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Original Article

Antihyperglycemic activity of agarwood leaf extracts in STZ-induced diabetic rats and glucose uptake enhancement activity in rat adipocytes

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Abstract

Agarwood leaf extract was found to possess antipyretic, laxative and antimicrobial activities. A trial of one diabetic patient taking long term agarwood leaf tea was found to have blood glucose returned to normal. In this work, the effects of methanol, water and hexane crude extracts of agarwood leaf on hyperglycemia in streptozotocin-induced diabetic rats were investigated. Only methanol and water extracts at the dose of 1 g/kg body weight lowered the fasting blood glucose levels, 54 and 40%, respectively. The results were comparable to 4 U/kg body weight of insulin (73%). In *in vitro* experiment, the effect of the methanol and water extracts at the concentration of 10 μg/mL enhanced glucose uptake activity on rat adipocytes by 172±10 and 176±21% of the control, respectively. The glucose uptake enhancement activity is comparable to 1.5 nM insulin (166±16%). The thin-layer chromatographic characteristics of all extracts were documented. The findings suggest that agarwood leaf is a promising potential antidiabetic agent.

Keywords: agarwood, anti-hyperglycemic activity, anti-diabetes mellitus, glucose uptake, rat adipocytes.

1. Introduction

At present, herbs are widely used to control diabetes mellitus (Ranilla *et al.*, 2010; Loarca-Pina *et al.*, 2010; Chee *et al.*, 2007). Some antidiabetic herbs have been reported to exert the activity by enhancing insulin secretion (Gray *et al.*, 1999), or by mimicking insulin activity (Gray *et al.*, 1998). Aquilaria or agarwood (*Aquilaria sinensis* Lour., Thymelaeaceae) is now widely cultivated for its resin. Various parts of *Aquilaria spp.* were reported to have several pharmacological activities; the aqueous extract of *A. agallocha* stem decreases hypersensitivity (Kim *et al.*, 1997), the benzene extract of agarwood possesses potent central nervous system depressant activities (Kim *et al.*, 1997). Jinkoh-eremol and agarospiral from agarwood given by peritoneal and intracereboventricular administration exert positive effects on the central nervous system and decrease both methamphetamine- and apomorphine-induced spontaneous locomotion in mice (Okugawa *et al.*, 1993; Okugawa *et al.*, 1994). Ethyl acetate extract of *Aquilaria agallocha* inhibits nitrite-induced methaemoglobin formation (Miniyar *et al.*, 2008). Oral administration of acetone extract of agarwood (*A. sinensis*) leaves was found to have laxative activity in mice (Hideaki *et al.*, 2008). Some other activities of agarwood extracts are antipyretic, anti-inflammatory (Zhou *et al.*, 2008) and antimicrobial (Dash *et al.*, 2008). There was a report of one diabetic patient who drank water infusion of agarwood leaf instead of water for 6 months. The blood glucose of this patient decreased from 184 mg/dL to 128 mg/dL. After that,

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the tea was taken twice a day, once in the morning and once at bed time, the blood glucose level decreased further to normal (117 mg/dL) (Akrarapholchote, 2008). However, there is no other evidence or systematic clinical study to confirm this antihyperglycemic activity.

Diabetic mellitus is a heterogeneous disease characterized by high blood glucose levels and dyslipidemia. A major metabolic defect associated with diabetes is the failure of peripheral tissues in the body to properly utilize glucose, thereby resulting in chronic hyperglycemia (Rother, 2007). At present, it is well established that adipose tissue and skeletal muscle are the primary targets of insulin-stimulated glucose uptake (Sophie *et al.*, 2009). Glucose is transported into most tissues by facilitated diffusion or glucose transporters (Sophie *et al.*, 2009). There are several members of glucose transporters distributed specifically or nonspecifically in various parts of organs and tissues. GLUT4 is present in insulin-responsive tissues such as skeletal muscle, adipose tissue, and the heart. GLUT4 is insulin-sensitive and has high affinity to glucose (Cushman *et al.*, 1980). GLUT12 found in MCF-7 breast cancer cells is another GLUT sensitive to insulin and present in the heart, prostate, muscle and white adipose tissue similar to GLUT4 but its affinity to glucose has not yet been determined (Rogers *et al.*, 2002 and Stuart *et al.*, 2009). Naowaboot *et al.* (2009) have reported that the 1.0 g/kg body weight of ethanolic extract of mulberry leaf lowers blood glucose in streptozotocin (STZ)-induced diabetic rats by 23% and 15 μg/mL of the extract increases glucose uptake of adipocytes of diabetic rat by 54±13% over the control group.

In the present study, hexane, methanol and water extracts of *A. sinensis* were tested on STZ-induced diabetic rats. The glucose uptake enhancement activity of hexane, methanol and water extracts were tested in normal rat adipocytes to check the possible mean of the anti-diabetes activity. Thin layer chromatographic (TLC) fingerprints of the extract were detected by UV absorption at 254 and 366 nm wavelengths and color reaction was detected by spraying with anisaldehyde-sulphuric acid reagent (Wagner *et al.*, 1996). Detection for antioxidant or radical scavenging activity of the crude extracts by spraying with 0.1 M DPPH was also performed since oxidative stress is involved in diabetes mellitus in many ways.

2. Materials and Methods

The chemicals used in this study were commercial grade for extraction and analytical grade for other tests. Bovine serum albumin fraction V (BSA), N-[2-hydroxylethyl] piperazine-N[2-ethanesulfonic acid] (HEPES), insulin powder, collagenase type 1 and cytochalasin B were purchased from Sigma Chemical (St. Louis, MO, USA). 2-Deoxy-D-[U-^{14}C] glucose (^{14}C-2-DG) and aqueous counting scintillant (ACS II®) were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.1 Plant extract

Leaves of *A. sinensis* were obtained from Nakhon Ratchasima Province, Thailand. This plant voucher (No. NAT001-002) was deposited at the Faculty of Pharmaceutical Sciences, Khon Kaen University and validated by comparing with the voucher specimen, BKF No. 140455 in the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand.

Dried leaf powder, 2 kg, was extracted sequentially with hexane, ethyl acetate and methanol. Each extraction was carried out three times with 6 L of solvent. Water extraction was carried out by infusion the dried powdered leaves (150 g) in boiling water (2.8 L) for 30 min, filtering through cotton cloth, and then lyophilizing. All extracts were kept at -20°C until used. TLC-fingerprints were performed to characterize the extracts.

2.2 TLC-fingerprints

2.2.1 Chromatographic system

Silica gel GF 254 pre-coated plate (Merck, Germany) was used as a stationary phase. The mobile phase consisted of toluene : ethyl acetate : methanol : acetic acid (1 : 2 : 1 : 0.04). The chromatograms were detected under the UV light at 254 nm and 366 nm) and sprayed with anisaldehyde-sulphuric acid reagent or 0.1M DPPH.

2.2.2 Sample preparation

For freshly prepared solution, 2 g of leaf powder was sonicated with 20 mL of methanol for 1 h and filtered. For methanolic, water, and hexane extracts, 0.3 g of each extract was separately dissolved in 2 mL of the corresponding solvent used in the preparation of the extracts. The solutions were centrifuged at 2,500 rpm for 5 min at 4°C. Supernatant of each solution was separately spotted on a TLC plate.

2.3 Hypoglycemic activity of the extracts

2.3.1 STZ induced diabetes in rats

Male Sprague-Dawley rats (200-280 g) were purchased from the National Laboratory Animal Center, Mahidol Universitiy (Nakhon Pathom, Thailand). They were maintained in an air conditioned room (25±1°C), with a 12 h light - 12 h dark cycle and fed with standard diet (C.P. mouse feed, Bangkok, Thailand) and water *ad libitum*. All procedures were complied with the National Standards for the care and use of experimental animals and were approved by the Animal Ethics Committee of Khon Kaen University, Khon Kaen, Thailand (Rec. No: AEKKU28/2551). Animals were housed in the Animal Transit Room, Faculty of Medicine, Khon Kaen University (Khon Kaen, Thailand) for 7 days

before starting the experiment. Animals were diabetes-induced by a single intraperitoneal injection of 45 mg/kg body weight of STZ dissolved in 0.1 M citrate buffer (pH 4.5). After 7 days of STZ injection, venous blood was collected from rat tail to determine fasting blood glucose level. Only the rats with fasting blood glucose over 200 mg/dL were considered diabetic and were included in the experiments.

2.3.2 Experimental design

The rats were divided into five groups with six rats in each group as follows: Group I: diabetic control rats orally administered with distilled water; Group II: diabetic rats subcutaneously injected with 4 U/kg/day insulin (Mixedtard®) and Group III-V: diabetic rats orally administered with 1 g/kg body weight/day of methanol, water and hexane extracts, respectively. The treatments were continued for 1 week. Fasting blood glucose levels of all rats were determined by a glucometer (Accu-Chek Advantage II, Roche Diagnostics, Mannheim, Germany). During fasting, rats were deprived of food overnight for 12 h but had free access to water.

2.4 Effects of agarwood leaf extracts on glucose uptake by rat adipocytes

2.4.1 Preparation and isolation of adipocytes

White adipocytes were isolated from the epididymal fat pad of normal rats by the method described by Rodbell (1964). The overnight fasted rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). The epididymal fat pad was removed, minced and digested by incubating with 1 mg/mL of collagenase type 1 in Krebs-Ringer bicarbonate buffer (KRBB; pH 7.4) with 25 mM HEPES, 1% w/v BSA and 1.11 mM glucose for 45 min at 37°C in a shaking water bath set at 120 cycles/min. The fat cell suspension was filtered through a 500 μm nylon mesh to separate the adipocytes from any undigested tissue fragments and washed 3 times with KRBB without glucose by floatation (accumulation of a thin cell layer on the top of the medium after centrifugation at 1,500 rpm for 1 min). Finally, the cells were suspended in KRBB without glucose to make 40% packed cell volume (approximately 10^5-10^6 cells/mL). Uptake of glucose was measured by using ^{14}C-2-DG as a tracer.

2.4.2 Experimental design

One milligram of each extract was reconstituted in 1 mL of KRBB without glucose and serially diluted to make appropriate concentration. Two hundred microliters of cell suspension was transferred to micro-tube and incubated for 15 min at 37°C in a shaking water bath. Fifty microliters of KRBB without glucose or 50 μL of insulin (1.5 nM, final concentration) or 50 μL of the extracts (1, 3, 10 and 30 μg/mL of final concentrations) was then added to those aliquots of cell suspensions for basal, insulin and the extract groups, respectively. Finally, 50 μL of ^{14}C-2-DG (0.4 μCi/mL, dissolved in KRBB with 1.11 mM glucose) was then added to each treatment group. All the treatments were done in triplicate. After incubation for 15 min at 37°C, the reaction was terminated by adding 10 μL of cytochalasin B (20 μM) and kept at 4°C for 10 min. The fat cells were then washed 3 times in 1.5 mL cold KRBB with glucose, filtered through Whatman® microfiber filters and placed in scintillation vials filled with 3 mL of aqueous scintillation cocktail. The radioactivity was counted by a Beckman LS6500 Liquid Scintillation Counter. Glucose uptake value was corrected for non-specific uptake by subtracting the activity of cells pretreated with 20 μM cytochalasin B from the measured activity.

2.5 Statistical Analysis

All results are expressed as mean ± SEM. Comparisons of blood glucose levels between base line and after treatments were performed using Student's paired *t*-test. For glucose uptake, the values among groups were compared and tested by analysis of variance (ANOVA) followed by Student Newman-Keuls test to show specific group differences. A *P* value less than 0.05 was considered statistically significant.

3. Results

3.1 Extraction and characterization of extracts

The yields of hexane, ethyl acetate, methanol extracts were 0.9, 0.9 and 7.95% w/w, respectively. The yield of the water extract was 13.33% w/w. The TLCs of dried crude extracts and of freshly prepared powdered leaves in methanol are shown respectively in lanes 1, 2, 5 and 6 of Figure 1. Trolox and β–sitosterol were used as markers (lanes 3 and 4). Detections were carried out by examining under UV light at the wavelengths of 254 and 365 nm. Anisaldehyde-sulfuric acid was used to detect compounds which can be coupled by the reagent. DPPH was sprayed to detect the antioxidant activity of the extracts. Methanol and water extracts contained high radical scavenging antioxidant (Figure 1 C, lanes 1 and 5) but low antioxidant activities in hexane extract (Figure 1 C, lane 2).

3.2 Hypoglycemic activity

Effects of methanol and water extracts of agarwood on fasting blood glucose levels are shown in Table 1. Interestingly, 1.0 g/kg of methanol and water extracts, but not hexane extract significantly ($p<0.05$) reduced blood glucose levels by 54.29% and 40.54%, respectively. The oral administration of 1.0 g/kg of methanol extract reduced blood glucose levels in diabetic rats to almost the same degree as 4 U/kg of insulin. After the treatments had been discontinued for 1

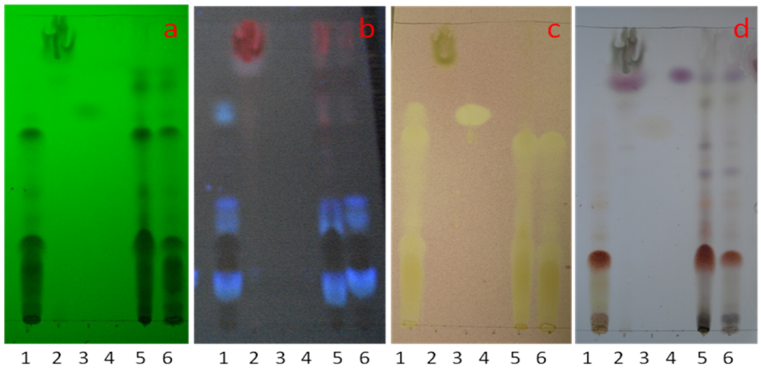

Figure 1. TLC fingerprints of agarwood crude extracts; a) under the uv light 254 nm; b) uv light 365 nm; c) sprayed with DPPH; d) sprayed with anisaldehyde-sulphuric acid reagent; lane 1 = water extract; lane 2 = hexane extract; lane 3 = trolox; lane 4 = β-sitosterol; lane 5 = methanol extract and lane 6 = freshly prepared solution.

Table 1. Effects of oral administration of agarwood leaf extracts on fasting blood glucose in STZ-induced diabetic rats

Groups	Mean fasting blood glucose			
	Pre-treatment (mg/mL)	After 1-week treatments		1 week after withdrawal (mg/mL)
		mg/mL	% change	
Control diabetic	314 ± 16	310 ± 20	+1.27	356 ± 25
Diabetic + Ins 4 U/kg	365 ± 28	97 ± 43[a]	-73.42[b]	335 ± 25
Diabetic + ME 1 g/kg	305 ± 22	141 ± 16[a]	-53.77	278 ± 26
Diabetic + WE 1 g/kg	257 ± 18	153 ± 18[a]	-40.46[b]	247 ± 23
Diabetic + HE 1 g/kg	280 ± 14	316 ± 26	+12.86	383 ± 23

[a] $p < 0.001$ by paired *t*-test comparing to the pre-treatment
[b] $p < 0.05$ by ANOVA test and followed by Student Newman-Keuls test to compare with the diabetic control group.
Ins: insulin, ME: methanol extract, WE: water extract, HE: hexane extract

week, the blood glucose of all of the diabetic rats returned to their pretreatment levels (Table 1), indicating the diabetic condition persisted throughout the period of the experiment.

3.3 Effects of agarwood leaf crude extracts on glucose uptake by rat adipocytes

Effects of methanol and water extracts to increase glucose uptake were similar to that of 1.5 nM insulin. However, the increases in glucose uptake of the adipocytes by both extracts were not concentration dependent (Table 2). The 10 mg/mL concentration of both extracts seemed to produce maximum increase in glucose uptake, whereas hexane extract exerted no activity (data not shown).

4. Discussion

The results from this study showed that the methanol and the water extracts had anti-hyperglycemic activity in STZ-induced diabetic rats, increased glucose uptake by adipocytes from normal rats and contained antioxidant activities. However, hexane extract had no anti-hyperglycemic activity.

The decrease in fasting blood glucose levels of the methanol and water extracts paralleled to their enhancement of glucose uptake by adipocytes. This implies that the anti-hyperglycemic activities of methanol and water extracts may be mediated via increasing glucose uptake by adipocytes similar to the effects exerted by insulin. It is very interesting that only 1 µg/mL of the extract exhibited uptake close to the positive control. Thus, doses lower than 1 µg/mL should be tried. At the concentrations of 3 and 10 µg/mL, both extracts provided slightly higher glucose uptake effects than 1.5 nM of insulin used as a positive control. However, the increase in glucose uptake was not concentration dependent at the concentrations tested. The concentration of 10 µg/mL of both extracts seemed to produce maximum effect. At the highest dose of both extracts tested, 30 µg/mL, the glucose uptake tended to decrease. The true concentration dependent effects of both extracts on glucose uptake may be

Table 2. Effects of agarwood leaf extracts on glucose uptake by rats adipocytes

Treatment	2-Deoxyglucose (% of negative control)	
	Methanol extract	Water extract
KRBB buffer (negative control)	100 ± 0	100 ± 0
insulin 1.5nM (positive control)	161 ± 6 *	172 ± 10*
extract 1 μg/mL	152 ± 7*	152 ± 14 *
extract 3 μg/mL	168 ± 12*	168 ± 14*
extract 10 μg/mL	172 ± 10*	176 ± 21*
extract 30 μg/mL	162 ± 12*	145 ± 15

Results are expressed as mean ± SEM of four independent experiments each of which performed in triplicate.
* $p < 0.05$ by ANOVA test and followed by Student Newman-Keuls test by comparing to buffer control group.

evidenced if lower concentrations are used and should be investigated in future study. Several plant extracts have been reported to have glucose uptake enhancing activity. Mulberry extract at 15 μg/mL concentration enhances glucose uptake of STZ-induced diabetic rat adipocytes by 54±13% (Naowaboot *et al.*, 2009) whereas 15 μg/mL of roselle (*Hibiscus sabdariffa*) extract increases glucose uptake to 111% in high fructose and fat fed diet diabetic rat (Yosaph, 2009). Gray et al. (1998 and 1999) also found that some herbal extracts, e.g. *Agaricus campestris* and *Coriandum sativum*, imitate insulin effects on *in vitro* glucose metabolism.

Although methanolic and water extracts of agarwood leaves exhibited antioxidant activities in the present study, their contribution to anti-hyperglycemic and enhancement of glucose uptake activities are not known. Antioxidants have been reported to be beneficial in the prevention or treatment of various diseases including diabetes (Maritim *et al.*, 2003). However, lowering blood glucose levels in STZ-induced diabetes by antioxidants in agarwood extract is unlikely, since the damage induced by STZ is a permanent one.

GLUT4 is the most active transporter for glucose uptake by insulin-responsive tissues present in adipocytes, skeletal muscle, and the heart (Holman *et al.*, 1994). The anti-hyperglycemic and glucose uptake enhancement activities of agarwood methanol and water extracts are similar to those of insulin, however, whether these effects are mediated via the mechanism involving GLUT4 or not is not known with certainty. Therefore, further investigation is warranted to uncover the exact mechanism of agarwood extracts as well as to isolate compound(s) responsible for the activity.

5. Conclusion

The methanol and water crude extracts have anti-hyperglycemic activity in diabetic rats, most likely to be associated with glucose uptake increasing mechanism. Lower doses of the extract should be tried in future study to establish the most appropriate dose for clinical trial. Agarwood leaf has shown to be a potential anti-diabetic and thus agarwood can be a promising source for anti-diabetic agent.

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