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IN-VITRO BIOLOGICAL ACTIVITIES OF FRACTIONS OF ETHYL ACETATE FRACTION OF THE WATER EXTRACT OF *ARTOCARPUS HETEROPHYLLUS* SENESCENT LEAVES

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ABSTRACT: *Artocarpus heterophyllus* belongs to the family Moraceae. In Sri Lankan traditional medicine the water extract of *A. heterophyllus* senescent leaves is used to control diabetes mellitus. The hypoglycemic activity of ethyl acetate fraction of water extract of senescent leaves has been reported. Ethyl acetate fraction (EA/W) was fractionated using sephadex LH-20 column chromatography using the solvent systems: dichloromethane/hexane (4:1), dichloromethane/acetone (3:2), dichloromethane/acetone (1:4), dichloromethane/ methanol (1:1) and methanol to produce five fractions. EA/W and fractions 1-5 were subjected to *in-vitro* antioxidant, α- glucosidase inhibitory, antiglycation and anti-inflammatory activity studies. Fraction 4 exhibited the highest DPPH radical scavenging activity (IC_{50} = 21.69 ± 0.31 µg/mL) and α- glucosidase inhibitory activity (IC_{50} = 0.4± 0.01 µg/ mL) which were greater than the standards gallic acid and acarbose respectively and EA/W. Fractions 3 and 4 showed higher antiglycation activity (IC_{50} = 0.44 ± 0.04 mg/mL and 0.30 ± 0.01 µg/mL respectively) and higher anti-inflammatory activity (IC_{50} = 24.4 ± 3.8 and 16.9 ± 0.1 µg/mL respectively) than any other fraction or EA/W. However, these were less than the standards rutin and ibuprofen respectively. The results will be of potential use for the development of a safe and effective anti-diabetic drug from senescent leaves of *A. heterophyllus*.

INTRODUCTION: *Artocarpus heterophyllus*, which belongs to the family Moraceae is a common tree in Sri Lanka. It is known as 'Kos' in Sinhalese. There are many ethno medical usages reported for various parts of the plant [1, 2, 3, 4, 5]. The water extract of senescent leaves of *A. heterophyllus*is used to control diabetes mellitus in Sri Lankan traditional medicine [6, 7].

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It has been shown that the aqueous extract of *A. heterophyllus* senescent leaves significantly lowered the fasting blood sugar levels and markedly improved glucose tolerance in animal models and in human subjects [8 - 10].

A recent study showed the hypoglycemic activity of the ethyl acetate fraction of the aqueous extract of *A. heterophyllus* senescent leaves was greater than that of tolbutamide, a sulphonyl urea drug commonly used for treatment of hyperglycemia [11]. Further, it has been reported that active compounds are soluble in ethyl acetate [12, 13]. Diabetes mellitus is a syndrome of chronic hyperglyceamia due to relative insulin deficiency, resistance or both [14]. The prevalence of the disease has increased all over

the world during the last twenty years and is of huge concern, not only in Sri Lanka but also worldwide [15, 16]. Long-term complications of diabetes mellitus include retinopathy, nephropathy, neuropathy, rheumatoid arthritis, osteoporosis and alzheimer's disease [14, 17, 18, 19]. Further, diabetic patients have an increased rate of atherosclerotic, cardiovascular, peripheral arterial and cerebro-vascular disease, hypertension and abnormalities of lipoprotein metabolism leading to mortality and morbidity [18].

Two types of medication are used in the western world for the management of diabetes mellitus. One is to maintain blood glucose levels while the other is to minimize its associated complications. However, controlling blood sugar levels still remains a challenge. We have already reported *in-vivo* hypoglyceamic and anti-diabetic activities of fractions of the ethyl acetate fractions of the aqueous extract of *A. heterophyllus* senescent leaves [20].

Here, we report *in-vitro* antioxidant, antiglycation, α-glucosidase inhibitory and anti-inflammatory activities of fractions of the ethyl acetate fraction of the aqueous extract of *A. heterophyllus* senescent leaves. It is of paramount importance that any remedy used in diabetes mellitus should have the capability to maintain blood glucose levels and minimize its associated complications. With that respect, the results will be of potential use for the development of a safe and effective anti-diabetic drug from senescent leaves of *A. heterophyllus*.

MATERIALS AND METHODS: DPPH (1,1-diphenyl-2-picrylhydrazyl) was purchased from Wako Chemicals Inc. (USA). *α*-Glucosidase enzyme was purchased from My Biosource Inc. (USA). Bovine serum albumin, methylglyoxal (MGO), *p*-nitrophenyl-*α*-D-glucopyranoside (pNPG), Hanks' balanced salt solution containing calcium chloride and magnesium chloride (HBSS), gallic acid (≥98 %) were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA).

Serum Opsonized Zymosan (SOZ) was purchased from Fluka Chemie (Buchs, Switzerland). 3-Aminophthalhydrazide (Luminol) was obtained from Research Organics Inc. (Cleveland, OH, USA). Other chemicals and reagents were of analytical grade and purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Water when used was distilled using GFL distillation apparatus. Microplate reader (SpectraMax 340PC384) and Luminometer (Labsystems, Helsinki, Finland) were used for respective assays.

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 with permission from the home owner. The material was authenticated by Mr. Isuru Kariyawasam of the Department of Botany, University of Sri Jayewardenepura and a 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, crushed using a mechanical blender and used for extractions.

Ethyl Acetate Fraction of the Water Extract of *A. heterophyllus* Leaves (EA/W): Crushed senescent leaves of *A. heterophyllus* (500 g) was extracted using distilled water (2500 mL) upon refluxing as previously reported [20]. The resulting extract was allowed to cool to room temperature. Excess ethanol was added to precipitate the high molecular weight fraction and extracted with ethyl acetate (300 mL × 6) also as previously reported [20]. Combined ethyl acetate extracts were evaporated under reduced pressure to obtain 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 (1.2 g) was fractionated using sephadex LH-20 column chromatography as previously reported [20]. Column was eluted with 5 different solvent systems 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 dichloro-methane/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 solvents were obtained and distribution of compounds were analyzed by TLC using ethyl acetate: dichloro-methane: methanol: formic acid (58:38:2:2) and ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:27) as solvent systems.

***In-vitro* Studies:** EA/W and fractions 1-5 were subjected for *in-vitro* antioxidant, antiglycation, α-glucosidase inhibitory and anti-inflammatory activity studies:

In each assay, initially the % inhibition of EA/W and fractions 1-5 were calculated using the following formula.

$$\% \text{ Inhibition} = [(A_o - A_1) / A_o] 100$$

A_1 = Absorbance/emission of sample

A_0 = Absorbance/emission of control

EA/W and fractions 1-5 were subjected for determination of IC_{50} values in the respective assay only if % inhibition was 50% or more at a concentration 0.5 mg/mL.

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Assay: The antioxidant activity was determined using the DPPH radical scavenging assay according to a previously published method [21]. A concentration series of EA/W (500 µg/mL to 15.625 µg/mL) was prepared in DMSO. DPPH solution of 0.3 mM was prepared using absolute ethanol. From each solution of EA/W, 5 μL aliquot was pipetted into a well in a 96-well microplate. Then 95 μL of DPPH solution was added and incubated for 30 min in the dark at 37 ^{0}C. The absorbance was measured at 517 nm. Gallic acid was used as the positive control. Negative control contained DMSO while blank contained ethanol. The IC_{50} values were calculated using the EZ-Fit Enzyme kinetics software program (Perrella Scientific Inc., Amherst, MA, USA) and compared to that of the positive control. Experiments were performed in triplicate. The same procedure was carried out for fractions 2, 3 and 4.

***α*-Glucosidase inhibition Assay:** *α*-Glucosidase inhibitory activity was determined using *α*-glucosidase inhibition assay according to a previously published method [22]. A concentration series of EA/W (7.8 μg/mL to 0.13125 μg/mL) was prepared in DMSO. Phosphate saline buffer of 50 mM was prepared. From each solution of EA/W 20 μL, buffer135 μL and enzyme 20 μL were pipetted into a well in a 96-well microplate and incubated for 15 min at 37 ºC. After incubation the absorbance was measured at 400 nm. Then 25μL of the substrate pNPG was added and after 30 minutes the absorbance was measured at 400 nm. Acarbose was used as the positive control.

Negative control contained DMSO. The IC_{50} values were calculated using the EZ-Fit Enzyme kinetics software program (Perrella Scientific Inc., Amherst, MA, USA) and compared to that of the positive control. Experiments were performed in triplicate. The same procedure was carried out for fractions 3, 4 and 5.

Antiglycation Assay: Antiglycation activity was determined using the antiglycation assay according to a previously published method [23]. A concentration series of fraction 3 (0.5 mg/mL to 0.125 mg/mL) was prepared using0.1 M phosphate buffer (pH 7.4). Bovine Serum Albumin (BSA) (10 mg/mL) solution was prepared using 0.1M phosphate buffer. Test sample (20 μL), BSA (50.0 μL), 14 mM MGO (50.0 μL) and phosphate buffer (80 µL) were pippetted into a well in a 96-well microplate and incubated under sterile conditions at 37 °C for 9 days. After which the fluorescence was measured at the excitation and emission wavelengths at 330 nm and 420 nm respectively.

Rutin was used as the positive control. Phosphate buffer (0.1 M) was used as the negative control. The IC_{50} values were calculated using the EZ-Fit Enzyme kinetics software program (Perrella Scientific Inc., Amherst, MA, USA) and compared to that of the positive control. Experiments were performed in triplicate. The same procedure was carried out for fractions 4. The experiment was carried out in triplicate.

Anti-inflammatory Assay: Anti-inflammatory activity was determined by oxidative burst assay using luminol-enhanced chemiluminescence assay according to a previously published method [24].

A 25 μL cell suspension of human whole blood (obtained from healthy volunteers working at the University of Karachi with consent) (diluted 1:200) in Hanks Balanced Salt Solution, containing calcium chloride and magnesium chloride (HBSS) was incubated with 25 μL of EA/W in HBSS at three different concentrations (10, 50 and 250 μg/mL) each in a separate well in a 96-well plate at 37 °C for 15 min. Medium HBSS with cell suspension was employed as the negative control.

Test was carried out in white half area of 96-well plate. Incubation was performed in the thermostat chamber of Luminometer. The ROS production was initiated by the addition of 25 μL of SOZ in HBSS (20 mg/mL) into each well except for the blank solution. Thereafter, 25 µL of intracellular reactive oxygen species detecting probe, luminol(7×10^5 M) was added into each reaction mixture except blank wells (containing only HBSS).

Chemiluminescence peaks were recorded using a Luminometer in terms of relative light units (RLU). Ibuprofen was used as the positive control. The percentage of inhibition was calculated in comparison to the negative control in the maximum luminescence (the height of the peak). IC_{50} value was calculated using the EZ-Fit Enzyme kinetics software program (Perrella Scientific Inc. Amherst, MA, USA). The same procedure was carried out for fractions 1 - 4.

Statistical Analysis: The results are represented as the $IC_{50} \pm SEM$. Every statistical analysis was performed with one-way ANOVA, followed by student *T* test using Minitab 17.0 software. Differences were accepted as statistically significant at $P \leq 0.05$.

RESULTS AND DISCUSSION: EA/W and fractions 1-5 obtained from Sephadex LH- 20 column were tested for their *in-vitro* antioxidant activity through DPPH radical scavenging activity, anti-diabetic activity through *α*-glucosidase inhibition and antiglycation assays and anti-inflammatory activity through oxidative burst assay. EA/W and fractions 1-5 were subjected for determination of IC_{50} values only if % inhibition was 50% or more at concentration 0.5 mg/mL in the respective assay. The results of *in-vitro* DPPH radical scavenging, α-glucosidase inhibition, antiglycation and oxidative burst assays are given in **Table 1**.

TABLE 1: IC_{50} VALUES OF EA/W AND FRACTIONS IN DPPH RADICAL SCAVENGING, *α*-GLUCOSIDASE INHIBITION, ANTIGLYCATION AND OXIDATIVE BURST ASSAYS

Sample/ standard	Assay			
	DPPH radical scavenging	*α*-Glucosidase inhibition	Antiglycation	Oxidative burst
	(µg/mL)	(µg/mL)	(mg/mL)	(µg/mL)
EA/W	29.26 ± 0.71	1.90 ± 0.57	nd	27.4 ± 2.0
Fraction 1	nd	nd	nd	71.3 ± 7.6
Fraction 2	108.34 ±0.45	Not determined*	nd	70.7 ± 10.2
Fraction 3	29.31 ± 0.45	51.00 ± 0.98	0.44± 0.04	24.4 ± 3.8
Fraction 4	21.69 ± 0.31	0.40 ± 0.01	0.30± 0.01	16.9 ± 0.1
Fraction 5	nd	8.60 ± 0.57	nd	nd
Gallic acid	23.46 ± 0.43			
Acarbose		0.54± 0.01		
Rutin			0.18± 0.01	
Ibuprofen				11.8 ± 1.91

All values are presented as $IC_{50} \pm SEM$, $n = 3$. nd = not determined as % inhibition is less than 50% at concentration 0.5 mg/ mL in the respective assay.

Antioxidant Activity: Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced due to normal cellular metabolism. These are playing dual role in the body, hence they can be harmful or beneficial to living systems. Beneficial effects occur at low or moderate concentrations of ROS or RNS. Over production of ROS or RNS or deficiency in antioxidants leads to a state of imbalance between ROS/RNS and antioxidants and causes a state called oxidative stress. At this stage high concentration of ROS/ RNS causes oxidative damage to DNA, lipids, and proteins which results in degradative effects that contributes to human diseases such as diabetes mellitus, cancer, cardiovascular diseases, atherosclerosis, arthritis, and neurodegenerative diseases [25].

Increasing evidences have suggested that while oxidative stress plays a major role in the pathogenesis of diabetes mellitus it also appears to be the pathogenic factor in underlying diabetic complications [26, 27, 28, 29, 30, 31, 32]. Free radical scavengers or antioxidants provide protection of cells against oxidative damage [25]. The results of DPPH radical scavenging assay are given in **Table 1**. Fraction 4 exhibited highest DPPH radical scavenging activity ($IC_{50} = 21.69 \pm 0.31$ μg/mL) which was greater than that of EA/W ($IC_{50} = 29.26$

± 0.71 μg/mL). DPPH radical scavenging activity of fraction 4 was also greater than that of gallic acid (IC_{50} = 23.46 ± 0.43). DPPH radical scavenging activity of fraction 3 (IC_{50} = 29.31 ± 0.45 μg/mL) was similar to that of EA/W. Gallic acid is a commonly used standard for antioxidant assays [21]. According to these results, fraction 4 possesses better antioxidant activity than gallic acid.

α-Glucosidase Inhibition Assay: α-Glucosidase is a membrane bound enzyme located in the epithelium of the small intestine that breaks down carbohydrates to glucose by catalyzing the cleavage of $\alpha(1 \rightarrow 4)$ bonds present in carbohydrates [22]. Compounds capable of inhibiting the intestinal α-glucosidase enzyme can slow the digestion of carbohydrates thereby reducing the uptake of carbohydrate by the small intestine [33].

The results of α-glucosidase inhibitory assay are given in **Table 1**. According to the results obtained, fraction 4 showed the highest α-glucosidase inhibitory activity (IC_{50} = 0.40±0.01 µg/mL), which was greater than that of EA/W (IC_{50} = 1.90 ± 0.57). The α-glucosidase inhibitory activity of fraction 4 was also greater than that of the acarbose(IC_{50} = 0.54 μg/mL). Acarbose is a commonly used standard for α-glucosidase inhibition assays [22]. According to these results, fraction 4 possesses better α-glucosidase inhibitory ctivity than acarbose.

Antiglycation Assay: Glucose reacts with amino groups on plasma proteins during long standing hyperglycaemic state in diabetes mellitus. This occurs under normal physiological conditions in the human body through a non-enzymatic process known as glycation [23, 34]. Protein glycation and formation of advanced glycation end products (AGEs) play a significant role in the pathogenesis of diabetic complications that include retinopathy, neuropathy, nephropathy and cardiovascular disease [34]. In addition, AGEs also lead to other diseases such as rheumatoid arthritis, alzheimer's disease, osteoporosis and even aging [35, 36].

The results of antiglycation assay are given in **Table 1**. According to the results obtained fractions 3 and 4 showed similar antiglycation activities with IC_{50} values 0.44 ± 0.04 and 0.30 ± 0.01 mg/mL respectively. However, the antiglycation activity of these fractions was less than that of rutin, which exhibited an IC_{50} value of 0.18 ± 0.01 mg/mL. Rutin is a commonly used standard in antiglycation assays. According to these results, fractions 3 and 4 possess antiglycation activity but not as much as the standard used.

Anti-inflammatory Activity: Diabetes mellitus is associated with elevated levels of inflammation. Further, poorly controlled diabetes mellitus involves increased susceptibility to infections [14, 37]. Oxidative burst is an important step in bacterial killing. It involves a series of metabolic events that take place when phagocytes are stimulated, resulting in the production of superoxide, hydrogen peroxide, and other more potent oxidizing radicals. This can be quantified by chemiluminescence assay. Chemiluminescence is based on the amplification of natural luminescence emitted when ROS are released during phagocytosis.

The results of oxidative burst assay using chemiluminescence are given in **Table 1**. Fractions 3 (IC_{50} = 24.4 ± 3.8μg/mL) and 4 (IC_{50} = 16.9 ± 0.1μg/mL) exhibited better anti-inflammatory activity than EA/W (IC_{50} = 27.4 ± 2.0 μg/mL) and lesser activity than ibuprofen (IC = 11.8 ± 1.91 µg/mL), a widely used drug for inflammatory diseases. According to these results, fractions 3 and 4 possess anti-inflammatory activity but not as much as the standard used.

CONCLUSION: EA/W and fractions 1-5 of the ethyl acetate fraction of the aqueous extract of *A. heterophyllus* senescent leaves were subjected for *in-vitro* antioxidant, α-glucosidase inhibitory, antiglycation and anti-inflammatory activity studies. Of all the fractions tested, fraction 4 gave best results in antioxidant and α-glucosidase inhibitory activity studies while fractions 3 and 4 gavebest results in antiglycation and anti-inflammatory activity studies. Further, we have already reported *in-vivo* hypoglyceamic and anti-diabetic activities of fractions 3 and 4 [20].

It is of paramount importance that any remedy used in diabetes mellitus should have the capability to maintain blood glucose levels and minimize its associated complications. With that respect, these results will be of potential use for the development of a safe and effective anti-diabetic drug from senescent leaves of *A. heterophyllus*.

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CONFLICT OF INTEREST: The authors declare that they have no conflict of interest.

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