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Original Article

Evaluation of effect of alcoholic extract of heartwood of *Pterocarpus marsupium* on *in vitro* antioxidant, anti-glycation, sorbitol accumulation and inhibition of aldose reductase activity

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ABSTRACT

Rising popularity of phytomedicines in various diseased conditions have strengthened the significance of plant-research and evaluation of phytoextracts in clinical manifestations. *Pterocarpus marsupium Roxb.*, a medicinal plant, known for its anti-oxidant and anti-diabetic activity is a rich source of phytochemicals with antihyperglycemic and antihyperlipidemic activities. However, its possible role in diabetic complications is not evaluated yet. The present study explores the possible role of alcoholic extract of heartwood of *P. marsupium* in the treatment of long-term diabetic complications. The alcoholic extract of *P. marsupium* was evaluated for advanced glycation-end-products formation, erythrocyte sorbitol accumulation and rat kidney aldose reductase enzyme inhibition at the concentration of 25–400 μg/ml using *in-vitro* bioassays. Also the phytoextract at the concentration of 10–320 μg/ml was evaluated for its antioxidant potential by *in-vitro* antioxidant assays which includes, determination of total phenol content; reducing power assay; nitric oxide scavenging activity; superoxide radical scavenging activity; total antioxidant capacity; total flavonoid content; DPPH scavenging activity; and hydrogen peroxide scavenging activity.

The alcoholic extract of *P. marsupium* across varying concentrations showed inhibitory effect as evident by IC_{50} on advanced glycation-end-products formation (55.39 μg/ml), sorbitol accumulation (151.00 μg/ml) and rat kidney aldose reductase (195.88 μg/ml). The phytoextract also exhibited high phenolic and flavonoid contents with promising antioxidant potential against the antioxidant assays evaluated. The present investigation suggests that the phytoextract showed prominent antioxidant, antiglycation property and, inhibited accumulation of sorbitol and ALR enzyme, thus promising a beneficial role in reducing/delaying diabetic complications.

1. Introduction

Metabolic pathway of the body results in continuous exposure to oxygen-centred free radicals and other reactive oxygen species (ROS), which produced *in vivo*. In diabetic conditions metabolic abnormalities may leads to overproduction of ROS in endothelial cells of both large and small vessels. This imbalance in ROS is thought to contribute to the pathogenesis of various diabetic complications including neuropathy, nephropathy, and retinopathy.[1–3] It is hypothised that the combined effects of increased levels of reactive oxygen species (ROS) and decreased capacity of

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the cellular antioxidant defence system plays important role to potentiate oxidative stress in diabetes. One of the mechanistic approach by which this oxidative stress in diabetes causes complications is the activation of polyol pathway and by increasing the formation of advanced glycation end products (AGEs). The polyol pathway consists of two enzymes. The first enzyme, aldose reductase (AR), reduces glucose to sorbitol with the aid of its co-factor NADPH, and the second enzyme, sorbitol dehydrogenase (SDH), with its co-factor NAD, converts sorbitol to fructose. Augmented cellular sorbitol and fructose levels boost the generation of AGEs. Accumulation of sorbitol leads to osmotic swelling, changes in membrane permeability and also oxidative stress culminating in tissue injury. Suppressing the metabolism of glucose via the polyol pathway by inhibiting AR is a potential way to prevent the secondary complications in diabetes.[4–6] Involvement of the polyol pathway and oxidative stress in the etiology of diabetic complications requires inhibition of both processes. Therefore, bifunctional compounds with joint antioxidant/aldose reductase inhibitory (AO/ARI) activities would be dually beneficial.

Several plants with rich source of flavonoids have been studied for aldose reductase inhibitory activity.[7,8] There is an increasing interest developing in natural antioxidants, e.g., polyphenols, present in medicinal and dietary plants, which might help in preventing oxidative damage. Certain herbal plants reported to have both aldose reductase inhibitory and antioxidative activities would have a better therapeutic value than other treatments for overcoming the diabetes mellitus.[9–11]

One of the such plant *Pterocarpus marsupium Roxb.*, from the family *Leguminosae* also known as “vijaysar” or “Bijasar” is a large tree that commonly grows in the central, western, and southern parts of India and in Sri Lanka. Various parts of the *P. marsupium* tree (heartwood, leaves and flowers) have long been used for their medicinal properties in Ayurveda.[12] The heartwood is used in the treatment of inflammation and reported to have medicinal importance in the management of diabetes since long.[13] The flavonoids and phenolic contents present in the tree viz., marsupiun, pterosupin, and liquiritegenin are reported to posses antihyperglycemic and antihyperlipidemic activities.[12–14] So many compounds isolated from the different parts of the *P. marsupium* extracts may serve as a potential source of natural antioxidant as well as for the treatment of diabetes.[15–20] Therefore, in the present study it was planned to evaluate the effect of alcoholic extract of heartwood of *P. marsupium* against aldose reductase, sorbitol accumulation, AGE inhibition and antioxidant assays so that its antioxidant and antidiabetic potential could be find out.

2. Materials and methods

2.1. Plant collection, identification and extract preparation

Heartwood of *P. marsupium* was procured from Shashi Phytochemical Industries, Alwar and further authenticated by the botanist of the Central research institute of homoeopathy, Noida. The *P. marsupium* heartwood was shade dried at room temperature and the dried wood was powdered and stored in airtight container till further use. Hundred gram powder of *P. marsupium* heartwood were packed in a soxhlet apparatus and extracted with 400 ml of absolute ethanol. The ethanol extracts were concentrated in a rotary evaporator (Hei-VAP-G1 Diagonal Heidolph, Germany). This crude extract was further used for the assessment of antioxidant activity.

2.2. Chemicals

Ascorbic acid; aluminum chloride, ferric chloride (FeCl3); Folin-Ciocalteu; bovine serum albumin (BSA); potassium persulphate; 2,2-diphenyl-1-picrylhy-drazyl (DPPH); nitro blue tetrazolium (NBT); phenazine methosulphate (PMS); sulphosalicylic acid; thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Sigma chemicals (St. Louis, MO, USA). All other chemicals procured from Sysco Research Laboratory (Mumbai, India). Methanol (99.8%) used were of analytical grade and purchased from Merck Life Science Private Limited, Mumbai, India.

2.3. In vitro studies antioxidant assays

The extract was dissolved in 95% methanol to make a concentration of 1 mg/ml and then diluted to prepare the series concentrations for antioxidant assays. Reference chemicals were used for comparison in all assays.

2.3.1. Determination of total phenol content

Total phenolic content in the alcoholic extract of *P. marsupium* was determined with Folin-Ciocalteau colorimetric method described using gallic acid as a standard phenolic compound.[21,22] Sample was diluted appropriately to obtain absorbance in the range of calibration curve. An aliquot of 1 ml of sample solution was mixed with 1 ml of Folin–Ciocalteau reagent. Three minutes later 3.0 ml of 2% sodium carbonate was added and the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance of the resulting blue color was measured at 765 nm with a UV–VIS spectrophotometer (UV 3000+, Labindia, Mumbai, India). Quantification was done with respect to the standard curve of gallic acid (10–100 μg/ml). The results were expressed as gallic acid equivalents (GAE), milligrams per 100 g of dry weight (dw). All determinations were performed in triplicate (n = 3).

2.3.2. Estimation of total flavonoid content

Aluminum chloride colorimetric technique was used for flavonoid estimation.[23] Quercetin, 10 mg was dissolved in 10 ml of methanol to get 1000 μg/ml solution and was used as standard. Aliquots ranging from 0.01 to 0.08 ml from the above stock solution were taken in different tubes. To each tube 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water was added. The reaction mixture was kept at room temperature for 30 min. The absorbance of the resulting solutions was measured at 415 nm against reagent blank. Methanol served as blank. The calibration curve was prepared by plotting absorbance against concentration (10–100 μg/ml). 10 mg of extracts were dissolved in 10 ml of methanol to get 1 mg/ml solutions respectively. The concentration of total flavonoid in the test sample was determined from the calibration curve. The total flavonoid content in the extract was expressed as quercetin equivalent (mg RE/g extract) and calculated by the following equation:

$$C = (c \times V)/m$$

where C = total content of flavonoid compounds, mg/g plant extract, in quercetin equivalent, c = the concentration of quercetin established from the calibration curve in mg/ml, V = the volume of extract in ml, and m = the weight of crude plant extract in g.

2.3.3. DPPH scavenging activity

The DPPH free radical scavenging activity of the alcoholic extract of *P. marsupium*, based on the scavenging of the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical was determined.[24,25] It is a discoloration assay, which is evaluated by the addition of the antioxidant to a DPPH solution in methanol and the ability to scavenge the stable free radical of DPPH was measured in the absorbance at 517 nm. The different concentrations

(10–320 μg/ml) in each reaction set were mixed with 1.0 ml of 0.1 mM of DPPH in methanol. The mixture was incubated in the dark for 30 min at room temperature. Degree of inhibition of DPPH by monitoring the decrease in absorbance measured at 517 nm. Ascorbic acid was used as positive control. Radical scavenging activity was expressed as inhibition percentage of free radical by the sample and was calculated using the following formula:

$$\% \text{ Inhibition} = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 was the absorbance of control (blank without sample) and A_t was the absorbance in presence of sample. All the tests were performed in triplicate and graph was plotted with mean values.

2.3.4. Hydrogen peroxide scavenging activity

The activity was determined according to the method described previously.[26,27] An aliquot of 40 mM H_2O_2 solution (0.6 ml) was mixed with various concentrations (10–320 μg/ml) of alcoholic extract of *P. marsupium*. To the mixture 2.4 ml of phosphate buffer (0.1 M, pH 7.4) was added and the mixture was shaken vigorously and incubated at room temperature for 10 min. Then, the absorbance of the reaction mixture was determined at 230 nm. Ascorbic acid was used as positive control. The H_2O_2 scavenging activity was calculated as follows:

$$\% \text{ Inhibition} = 1 - \left(\frac{A_1 - A_2}{A_0}\right) \times 100$$

where A_0 is the absorbance of the control (water instead of sample), A_1 is the absorbance of the sample and A_2 is the absorbance of the sample only (phosphate buffer instead of H_2O_2 solution). The IC_{50} value represented the concentration of the compounds that caused 50% inhibition of H_2O_2.

2.3.5. Reducing power assay

The ferricyanide-ferric chloride method[28] was adopted for evaluating the reducing power of alcoholic extract of *P. marsupium*. Different concentrations (10–320 μg/ml) of samples (2.5 ml) were mixed with 2.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide and the mixture was incubated in a water bath (50 °C for 20 min). After incubation, 2.5 ml of 10% trichloroacetic acid (w/v) was added and the mixture centrifuged (Remi-C24bL, Remi Laboratories Instruments, Mumbai, India) at 1000 rpm for 8 min. The supernatant (5 ml) was mixed with 5 ml of distilled water and 1 ml of 0.1% of ferric chloride and the absorbance was measured spectrophotometrically at 700 nm. The assay was carried out in triplicate and the results expressed as mean values ± standard deviations. Ascorbic acid was used as positive control.

2.3.6. Nitric oxide scavenging activity

At physiological pH, aqueous solution of sodium nitroprusside spontaneously generates nitric oxide[29] which interacts with oxygen to produce nitric ions that can be estimated by using Griess reagent. Scavengers of nitric oxide compete with oxygen leading to reduce production of nitric oxide. The reaction mixture of 5 mM Sodium nitroprusside in phosphate buffer saline (PBS) and 3.0 ml of different concentrations (10–320 μg/ml) of the alcoholic extract of *P. marsupium* was incubated at 25 °C for 150 min. After incubation, the samples were added to Greiss reagent (1% sulphanilamide, 2% H_3PO_4 and 0.1% napthylethylenediamine dihydrochloride). The pink chromophore generated during the diazotization of nitrite with sulphanilamide and subsequent coupling with napthylethylenediamine was measured at 546 nm. Ascorbic acid was used as positive control. The percentage of inhibition was measured by the following formula:

$$\% \text{ Inhibition} = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 was the absorbance of the control (blank, without sample) and A_t was the absorbance in the presence of the sample. All the tests were performed in triplicate and the graph was plotted with the mean values.

2.3.7. Superoxide radical scavenging activity

The activity was measured by the reduction of NBT (nitroblue tetrazolium reagent) method as previously reported.[30] The method is based on generation of superoxide radical (O^{2-}) by auto-oxidation of hydroxylamine hydrochloride in presence of NBT, which gets reduced to nitrite. Nitrite in presence of EDTA gives a color that was measured at 560 nm. The reaction mixture contains 1 ml of nitroblue tetrazolium (NBT) solution (l M NBT in 100 mM phosphate buffer, pH 7.4), 1 ml NADH solution (l M NADH in 100 mM phosphate buffer, pH 7.4) and 0.1 ml of the extracts (10–320 μg/ml) and ascorbic acid (0.01–0.32 mg/ml) were mixed. The reaction was started by adding 100 μl of PMS solution (60 μM PMS in 100 mM phosphate buffer, pH 7.4) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm was measured against blank samples, containing all the reagents except the PMS. The ascorbic acid used as positive control. The positive control and negative controls were subjected to the same procedures as the sample. In case of negative control only solvent was added, while sample was replaced with ascorbic acid during the measurement of positive control. All measurements were made in triplicate. The abilities to scavenge the superoxide radical were calculated using the following equation;

$$\% \text{ Inhibition} = \frac{A_0 - A_t}{A_0} \times 100$$

where A_0 was the absorbance of the control (blank, without sample) and A_t was the absorbance in the presence of the samples. All the tests were performed in triplicate and the graph was plotted with the mean values.

2.3.8. Total antioxidant capacity (TAOC)

The assay is based on the reduction of Mo (VI) to Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acidic pH.[31] An aliquot of 0.3 ml of alcoholic extract of *P. marsupium* were mixed with 3 ml of the reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). In case of blank 0.3 ml of methanol was used instead of sample. The sample was incubated on boiling water bath at 95 °C for 90 min followed by cooling at room temperature. The absorbance was measured at 695 nm against a blank. Ascorbic acid (50–600 μg/ml) was used as a standard. Total antioxidant capacity was expressed as equivalents of ascorbic acid (μmol/g).

2.4. Anti-glycation activity

In vitro antiglycation activity of alcoholic extract of *P. marsupium* at different concentrations (25–400 μg/ml) was examined by testing their ability to inhibit the fluorescence of BSA in accordance with a previous method.[6,32] The reaction mixture of BSA (10 mg/ml), 1.1 M fructose in 0.1 M phosphate buffered-saline (PBS), pH 7.4 containing 0.02% sodium azide with or without sample was incubated in darkness at 37 °C for 1, 2, 3, and 4 weeks. AGE formation was measured by fluorescent intensity at an excitation wavelength

355 nm and emission wavelength 460 nm. Aminoguanidine (25–400 μg/ml) was used as a positive control for this study.

2.5. Erythrocyte sorbitol accumulation inhibition

5 ml of heparinised human blood was collected from overnight fasted healthy male volunteer and erythrocytes were separated from the plasma by centrifugation at 3000 g for 30 min. The cells were washed three times with isotonic saline at 4 °C and in the final washing the cells were centrifuged at 1500 g for 15 min to obtain a consistently packed cell preparation. The packed cells (1 ml) were then incubated in Krebs-Ringer bicarbonate buffer (pH 7.4) (4 ml) containing 55 mM glucose in the presence or absence of alcoholic extract of *P. marsupium* at (25–400 μg/ml) at 37 °C for 3 h. The erythrocytes were washed with cold saline by centrifugation at 2000 g for 5 min, precipitated by adding 6% of cold perchloric acid (3 ml) and centrifuged again at 2000 g for 10 min. The supernatant was neutralized with 2.5 M K_2CO_3 at 4 °C and used for sorbitol determination.[33,34]

2.6. Aldose reductase (ALR1) enzyme inhibition

Partial purification of ALR1 from rat kidney was carried out following the previously described methods.[33,34] Isolated kidney was homogenized in 3 volumes of 10 mM sodium phosphate buffer, pH 7.2 containing 0.25 M sucrose, 2.0 mM EDTA, 2.5 mM 2-mercaptoethanol. The homogenate was centrifuged at 10,000 g for 20 min and the supernatant was subjected to ammonium sulfate precipitation. Precipitate obtained between 45% and 75% saturation was dissolved in the above buffer. The supernatant was used as the source of ALR1. The activity of ALR1 was measured spectrophotometrically by monitoring the oxidation of NADPH at 340 nm as a function of time at 37 °C using glyceraldehyde as substrate. The assay mixture in 1 ml contained 50 mM sodium phosphate buffer of pH 7.2, 0.2 M ammonium sulfate, 10 mM DL-glyceraldehyde, 5 mM β-mercaptoethanol and 0.1 mM NADPH. Various concentrations (25–400 μg/ml) of alcoholic extract of *P. marsupium* were added to assay mixtures of ALR1 and incubated for 5 min before initiating the reaction by NADPH as described above. The percentage inhibition was calculated considering the activity in the absence of alcoholic extract of *P. marsupium* as 100%. The IC_{50} values were determined by linear regression analysis of the plot of percent inhibition versus inhibitor concentration.

3. Results

3.1. Total phenolic content

Total phenolic content of the alcoholic extract of *P. marsupium* was determined by using the Folin-Ciocalteu reagent and were expressed as GAE per gram of plant extract. The total phenolic contents of the extract was calculated using the standard curve of gallic acid ($y = 0.010x - 0.031$; $R^2 = 0.994$) (Fig. 1). The alcoholic extract of *P. marsupium* was found to contain total phenolic value of 90.9 ± 1.6 mg GAE/g of gallic acid.

3.2. Total flavonoid content

The total flavonoid content of alcoholic extract of *P. marsupium* was determined using $AlCl_3$ method. A standard curve of quercetin at different concentrations was obtained and the flavonoid content of the alcoholic extract was extrapolated and calculated (Fig. 2). The total flavonoid content of alcoholic extract of *P. marsupium* was calculated as 57.33 ± 1.5 mg QE/g extract (n = 3).

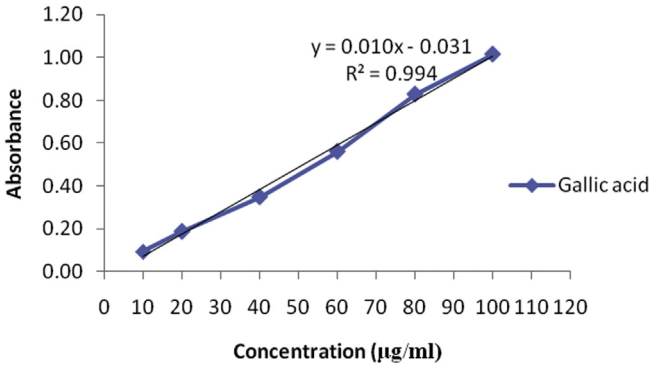

Fig. 1. Standard calibration curve of Gallic acid for estimation of total phenolic content.

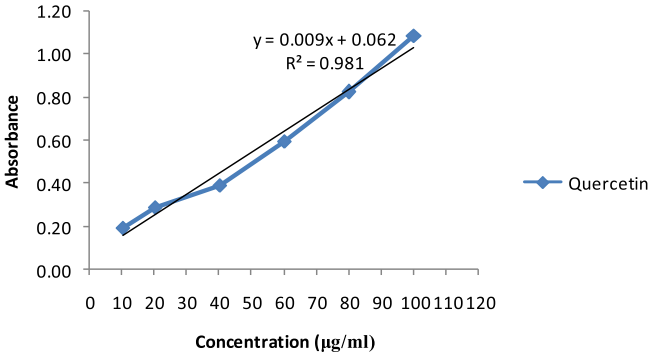

Fig. 2. Standard calibration curve of Quercetin for estimation of total flavonoid content.

3.3. DPPH radical scavenging activity

The free radical DPPH scavenging activity of alcoholic extract of *P. marsupium* was determined at various concentrations (10, 20, 40, 80, 160, 320 μg/ml) and compare with the standard ascorbic acid as mentioned in Fig. 3. It was observed that the extract scavenged free radicals in a concentration dependent manner and showed % inhibition in range of minimum at the concentration 10 μg/ml was 17.66 ± 1.94 and maximum 61.96 ± 0.58 at 320 μg/ml respectively. The scavenging activity of alcoholic extract and ascorbic acid in terms of IC_{50} is mentioned in Table 1. The IC_{50} value was defined as the concentration of sample that scavenged 50% of the DPPH. The standard ascorbic acid was found to have an IC_{50} of 43.47 μg/ml. Whereas, in comparison to standard ascorbic acid, alcoholic extract of heartwood of *P. marsupium* showed IC_{50} 203.77 μg/ml respectively.

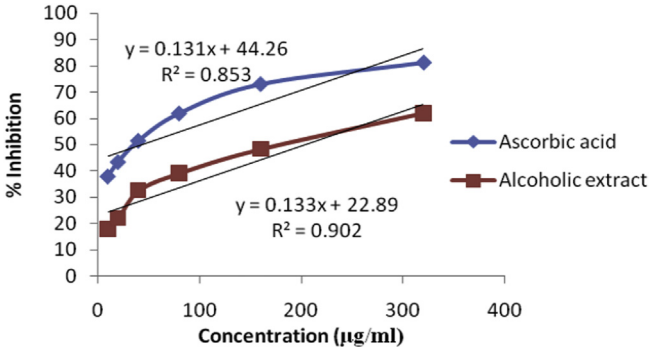

Fig. 3. DPPH radical scavenging activity of alcoholic extract of heartwood of *P. marsupium.*

Table 1
In vitro antioxidant activity of alcoholic extract of heartwood of *P. marsupium* in different antioxidant methods.

Antioxidant assays	IC_{50} (μg/ml)	
	Alcoholic extract of *P. marsupium*	Ascorbic acid
DPPH	203.77	43.47
Hydrogen peroxide scavenging activity	159.87	66.72
Nitric oxide scavenging activity	189.21	64.55
Superoxide radical scavenging activity	152.12	75.26

3.4. Hydrogen peroxide scavenging activity

The effect of alcoholic extract of *P. marsupium* on hydrogen peroxide radical scavenging activity is shown in Fig. 4. The alcoholic extract of *P. marsupium* showed significant antioxidant activity against H_2O_2 radical in a concentration (10–320 μg/ml) dependent manner and showed % inhibition in range of minimum at the concentration 10 μg/ml is 25.89 ± 0.81 and maximum 68.94 ± 0.52 at 320 μg/ml respectively. The scavenging activity of alcoholic extract and ascorbic acid in terms of IC_{50} is mentioned in Table 1. The standard ascorbic acid was found to have an IC_{50} of 66.72 μg/ml. Whereas, in comparison to standard ascorbic acid, alcoholic extract of heartwood of *P. marsupium* showed IC_{50} 159.87 μg/ml respectively.

3.5. Nitric oxide scavenging

The effect of alcoholic extract of *P. marsupium* on nitric oxide scavenging activity is shown in Fig. 5. The alcoholic extract of *P. marsupium* showed prominent nitric oxide scavenging activity in a concentration (10–320 μg/ml) dependent manner and showed % inhibition in range of minimum at the concentration 10 μg/ml was 22.88 ± 1.56 and maximum 62.76 ± 0.18 at 320 μg/ml respectively. The scavenging activity of alcoholic extract and ascorbic acid in terms of IC_{50} is mentioned in Table 1. The concentration of alcoholic extract needed for 50% inhibition (IC50) was found to be 189.21 μg/ml, whereas 64.55 μg/ml was needed for ascorbic acid.

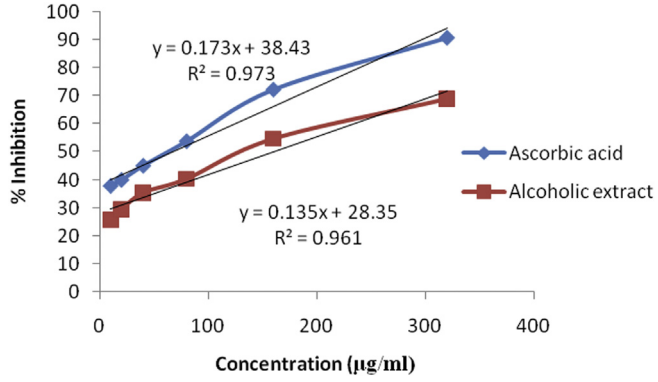

Fig. 4. H_2O_2 scavenging activity of alcoholic extract of heartwood of *P. marsupium*.

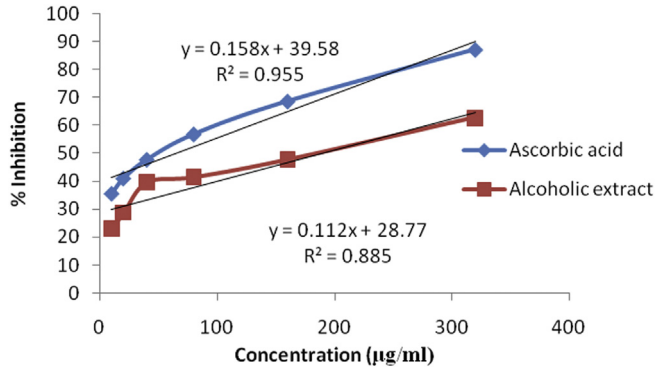

Fig. 5. Nitric oxide scavenging activity of alcoholic extract of heartwood of *P. marsupium*.

3.6. Superoxide radical scavenging activity

The percentage of superoxide anion scavenging activity of alcoholic extract of heartwood of *P. marsupium* was presented in Fig. 6. A maximum scavenging activity of alcoholic extract and ascorbic acid at 320 μg/ml was found to be 68.94 ± 0.517% and 75.49 ± 2.29%; IC_{50} value 152.12 μg/ml and 75.26 μg/ml respectively.

3.7. Total antioxidant activity

Total antioxidant capacity of the alcoholic extract of heartwood of *P. marsupium* was evaluated by the phosphomolybdenum method and expressed as ascorbic acid equivalents (AAE) per gram of plant extract. The method was based on the reduction of Mo (VI) to Mo (V) by the antioxidant compound and the formation of a green phosphate/Mo (V) complex with a maximal absorption at 695 nm. Total antioxidant capacity of the test samples was calculated using the standard curve of ascorbic acid ($y = 0.005x + 0.250$; $R^2 = 0.999$) (Fig. 7). Alcoholic extract of heartwood of *P. marsupium* was found to possess the total antioxidant capacity 162 ± 2.45 mg/g equivalent to ascorbic acid equivalents.

3.8. Reducing power activity

The reducing power of alcoholic extract and ascorbic acid are illustrated in Fig. 8. The increase in absorbance at 700 nm indicated better reducing power of test materials. The reducing power (A)

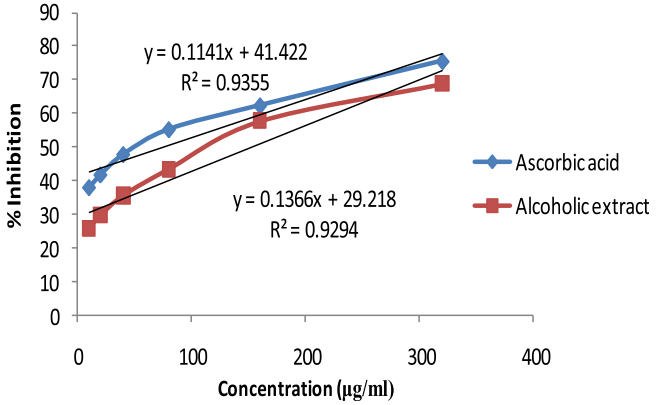

Fig. 6. Superoxide radical scavenging activity of alcoholic extract of heartwood of *P. marsupium*.

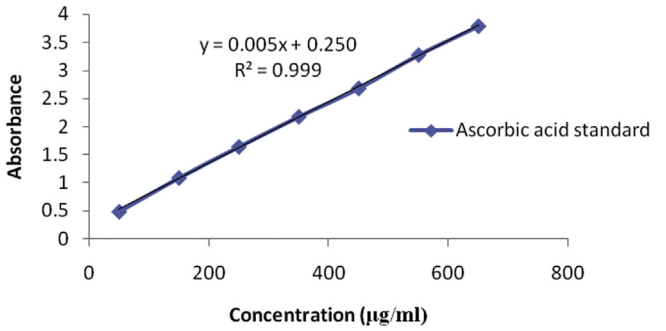

Fig. 7. Standard calibration curve of ascorbic acid for total antioxidant capacity activity.

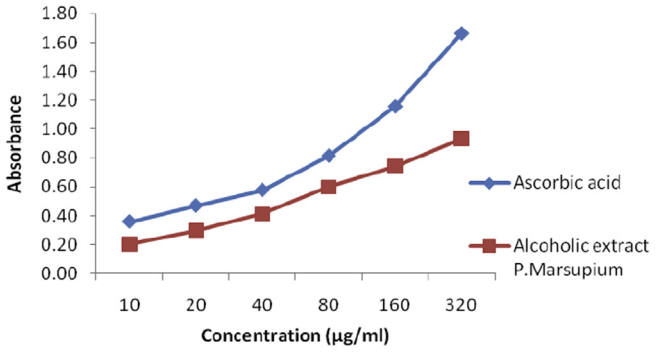

Fig. 8. Reducing power of alcoholic extract of heartwood of *P. marsupium*.

increased from 0.202 ± 0.021 to 0.932 ± 0.049 for alcoholic extract and 0.359 ± 0.051 to 1.661 ± 0.084 for ascorbic acid in a concentration-dependent manner (10–320 µg/ml) as the strength of the concentration doubled. The result indicated that the extracts may consist of polyphenolic compounds that usually show great reducing power.

3.9. AGEs inhibition activity

The formation of AGEs was monitored weekly by measuring fluorescence intensity of the BSA-fructose solutions (Fig. 9; Table 2). When BSA was incubated with fructose, a remarkable increase in fluorescence intensity was observed during 4 weeks of the experiment. After addition of alcoholic extract to reaction media containing BSA/fructose system, the fluorescence intensity was decreased in a concentration-dependent manner throughout the study period. A prominent inhibition of AGEs formation (93.38%) was observed in fructose-induced glycated BSA plus aminoguanidine (400 µg/ml). At 4th week of incubation, the percentage inhibitions of AGEs formation by extract of *P. marsupium* (25–400 µg/ml) was 38.49–84.78% and Aminoguadine was 40.95–93.38% respectively. At 4th week of incubation, the IC_{50} of AGEs formation by the extract of *P. marsupium* was 55.39 µg/ml and Aminoguadine was 35.77 µg/ml, respectively.

3.10. Erythrocyte sorbitol accumulation inhibition

Incubation of RBC with 55 mM glucose in the presence of alcoholic extract of *P. marsupium* under high glucose conditions lead to reduction in the accumulation of intracellular sorbitol in a dose dependent manner (Table 3). There was 50% reduction of sorbitol accumulation with alcoholic extract of *P. marsupium* was observed with IC_{50} 151.00 µg/ml and 105.12 µg/ml for ascorbic acid.

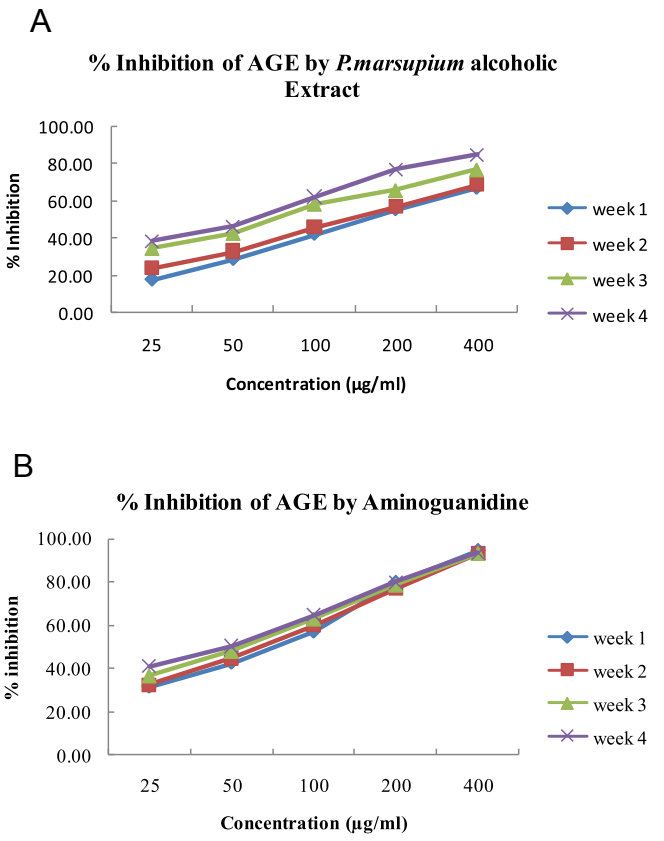

Fig. 9. (A & B): Effect of alcoholic extract of *P. marsupium* and Aminoguanidine on formation of fluorescent advanced glycation end products (AGEs) in BSA incubated with fructose. Values are Mean ± SEM.

Table 2
Results of AGE inhibition activity of alcoholic extract of heartwood of *P. marsupium* using aminoguadinine as standard.

	IC_{50} (µg/ml)	
	Alcoholic extract of *P. marsupium*	Aminoguadinine
Week 1	221.09	87.65
Week 2	195.43	79.74
Week 3	100.55	58.23
Week 4	55.39	35.77

3.11. Aldose reductase inhibitory activity

The inhibitory effect of alcoholic extract of *P. marsupium* and quercetin on the ALR1 enzyme partially purified from kidney of Sprague Dawley rat is summarized in Table 3. Alcoholic extract of *P. marsupium* was tested for their inhibitory activity against rat kidney ALR and IC_{50} value was calculated along with that of quercetin, a known ALR inhibitor (IC_{50} value of quercetin is 4.79 µg/ml). Alcoholic extract of *P. marsupium* inhibited ALR with IC_{50} value 195.88 µg/ml, respectively.

4. Discussion

Enhanced oxidative stress and the changes in antioxidant capacity plays a vital role in the pathogenesis of long term diabetic complications by increasing aldose reductase (AR) enzyme levels in polyol pathway and also by increasing the formation of advanced glycation end products (AGEs) which are observed in both clinical and experimental DM.[5,6] In the first step of the polyol pathway,

Table 3
Effect of alcoholic extract of *P. marsupium* on ALR inhibition and sorbitol accumulation inhibition assays.

Conc. (μg/ml)	Sorbitol accumulation inhibition (% inhibition ± S.D.)		ALR inhibition assay (% inhibition ± S.D.)	
	Alcoholic extract of *P. marsupium*	Ascorbic acid	Alcoholic extract of *P. marsupium*	Quercetin
25	17.98 ± 1.49	31.36 ± 1.50	20.17 ± 0.58	37.50 ± 2.07
50	40.95 ± 0.96	40.83 ± 1.12	30.51 ± 2.15	52.51 ± 2.20
100	53.01 ± 1.75	57.59 ± 0.93	41.42 ± 1.13	66.76 ± 0.57
200	63.41 ± 1.73	70.05 ± 0.05	59.59 ± 1.23	77.92 ± 2.36
400	76.16 ± 1.50	79.95 ± 0.07	72.18 ± 2.15	88.52 ± 2.87
IC_{50} (μg/ml)	151.00	105.12	195.88	4.79

Values are mean ± SEM for n = 3.

conversion of glucoses into sorbitol is catalysed by AR in the presence of the cofactor NADPH. In later stage, sorbitol is converted to fructose by sorbitol dehydrogenase enzyme. Augmented cellular sorbitol and fructose levels boost the generation of AGEs.[7] AGEs are heterogeneous group of molecules formed from the non-enzymatic reaction of reducing sugars with free amino groups of proteins, lipids, and nucleic acids and their formation is markedly accelerated in diabetes because of the increased availability of glucose.[35] Suppressing the metabolism of glucose via the polyol pathway by inhibiting AR is a potential way to prevent the complications noted above.[9]

P. marsupium (PM) is one such plant that has been used for over thousands of years as a treatment of different diseases. It has been scientifically reported for hypolipidemic, hepatoprotective, anti-ulcer, anti-inflammatory, and anti-diabetic activity. Phytochemical testing showed the presence of carbohydrates, glycosides, saponins, tannins, flavonoids, alkaloids and phenolic compounds, saponins, fixed oils and fats from various parts of the plant. It is also a rich source of polyphenolic compounds.[14] Epicatechin a flavonoid; marsupsin, pterosupin, pterostilbene an important phenolic constituents, 5, 7, 2—4 tetrahydroxy isoflavone 6-6 glucoside, a glycoside and potent anti-oxidant is assumed to be the main compound responsible for anti-diabetic effect.[13,14] Due to the close link between AGE formation and AR-related polyol pathway and reported activity of *P. marsupium* as antioxidant and anti-diabetic[15,17,18,20] in the present study, the role of alcoholic extract on antioxidant potential, ALR inhibition, sorbitol accumulation and inhibition on AGE products by different *in-vitro* methods was evaluated.

The alcoholic extract showed high content of phenolic content and total flavonoid when analyzed. Also high antioxidant activity was observed of alcoholic extract in different antioxidant assays in a concentration dependent manner. This relationship indicated that the free radical scavenging activity of the plant extracts was associated with the phenolic compounds as reported in several studies also.[12,15,16,18] This result agreed with previous studies reporting that phenolic compounds in various plant extracts are the major constituents with free radical scavenging property to donate a hydrogen atom from their phenolic hydroxyl groups.[22,23] In correspondence to this, the alcoholic extract also showed a significant inhibition of AGEs formation upto 4th week of treatment in a concentration dependent manner. It has been reported that most antiglycation agents from the edible plants have been reported to possess phenolic compounds and flavonoids. The inhibitory activity of flavonoids against protein glycation was strongly related to their scavenging effect on free radicals derived from the glycoxidation process.[36] The findings of the study indicate that high phenolic compounds and flavonoid contents of alcoholic extract may contribute to antiglycation activity by decreasing the ROS formation or by scavenging the ROS formed *in vitro* by auto-oxidation of sugars and/or oxidative degradation of amadori products.

In polyol pathway, conversion of glucoses into sorbitol is catalysed by AR in the presence of the cofactor NADPH which indicates direct correlation of AR and level of sorbitol.[33] Therefore, we assessed accumulation of sorbitol in RBC under high glucose conditions (ex vivo) to understand the significance of *in vitro* inhibition of ALR1 by alcoholic extract. Incubation of RBC with 55 mM glucose in presence of alcoholic extract resulted in reduction in the accumulation of intracellular sorbitol in a dose-dependent manner in comparison to ascorbic acid. This is observed with the results when the alcoholic extract evaluated for its effect on sorbitol accumulation, the observed IC_{50} was 151.00 μg/ml while 105.12 μg/ml was for ascorbic acid respectively. While compared to standard drug quercetin IC50 values 4.79 μg/ml, alcoholic extract of *P. marsupium* displayed modest IC50 195.88 μg/ml for their inhibitory activity against rat kidney ALR. These results not only substantiate the inhibition of ALR by alcoholic extract but also indicate the significance of alcoholic extract of *P. marsupium* in terms of preventing the accumulation of intracellular sorbitol.

To get the beneficial effect on consistent glycaemic control to prevent the onset and development of diabetic complications, natural sources with high phenolic and flavonoid content with high in antioxidant, antiglyecation potential along with inhibition of sorbitol accumulation and ALR inhibition would offer many advantages. Therefore, extensive research is required to identify such type of natural products that could be of potential agents for their therapeutic value to prevent diabetic complications.

5. Conclusion

In conclusion, the present investigation suggests that the alcoholic extract showed remarkable antioxidant activity and/or free radical scavenging activity, antiglycation, property and inhibit the accumulation of sorbitol and ALR enzyme may be due to the presence of the different type of constituents specifically phenolic and flavonoid contents. Multiple properties of the extract might make it more effective to reduce or delay diabetic complications subjected to confirmation with future *in vivo* evaluation to complete these findings.

Declaration of interest

The authors report no conflicts of interest.

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