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A study of the chemistry of *Artocarpus heterophyllus* Lam. leaves and identification of active antioxidant and hypoglycemic fractions/ compounds

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Introduction

Diabetes mellitus is a syndrome of chronic hyperglycemia due to relative insulin deficiency, resistance, or both.[1] During the last twenty years, the prevalence of the disease has increased all over the world and it now affects all populations. The International Diabetes Federation (IDF) estimated that in 2013 worldwide 387 million people were suffering from diabetes mellitus with projections that the number will increase to 592 million by 2035.[2] Long-term complications of diabetes include retinopathy with potential loss of vision, nephropathy leading to renal failure, peripheral neuropathy with risk of foot ulcers leading to amputations and Charcot joints, autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms and sexual dysfunction.[3] Further, diabetic patients have an increased rate of atherosclerotic, cardiovascular, peripheral arterial and cerebrovascular disease, hypertension and abnormalities of lipoprotein metabolism.[3] Mortality and morbidity are increased due to these complications. Currently insulin and various oral antidiabetic agents are used as monotherapy or in combination to achieve better glycemic regulation. Many of these oral antidiabetic agents have several serious adverse effects and most of drugs show development of resistance.[4] It is still a challenge to manage diabetes mellitus without any side effects. The search for new drugs which are safer and more effective is an active area of research. *Artocarpus heterophyllus* is a well-known plant which belongs to family Moraceae and its leaves are used ethnomedically in Sri Lanka for the control of diabetes mellitus. The antidiabetic and hypoglycemic effects of the aqueous leaf extract have been demonstrated in animal models and in human subjects.[5-7] The hypoglycemic effect of the ethyl acetate fraction of aqueous leaf extract has also been demonstrated in animal models.[8-10] In this paper, we report the potential of *A. heterophyllus* leaves for the treatment of diabetes mellitus through its hypoglycemic activity of the fractions of ethyl acetate fraction of the water extract and its chemical analysis.

Methodology

Plant material

Senescent leaves of *A. heterophyllus* were collected from a plant (cultivar *Waraka*) growing in a home garden in Wijerama in the Colombo district. Voucher specimen (B7/006(SJP)) has been deposited in the herbarium, Department of Botany, University of Sri Jayewardenepura. The leaves were washed, air-dried for 3 hours and crushed using a mechanical blender and was used immediately for extractions.

Extraction

A sample of senescent leaves of *A. heterophyllus* (500 g) was extracted by refluxing with 2500 mL of distilled water in a 5 L round bottom flask for 4 hours. After filtration, the extract was concentrated and excess ethanol was added to precipitate the high molecular weight fraction. The mixture was filtered, concentrated and extracted with ethyl acetate. Combined ethyl acetate extract was concentrated to produce a brownish-black sticky solid (1.2 g) (EA/W). This was repeatedly done to obtain 14 g of EA/W.

Fractionation of EA/W

EA/W (14 g) was fractionated by Sephadex LH-20 column chromatography. Column was eluted with 5 different solvents and fractions were collected separately. Fraction 1 was eluted with dichloromethane/hexane 4:1 (1 L) and fraction 2, 3, 4 and 5 were eluted with dichloromethane/acetone 3:2 (1 L), dichloromethane/acetone 1:4 (0.9 L), dichloromethane/ methanol 1:1 (1 L) and methanol (1 L) respectively. The weights of each fraction after evaporation of the solvent were obtained. Distribution of compounds were analyzed by TLC (Silica (GF 254) with solvent systems; ethyl acetate: dichloromethane: methanol: formic acid (58:38:2:2) and ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:27).

***In vivo* studies**

Animal model

Male Wistar rats approximately 8 - 12 weeks old, weighing 150 - 300 g were used for the study. The rats were maintained and handled in accordance with the standard guide for the care and use of laboratory animals and necessary skills for rat handling were obtained before commencing the research. Rats were individually identified by colour markings on their body.

For *in vivo* studies on normoglycemic rats, 42 rats were divided into 7 groups (groups A–G) and for *in vivo* studies in the diabetic model experiment 48 rats were divided into 8 groups (groups H – O). The animals used were kept fasted overnight before commencement of experiment.

Induction of diabetes mellitus

Rats in groups H – O were diabetized using nicotinamide (120 mg/kg body weight) and streptozotocin (60 mg/kg body weight) according to a previously published method.[11-15]

Effect on blood glucose levels in normoglycemic rats

Animals in groups A – G were fasted overnight and fasting blood glucose levels of the rats were measured. After that group A served as control and received water. Group B received a single dose of 50 mg/ kg body weight EA/W. Groups C, D, E, F and G received a single dose of fraction 1,2,3,4 and 5 (50 mg/ kg body weight), respectively which were obtained from Sephadex LH-20 column. All samples were given orally. After that blood glucose levels were measured at 1 hour, 2 hours and 3 hours after administration of sample. Blood was obtained from the tail vein. Blood glucose was measured using a glucometer (Accu-Check glucometer).

Effect on fasting blood glucose levels in diabetic rats

Same procedure as above was carried out for groups H –N and Group O rats were administered with glibenclamide at 5 mg/ Kg body weight.

Effect on glucose tolerance in normoglycemic rats

After 2 weeks of the experiment using normoglycemic rats, animals in groups A – G were kept fasted overnight and fasting blood glucose levels of the rats were measured. After that group A served as control and received water. Group B received a single dose of 50 mg/kg body weight EA/W. Groups C, D, E, F and G received a single dose of fraction (50 mg/kg body weight) 1, 2, 3, 4 and 5, respectively which were obtained from Sephadex LH-20 column. All samples were given orally. After 1 hour blood glucose level was measured and a glucose load (3 g/kg body weight) was administrated. After that blood glucose levels were measured at 1 hour, 2 hours, and 3 hours after the administration of glucose load. Blood was obtained from the tail vein. Blood glucose was immediately measured using a glucometer (Accu-Check glucometer).

Effect on glucose tolerance in diabetic rats

After 2 weeks of the experiment in diabetic rats, animals in groups H – O were kept fasted overnight and fasting blood glucose levels of the rats were measured. Same procedure was carried out for groups H –N while Group O rats were administered with glibenclamide at 5 mg/ Kg per body weight.

***In vitro* studies**

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, superoxide anion radical scavenging assay, α- glucosidase enzyme inhibition assay,

antiglycation activity and anti-inflammatory activity of EA/W and fractions 1-5 were carried out according to previously published methods.[11-15] All the experiments were carried out in triplicate.

Compound Isolation

Fractions 3 and 4 showed the highest activity in *in vivo* and *in vitro* assays. These two fractions also showed similar chemical profiles in thin layer chromatographic studies. Thus, fractions 3 and 4 were combined and subjected to compound isolation. Combined fraction (5.0 g) was chromatographed on MCI gel column chromatography to produce 17 fractions (M1 – M17). Fraction M3 (1.2 g) upon further chromatography produced compound 1 (3.3 mg) and 2 (6.2 mg). Fractions M2 (0.2 g) and M4 (1.0 g) upon further chromatography produced compound 3 (3.1 mg) and compound 4 (4.3 mg), respectively. All compounds were characterized by ^{1}H NMR, ^{13}C NMR, IR, UV-visible spectroscopy, EI and FAB spectrometry in positive ion mode.

Results and Discussion

EA/W and fractions 1-5 were screened for *in vivo* hypoglycemic and antidiabetic activities. Treatment of the rats with nicotinamide and streptozotocin resulted in elevated plasma glucose levels consistent with diabetes (>120 mg/ dL), whereas non-treated rats without nicotinamide and streptozotocin had normal glucose levels (< 120 mg/dL). Each fraction was tested for its effect on the blood glucose levels of fasted normal and diabetic rats. None of the fractions caused hypoglycemia on normal rats. However, there was significant ($p < 0.001$) reduction in the blood glucose level during the first three hours after giving the fractions 3, 4 and 5 to diabetic rats (Figure 1). Figure 2 clearly show that the highest reduction occurs at second hour after administration of the fractions. Further, it shows fraction 3, 4 and 5 have significant reduction ($p < 0.001$) with respect to control group. Of the three fractions, fraction 4 showed the highest hypoglycemic activity. Fractions 3, 4 and 5 were also the most active in the glucose tolerance test carried out on both normoglycemic and diabetic rats (Figures 3 and 4). However, fraction 3 was the most active in this assay. In both assays fractions 3, 4 and 5 at 50 mg/kg body weight showed activity of the same order as glibenclamide at 5 mg/kg body weight. Fraction 4 was found to be the most active fraction in all the *in vitro* assays.

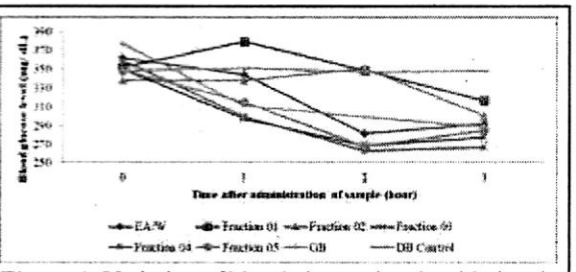

Figure 1: Variation of blood glucose levels with time in diabetic rats after administration of fractions of EA/W. (Value at t=0 correspond to fasting blood glucose level)

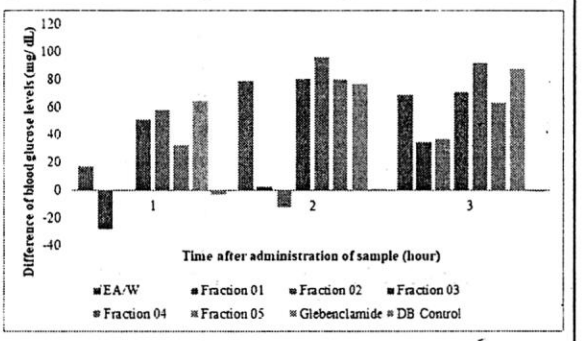

Figure 2: Reduction in blood glucose levels with time in diabetic rats after administration of fractions of EA/W.

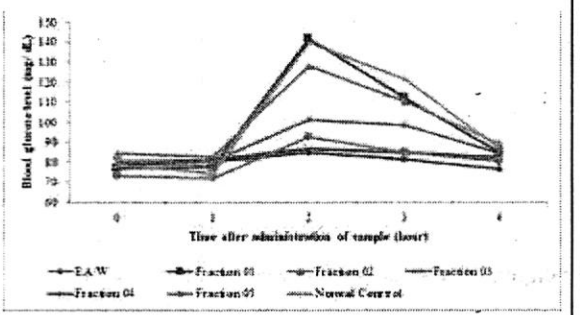

Figure 3: Variation of blood glucose levels of normoglyceamic rats with the time after administration of sample followed by glucose load.
Value at t=0 correspond to fasting blood sugar level.

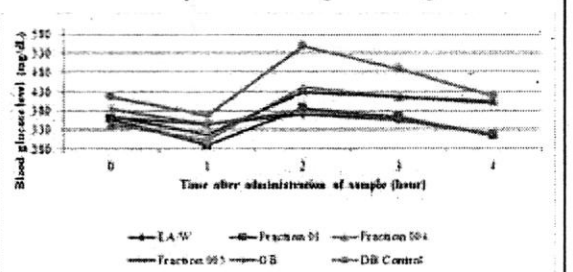

Figure 4: Variation of blood glucose levels of diabetic rats with time after administration of sample followed by glucose load.

Although the composition of the three fractions were different from each other and from EA/W as

analyzed by thin layer chromatography, there are no significant difference ($p>0.01$) in the activities. This suggests that EA/W contains many different compounds having approximately the same activity. When comparing all the *in vivo* results we can clearly see that optimum time of action is at the end of the 2nd hour after administration of sample.

EA/W and five fractions were subjected for *in vitro* antioxidant, antiglycation, α-glucosidase inhibition and anti-inflammatory assays the results of which are given in Table1. Fraction 4 was found to be the most active fraction in all the *in vitro* assays. These results indicate that the hypoglycemic activity of *Artocarpus heterophyllus* senescent leaves may be partly due to α–glucosidase inhibiting activity. Further its antioxidant activity and antiglycation activity (even though lower than rutin) may contribute to reducing the complication arising from diabetes mellitus.

Table 1: *In vitro* biological activity results of EA/W and its fractions

	DPPH radical scavenging assay IC_{50}(µg/ mL)	**Superoxide radical scavenging assay % Inhibition**	**Antiglycation assay IC_{50}(µg/ mL)**	**α- Glucosidase inhibitory assay IC_{50}(µg/ mL)**	**Anti-inflammatory assay. IC_{50}(µg/ mL)**
EA/W	29.26 ± 0.71	90.60	Not active	1.9 ± 0.57	27.4 ± 2.0
Fraction 1	Not active*	<50	Not active	Not active*	71.3 ± 7.6
Fraction 2	108.34 ±0.45	<50	Not active	Not active*	70.7 ± 10.2
Fraction 3	29.31 ± 0.45	97	0.44 ± 0.01	51.0 ± 0.98	24.4 ± 3.8
Fraction 4	21.69 ± 0.31	98	0.30 ± 0.02	0.40 ± 0.01	16.9 ± 0.1
Fraction 5	Not active*	<50	Not active	8.60± 0.57	>100
Gallic acid	23.46 ± 0.43	-	-	-	-
Quercetin		100			
Rutin	-	-	0.18 ± 0.01	-	-
Acarbose	-	-	-	0.54± 0.01	-
Ibuprofen	-	-	-	-	11.8 ± 1.9

*Percentage inhibition was < 50 at 05 mg/mL concentration and hence IC_{50} value was not calculated
All values are expressed as IC50 ± SEM, n=3

The EA/W fraction was subjected to repeated column chromatography over Sephadex LH-20, MCI gel, preparative TLC and preparative HPLC to yield compounds 1, 2, 3 and 4 (Figure 7). Their structures were confirmed as 3, 4-dihydroxy-7-ene-megastigman-9-one (1), 13-Hydroxy-3-oxo-α-ionol (2), 4-hydroxy benzoic acid (3) and 3,4- dihydroxy benzoic acid (4), respectively by ^{1}H NMR, ^{13}C NMR, IR and UV-visible spectroscopy, EI and FAB spectrometry in positive ion mode. NMR spectroscopic data of compounds 1-4 are given in Table 2.

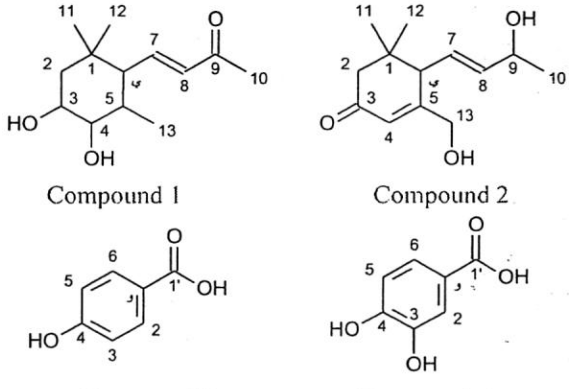

Figure 7: Structures of compounds 1-4