] was followed with slight modifications. Test samples of the plant extracts, in triplicate, were prepared as follows: one hundred (100) mg each of the crude extract/fraction was initially dissolved in 10mL of the solvent dimethyl sulfoxide (DMSO) and further diluted with boiled filtered sea water to produce the required concentrations. Appropriate amounts of extracts (5000-, 500-, 50- and 5- µl) for dosages of 5000-, 1,000-, 100- and 10-ppm, respectively were transferred to vials containing small filter paper discs, air-dried overnight to evaporate the solvent, and further dried under nitrogen gas. Ten brine shrimp larvae were transferred to each sample vial and boiled filtered seawater was added to make 5 ml final volume in each vial. Tests for each concentration were done in triplicate. Dimethyl sulfoxide (DMSO) and podophylotoxin were used as the control set up for the four concentrations in triplicates. All the vials were maintained under illumination. The number of dead and alive nauplii was counted after 6 and 24 h. The results were evaluated and the acute and chronic LC50 values were determined using the Reed-Muench method.

Induction of mice to hyperglycemic condition

Induction of mice to hyperglycemic condition was done using a modified procedure used byKlein et al. (2007) [6]. Hyperglycemia was induced in healthy mice that were fasted for 6 hours by a single intraperitoneal injection of streptozotocin (STZ) (Sigma-Aldrich Co., Missouri, USA), which was dissolved in 3 mM citrate buffer with pH 4.5 at a dose of 50 mg/kg body weight. Streptozotocin (2-deoxy-2 ({[methyl(nitroso) amino]carbonyl}amino)-β-D-glucopyranose) acts as an alkalyting agent of DNA in β cells of the Islets of Langerhans in the pancreas, and is a better alternative in inducing hyperglycemia in mice [24]. Fasted mice remained active and appeared normal with their behavior. After 48 hours of STZ injection, mice that exhibited plasma glucose level of >180 mg/dl were included in the experiment that tested the effect of S. alba leaf extract. Diabetes was confirmed by the determination of tail vein blood glucose levels at 0, 6 and 12 hours three days after administration of STZ. The blood sugar concentration in mg/dl was determined using a glucometer (Omnitest®, Melsungen, Germany).

Treatment groups

Following a completely randomized design, 24 IRC-mice were used and assigned into the following four treatment groups, each replicated six times with one replicate comprising one mouse:

a) Group I (Negative Control) - Healthy mice with normal blood glucose level and were not given sample extract injection.

b) Group II (Positive Control 1) - Healthy mice with normal blood glucose level, but received sterile normal saline via intraperitoneal injection.

c) Group III (Positive Control 2) - Healthy mice with normal blood glucose level, but were given tannin-containing polar extract via intraperitoneal injection.

d) Group IV (Hyperglycemic Group) - STZ-induced hyperglycemic mice that were given intraperitoneal injection of tannin-containing polar extract of the same dose as applied to Group III.

Albino male mice strain-IRC with a weight range of 20-25 grams were obtained from the National Science Research Institute of University of the Philippines - Diliman, Quezon City. Permit to conduct animal research was taken from the Bureau of Animal Industry-Animal Welfare Association (BAI-AWA), Quezon City, Philippines. All experimental animals were acclimatized for one week in their respective cages made of plastic embedded with wood shavings and covered with a metal screen on top. One mouse was assigned to a cage and mice were fed ad libitum with water and pellets bought from agricultural supply containing crude protein, 16%; crude fiber 10%; crude fat 3% and moisture 13%. The administration of tannin-containing polar fraction from leaf extract of S. alba was done to non-diabetic and STZ-induced diabetic mice (Groups III and IV, respectively) through intraperitonial injection once daily for 9 days using tuberculin syringe with ultrafine needle. Glucose level changes were observed within 0-, 6-, 12-hour period after mice were fasted for 12 hours prior to experimentation.

Measurement of blood glucose levels

All blood sugar levels were monitored using a glucometer (Omnitest®, Melsungen, Germany) at 0, 6 and 12 hours after injection of the leaf extract during diabetic condition. Blood sugar levels were determined prior to mouse induction to diabetes. To measure blood glucose level, the slit of the glucometer strip was filled completely with drops of blood that was obtained from the tail of the animal. This was then inserted into the glucometer for reading of the glucose content in the blood expressed in mg/dl.

Statistical analysis

A one-way analysis of variance was computed to determine variations of blood glucose readings across days and treatment groups and interactions between days and treatment groups. Blood glucose levels across treatments were averaged and standard error among replicates was computed. Tukey's Honestly Significant Difference test was used to compare average values among treatments. All statistical analyses used SPSS version 11 [25].

RESULT

Acute (6 hours) and chronic (24 hours) exposures to the crude methanol extract of S. alba leaves and fraction recovered through liquid separation showed varying results (Table 1). The LC50 values for the crude extract were 817.5 ppm and 515.8 ppm for both acute and chronic exposures, respectively, indicating mild toxicity. In contrast, LC50 for the liquid separation fraction was >1000 ppm for both acute and chronic exposures suggesting no sign of toxicity to the brine shrimps.

Table 1. Acute (6 hours) and chronic (24 hours) toxicity test results of the S. alba leaf crude methanol extract and fraction recovered through liquid separation against the brine shrimp Artemia salina.

Extract	Dose (ppm)	Log Dose	After 6 hours % Mortality		After 24 hours % Mortality	
Crude	5,000	3.0	56.8	LC_{50} =	74.4	LC_{50} =
	1,000	2.7	15.2	817.5	29.6	515.8
	100	2.0	0.0	ppm	6.6	ppm
	10	1.0	0.0		0.0	
Liquid Separation	5,000	3.0	18.8	LC_{50} =	32.4	LC_{50} =
	1,000	2.7	3.6	>1000	7.4	>1000
	100	2.0	0.0	ppm	1.2	ppm
	10	1.0	0.0		0.9	

Figure 1A shows the variation of the mean fasting blood sugar for 9 days among groups before the tannin-containing polar fraction from *S. alba* leaf extract was injected. Group IV (hyperglycemic mice) showed the highest mean (340.3 mg/dl) on day 3. Groups I, II and III are all in the normal range of blood sugar with highest mean values of 108.7, 83.3 and 90.7 mg/dl, respectively. Blood sugar levels varied significantly (F = 29.70, df = 3, p< 0.000) among the different groups of mice before the injection of tannin-containing polar fraction from *S. alba* leaf extract injection. This was attributed to the high levels of blood sugar in STZ-induced hyperglycemic mice compared to the other mice groups that were non-diabetic. However, blood sugar levels in mice across days did not vary significantly (F = 0.56, df = 8, p> 0.80), and there were no interactions between mice groups and observation period (F = 0.56, df = 24, p> 0.96).

A decrease of mean blood sugar level among diabetic mice was shown 6 hours after the tannin-containing polar fraction from *S. alba* leaf extract was injected (Figure 1B). Over 9 days, the highest observed mean in Group IV mice was 247.3 mg/dl and the lowest was 42.7 mg/dl on day 8. Based on values at 0 hours from Group IV mice, the average decrease over 9 days in blood sugar levels was 39.6 ± 31.7 SD %. The lowest value was comparable to the lowest mean value of 34 mg/dl in healthy untreated mice (Group I). Control Groups I and II and the non-diabetic group (Group III) that received the tannin-containing polar fraction from *S. alba* leaf extract injection remained at normal mean blood sugar levels with maximum values of 108.0, 94.3, 72.7 mg/dl, respectively. Six hours after injection tannin-containing polar fraction from *S. alba* leaf extract, the blood sugar levels in STZ-induced hyperglycemic mice were still significantly higher than those in other groups (F = 13.68, df = 3, p< 0.000), but a slight decrease in blood sugar was already observed among these hyperglycemic mice. Blood sugar levels in mice were similar across days (F = 1.34, df = 8, p> 0.23) and there was no interaction between blood sugar values by days and groups of mice (F = 1.59, df = 24, p> 0.06).

Decrease in mean levels of the blood sugar among diabetic and non-diabetic mice became more pronounced 12 hours after the injection of tannin-containing polar fraction from *S. alba* leaf extract, and the drop of blood sugar among diabetic mice was almost toward the normal level (i.e., range over 9 days = 40.5-192.0 mg/dl) (Figure 1C). In reference to values at 0 hours from Group IV mice, the average decline in blood sugar levels was 56.4 ± 25.0 SD %. Blood sugar levels of diabetic mice were still significantly higher than the non-diabetic groups though a large decrease in blood sugar was observed among diabetic mice 12 hours after the tannin-containing polar fraction from *S. alba* leaf extract injection (F = 6.52, df = 3, p< 0.001). However, as the days progressed, the diabetic group showed a statistical interaction with the non-diabetic mice in terms of blood sugar level (F = 1.80, df = 24, p< 0.05). This result indicates that as days went by, the blood sugar of hyperglycemic mice dropped toward a normal mean levels (Group I), which is 57.3-106.3 mg/dl. This implies that the tannin-containing polar fraction from *S. alba* leaf extract

which was injected to the hyperglycemic mice shows a hypoglycemic property, thus resulting to a significant decrease in the blood sugar. Blood sugar values within each treatment group, however, remained comparable across days (F = 1.75, df = 8, p> 0.10).

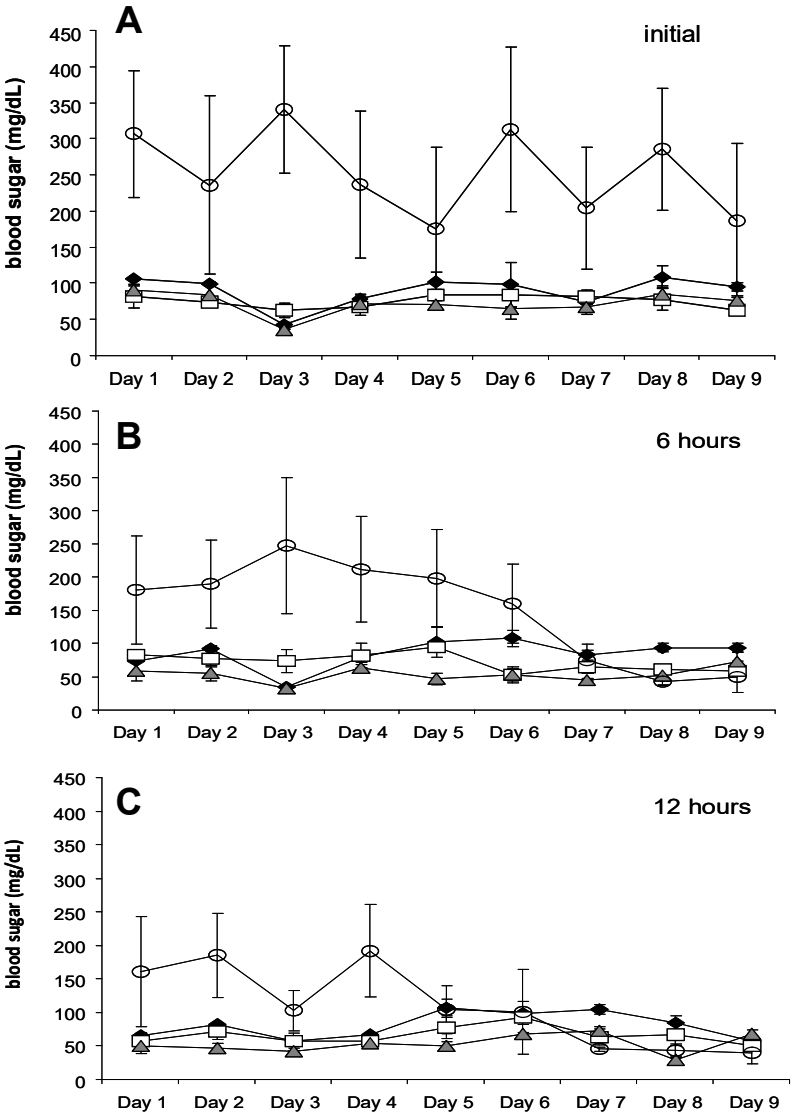

Figure 1. Mean blood sugar level in IRC mice over nine (9) days observation period: (A) before injection of tannin-containing polar extract from *S. alba* leaves (values are equivalent to a six-hour fasting blood sugar); (B) six hours after injection of the extract; and (C) 12 hours after injection of the extract. Error bars = standard error. Legend: diamond = Group I (normal non-diabetic uninjected IRC mice); square = Group II (normal non-diabetic IRC mice injected with sterile normal saline alone); triangle = Group III (normal non-diabetic IRC mice injected with tannin-rich *S. alba* extract); circle = (Group IV) STZ-induced hyperglycemic IRC mice, injected with tannin-containing polar *S. alba* extract.

DISCUSSION

The LC_{50} for the crude extract of both acute and chronic exposures indicates mild toxicity[21, 22,23]. In contrast, LC_{50} for the liquid separation (tannin-containing) fraction for both acute and chronic exposures was>1000 ppm suggesting no sign of toxicity to the brine shrimps[21,22]. We attribute this to the removal of the phenolic and alkaloid compounds and other secondary metabolites during further purification of the extract through liquid separation [26]. Phenols and alkaloids are identified to be toxic to many animals[27].

The pattern of hypoglycemic effect observed in this study is similar to those reported by Nabeel *et al*. (2010)[14] on the mangrove species, *Ceriops decandra,* ethanolleaf extract showing the hypoglycemic

property among alloxan-induced diabetic rats. However, the main difference is that this study used less than half the dose (50 mg/kg) of the extract used by Nabeel et al. (2010)[14], and the dramatic hypoglycemic effect of *S. alba* extract was relatively rapid as the blood sugar decrease was shown within 6 hours.

Recently, we reported the blood sugar lowering property of complex polysaccharides from *S. alba* extract [17]. We surmise that the tannins in our extract may belong to the polysaccharides we found in our earlier study. However, whether both molecules are the same and not, the present study has demonstrated that tannins are as hypoglycemic as polysaccharides. The abundance of tannins in the mangrove tree, *S. alba,* is not surprising because the species is a close relative of *Lagerstroemia speciosa* in the Family Lythraceae [19,20] with the latter species known for its tannic acid that have a stimulatory property in the glucose uptake and adipocyte differentiation of 3T3 L1 cultured cells [28]. In particular, the same tannic acid molecule has the ability to induce phosphorylation of insulin receptor IR and Akt as well as translocation of glucose transporter GLUT 4 whilst inhibiting the expression of key genes for adipogenesis in 3T3 L1 cultured cells[28]. Another possible hypoglycemic mechanism is the control of starch breakdown via the inhibitory effect of tannins on pancreatic □-amylase[10] (Sudha et al., 2011). Since most antidiabetic medicinal plants revealed the presence of polysaccharides, alkaloids, proteins, cardiac glycosides, flavonoids, saponins and steroids apart from tannins[29] (Ponnusamy et al., 2011), it is very likely that tannins show synergistic effect with other bioactive compounds with an ultimate outcome of attenuating hyperglycemia.

CONCLUSION

In conclusion, this study observed and substantiated that the species of mangrove tree that belongs to Family Lythraceae, *Sonneratia alba* demonstrated non-toxic and hypoglycemic properties. It manifested significant decrease in the blood sugar level among those hyperglycemic-induced IRC-mice 6 hours after intraperitoneal injection. The active substance is associated with tannin-containing polar fraction from *S. alba* leaf extract. It is recommended that the identification and mechanism of action(s) of the tannin be further studied using bioassay-guided procedures.

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