Original Article

Antioxidant activity of *Gmelina arborea* Roxb. (Verbenaceae) bark extract: *In vivo* and *in vitro* study

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ABSTRACT

Context: *Gmelina arborea* Roxb (Family:Verbenaceae) is widely used in Sri Lankan traditional Ayurvedic medicine for long -term treatment of diabetes mellitus.

Aims: To investigate the *in vitro* and *in vivo* antioxidant activities of the aqueous bark extract of *G. arborea*.

Materials and Methods: The *in vitro* total antioxidant activities of the hot water bark extract of *G. arborea* were evaluated by 2,2'-diphenyl-2-picrylhydrazyl hydrate (DPPH), ferric reducing antioxidant potential (FRAP), and NO inhibition assays. The *in vivo* antioxidant activity was evaluated by the activities of liver enzymes, antioxidant enzymes, and extent of lipid peroxidation (LPO) in the liver of streptozotocin (STZ)-diabetic rats.

Results: *In vitro* antioxidant assays (DPPH, FRAP, and NO) clearly demonstrated the antioxidant potential of *G. arborea* extract. The *G. arborea* extract decreased LPO by 27%; activities of alanine aminotransferase, aspartate aminotransferase, and alkaline phosphatase decreased by 29%, 23% and 29%, respectively ($P < 0.05$). The liver reduced glutathione, activities of glutathione reductase, glutathione peroxidase, and glutathione S-transferase of plant extract treated diabetic rats increased to 606.47 ± 8.04 μg/g liver tissue, 7.92 ± 0.75, 8.56 ± 1.00, and 7.44 ± 1.42 nmol/min/mg protein, respectively ($P < 0.05$). The extract was more effective than glibenclamide in restoring the hepatic antioxidant enzymes in STZ diabetic rats.

Conclusions: The present investigation revealed that the bark extract of *G. arborea* exerts significant *in vivo* and *in vitro* antioxidant activities.

Key words: *Gmelina arborea*, hepatic oxidative stress markers, *in vitro* antioxidant assays, lipid peroxidation, streptozotocin-diabetic rats

Introduction

Medicinal plant extracts that possess antioxidant potentials have been found to be useful against cellular damage caused by increased oxidative stress.[1-3] *Gmelina arborea* Roxb. (common name: Et-demata, family: Verbenaceae) is valuable in Sri Lankan traditional medicine, and a decoction of the bark of G. *arborea* is successfully employed for long-term complications of diabetes mellitus by Ayurvedic physicians in Sri Lanka.[4,5] Extensive research have been done for the investigations on phytochemicals, antihyperglycemic, and *in vivo* toxic effects.[6,7] The present study was designed to determine the *in vitro* total antioxidant activities, the effect of bark extract of G. *arborea* on liver enzymes, hepatic oxidative stress markers in streptozotocin (STZ)-induced diabetic rats.

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Materials and Methods

Chemicals

All chemicals were of analytical grade and used without any purification.

Plant material

Bark parts of G. *arborea* were collected during May–June 2013 from the Southern region of Sri Lanka.

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Botanical identity was determined by the descriptions given by Jayaweera[4] and confirmed by comparing authenticated samples at National Herbarium, Royal Botanical Gardens, Peradeniya, Sri Lanka. A voucher specimen was preserved at the Department of Biochemistry, Faculty of Medicine, University of Ruhuna, Sri Lanka.

Preparation of the aqueous plant extract

The pieces of stem bark were cut into small pieces, dried at 40°C until a constant weight was reached and coarsely ground.

In vitro study

Powdered plant material (50.00 g) was dissolved in 400.0 mL of distilled water and refluxed for 4 hours. The concentration of the refluxed extract was 0.05 g/mL. A concentration series of the extract was prepared (1–500 μg/mL) for 2,2'-diphenyl-2-picrylhydrazyl hydrate (DPPH) assay and nitric oxide inhibition assays.

In vivo study

Powdered plant material (50.00 g) was dissolved in 400.0 mL of distilled water and refluxed for 4 hours. The mixture was strained through a cheese-cloth, and the final volume was adjusted to 50.0 mL. The dose of the extract was 1.00 g/kg.

Estimation of total polyphenol content

Total polyphenol content was estimated using Folin-Ciocalteu colorimetric method.[8] Plant extract (1.0 mL) was mixed with 95% EtOH (1.0 mL), distilled water (5.0 mL), and 50% Folin-Ciocalteu reagent (0.50 mL). The mixture was allowed to react for 5 minutes; 5% Na_2CO_3 (1.0 mL) was added to the resultant solution, mixed and placed in dark at 27°C for 1 hour. The absorbance of the resultant solution was measured spectrophotometrically at 725 nm. Quantification was done with respect to the standard curve of gallic acid at a range 0-50 mg/mL ($y = -0.0203 + 0.0101\times$). The results are expressed in gallic acid equivalents mgGAE/g of the dry weight.

Total flavonoid content

Total flavonoid content was estimated using the aluminum chloride method.[9,10] The plant extract (0.50 mL) was mixed with 95% EtOH (1.50 mL) followed by 10% $AlCl_3$ (0.10 mL), 1M potassium acetate (0.10 mL), and distilled water (2.80 mL). The resultant mixture was incubated at 27°C for 30 minutes. The absorbance of the reaction mixture was measured spectrophotometrically at 415 nm. The flavonoid content was calculated using standard calibration of quercetin solution at a range of 0-50 μg ($y = -0.0101 + 0.0072\times$). The results are expressed in quercetin equivalents μgQE/g of the dry weight.

2,2´-diphenyl-2-picrylhydrazyl hydrate radical scavenging activity

The total antioxidant activity was measured by the DPPH radical scavenging assay method.[11] Plant extract (1.00 mL) at different concentrations (1-500 μg/mL) was added to 0.004% DPPH solution (3.0 mL). The mixture was shaken vigorously, allowed to stand at 25°C in dark for 30 minutes. The decrease in absorbance of the resultant solution was measured spectrophotometrically at 517 nm, against a ethanol blank (A_{sample}). The absorbance of the DPPH solution alone was also measured at 517 nm ($A_{control}$). L-Ascorbic acid was used as the reference compound. The antioxidant activity is expressed in terms of IC_{50} (concentration of the extract/reference compound required to inhibit DPPH radical formation by 50%).

$$\% \text{ DPPH radical scavenging activity} = (A_{control} - A_{sample})/A_{control} \times 100\%$$

Ferric reducing antioxidant potential assay

The FRAP assay was performed according to the method of Benzie and Strain.[12] The FRAP working reagent (3.0 mL) and sample solution (100 μL) were mixed. The absorbance of the resultant solution was measured ($t = 0$) at 593 nm ($A_{sample\ t=o)}$ against a reagent blank. Thereafter, the sample was kept at 37°C for 4 minutes, and the absorption was measured at the same wave length after 4 minutes ($A_{sample\ t=4}$). The ascorbic acid (100 μM) was used as the standard compound and preceded as in the same way.

$$\text{FRAP value of the plant extract } (\mu M) = (A_{sample\ t=0\text{-}4})/(A_{standard\ t=0\text{-}4}) \times \text{FRA } P \text{ value of 1000 } \mu M \text{ ascorbic acid.}$$

Nitric oxide radical scavenging assay

Nitric oxide generated from SNP in aqueous solution at physiological pH interacts with the Griess reagent, and the absorbance of the chromophore was measured spectrophotometrically.[13] Also, 5 mM SNP (1.0 mL) was mixed with the plant extract (4.0 mL) at different concentrations (1-500 μg/mL) and incubated the resultant solution at 29°C for 2 hours. The incubated solution (2.0 mL) was mixed with the Griess reagent and measured the absorbance at 550 nm (A_{sample}) against distilled water blank. The absorbance of the control was also measured at the same wave length ($A_{control}$). L-Ascorbic acid was used as the reference compound. The antioxidant activity is expressed in terms of IC_{50} (micromolar concentration required to inhibit NO radical formation by 50%).

$$\% \text{ NO radical scavenging activity} = (A_{control} - A_{sample})/A_{control} \times 100\%$$

Development of the diabetic rat model

Streptozotocin dissolved in citrate buffer (0.1M, pH 4.4) at a dose of 65 mg/kg was administered intraperitonially to rats fasted for 12 hours. Thereafter, rats were maintained on 5% D-glucose solution for the next 24 hours. Rats were allowed to stabilize for 3 days; thereafter on the 4th day, blood samples were drawn from tail vein to determine the blood glucose concentration to confirm the development of diabetes mellitus. Rats with fasting blood glucose concentration of 12.0 mmol/L or above were considered as hyperglycemic and used for the experiments.[14]

Experimental design

Rats were randomly allotted to four groups of six animals per group. The group 1 and group 2 served as healthy and diabetic untreated (control) groups, respectively. The group 3 and group 4 diabetic rats received the aqueous bark extract of G. *arborea* at the optimum effective dose (1.00 g/kg) and glibenclamide (0.50 mg/kg) daily for 30 days, respectively. At the end of the study (on 30th day), blood was collected for the estimation of glycosylated hemoglobin (HbA_{1C}) and serum activities of liver enzymes. The liver tissues of rats were excised for the estimation of concentration of reduced glutathione, activities of antioxidant enzymes, extent of LPO.

Assessment of biochemical parameters

The ion exchange resin method as described by Abraham *et al*.[15] was followed for the estimation of glycosylated hemoglobin using spectrophotometric enzyme assay kit (Stanbio, USA). Fasting serum activities of liver enzymes, i.e., alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) were estimated using spectrophotometric enzyme assay kits.[16,17] The estimation of reduced glutathione (GSH), activities of glutathione reductase (GR, EC 1.6.4.2), glutathione peroxidase (GPx, EC 1.11.1.9), and glutathione S-transferase (GST, EC2.5.1.18) in the liver homogenates were done using reported protocols.[18-20] Further, the extent of LPO and total protein were estimated in liver homogenates by the formation of malondialdehyde (MDA) using thiobarbituric acid and Lowry methods, respectively.[21,22]

Assessment of liver histopathology

The liver tissues of test rats were fixed in 10% formalin. Tissues were processed routinely and embedded in paraffin wax. Sections were stained with hematoxylin and eosin.

Statistical analysis

The replicates of each sample were used for statistical analysis and the values were expressed as mean ± standard deviation in the *in vitro* study. The data were analyzed using analysis of variance (ANOVA), and the mean values for each group were compared by Dunnett's multiple comparison test in the *in vivo* study. The level of significance was set at $P < 0.05$.

Results

In vitro study

Total polyphenol and flavonoid contents of the G. *arborea* extract were 13.00 ± 1.10mg GAE/g of dry weight and 1.77 ± 0.1 μgQE/g of dry weight respectively. The scavenging ability of G. *arborea* on DPPH is shown in Figure 1 and compared with that of L- ascorbic acid. The scavenging effect of the extract and the reference on the DPPH radical is expressed as a percentage of inhibition. The IC_{50} values of the plant extract and the reference compound were 36.89 ± 1.23 μg/mL and 4.52 ± 0.11 μg/mL respectively. The reducing power of G. *arborea* was 8.98 ± 0.09 μM. The scavenging ability of G. *arborea* on NO is shown in Figure 2 and compared with the L-ascorbic acid. The extract of G. *arborea* was capable of scavenging NO in a dose-dependent manner. The IC_{50} values of the plant extract and the reference compound were 139.56 ± 4.20 μg/mL and 28.59 ± 0.80 μg/mL respectively.

In vivo study

As shown in Figure 3, plant extract-treated diabetic rats exhibited a remarkable glycemic control as evident by a reduction in the percentage of HbA_{1C} ($P < 0.05$). The effect

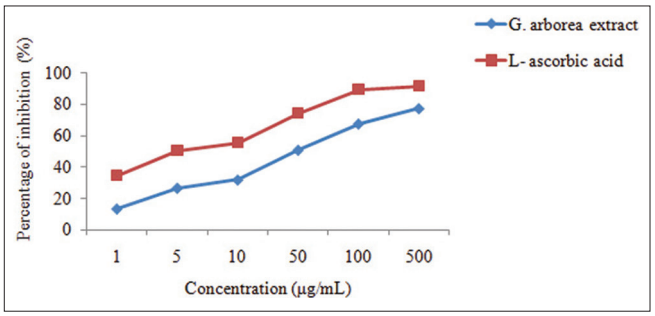

Figure 1: Percentage of inhibition in radical scavenging activity of *Gmelina arborea* extract and L-ascorbic acid at different concentrations (1-500 μg/mL)

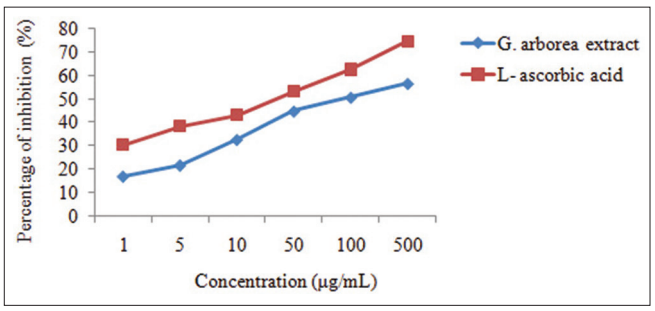

Figure 2: Percentage of inhibition NO radical scavenging activity of *Gmelina arborea* extract and L-ascorbic acid at different concentrations (1-500 μg/mL)

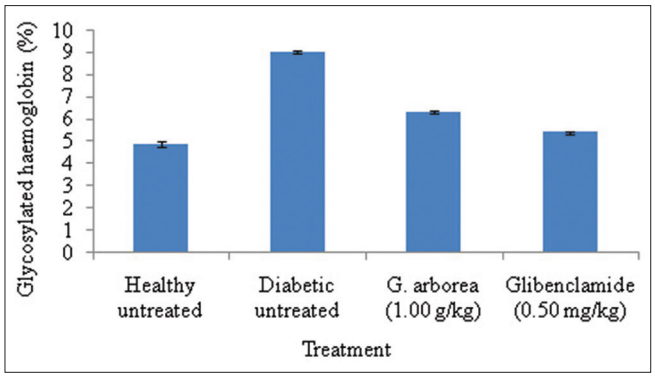

Figure 3: Effect of extract of *Gmelina arborea* on percentage of glycosylated hemoglobin. Data are expressed as mean ± SEM (*n* = 6/group)

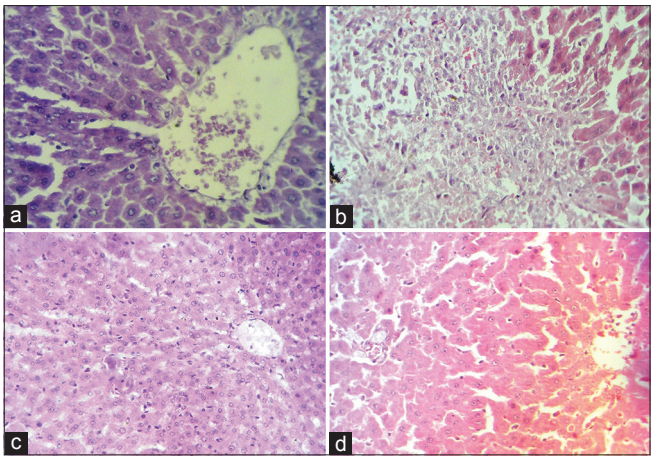

Figure 4: Photomicrographs (×400) of liver histopathology on hematoxylin and eosin-stained sections. (a) untreated healthy rats: Normal histological structure; (b) untreated diabetic rats: Focal necrosis with congestion; (c) *Gmelina arborea*-treated (1.00 g/kg) diabetic rats: No histological evidence of necrosis; (d) Glibenclamide-treated (0.50 mg/kg) diabetic rats: Moderate lymphocytic infiltrates with no necrosis

of the G. *arborea* bark extract on liver enzymes and hepatic oxidative stress markers in STZ-diabetic rats is shown in Tables 1 and 2, respectively. There was an elevation in the activities of ALT, AST, ALP, and the concentration of MDA in streptozotocin diabetic rats when compared with the untreated healthy rats. On the other hand, there was a reduction in the concentration of GSH, GR, GPx, and GST in the same treatment groups. Treatment of diabetic rats with the extract and glibenclamide decreased the activities of ALT (by 29%, 16%), AST (by 23%, 17%) and ALP (by 29%, 5%) ($P < 0.05$). The protective effect of the extract on LPO was also demonstrated; significant reduction in the concentration of MDA by 27% when compared with untreated diabetic rats ($P < 0.05$). The administration of the plant extract restored the concentration of GSH, activities of GR, GPx, GST to near normalcy (by 44%, 49%, 86%, 57%), and it was more effective than the attainment of above biochemical parameters by glibenclamide (by 35%, 20%, 38%, 12%).

As shown in Figure 4, liver histology was normal in untreated healthy rats. In contrast, untreated diabetic rats showed very early microvesicular fatty change in centrilobular areas of the liver, mild congestion, moderate lymphocytic infiltrates mostly around portal tract, increased fibrosis with paranchymal infiltrates and focal necrosis. The light microscopic appearance of the liver tissue in G. *arborea*-treated rats are in line with biochemical results, with a reduction in microvesicular fatty change, mild lymphocytic infiltrates, and no necrosis. Further, reduction in microvesicular fatty change and moderate lymphocytic infiltrates were also observed in glibenclamide-treated diabetic rats.

Table 1: Effect of *Gmelina arborea* on serum liver enzymes in streptozotocin induced diabetic rats after 30 days of treatment

Treatment	ALT (U/L)	AST (U/L)	ALP (U/L)
Healthy untreated	12.40±0.12	44.21±1.75	61.48±1.00
Diabetic untreated	36.64±2.24	90.08±0.07	165.69±2.52
G. arborea (1.00 g/kg)	26.02±0.57*	69.67±2.03*	117.48±3.59*
Glibenclamide (0.50 mg/kg)	30.67±1.03*	74.98±3.00*	156.65±2.30*

The values are expressed as mean±SEM (*n*=6/group). *Statistically significant from streptozotocin-induced diabetic control rats at *P*<0.05 (ANOVA followed by Dunnett's test). ALT: Alanine aminotransferase, AST: Aspartate aminotransferase, ALP: Alkaline phosphatase

Discussion

The chemical approaches facilitate the study of total antioxidant activity of medicinal plant extracts and the precise mechanisms of action of antioxidants. So far, numerous studies on antioxidant properties of many plant species have been conducted using different assay methods. The general recommendation is to employ at least three *in vitro* methods due to the presence of wide variety of oxidation systems.[23] In the present study, antioxidant activity was evaluated by three spectrophotometric methods; DPPH, FRAP, and NO assay. The IC_{50} value of the extract was calculated for DPPH and NO inhibition assays to compare the antioxidant activities at different concentrations and to obtain a more precise single value over a range of concentration of the plant extract as described by many authors.[24,25] The bark extract of G. *arborea* and the standard compound exhibited concentration-dependent radical scavenging activities in DPPH and NO inhibition assays. The DPPH assay is reported to be a direct and reliable method for the determination of radical scavenging activity, where the structure of electron donor (e.g. plant extract) is not known. DPPH assay method can afford data on reduction potential of the sample and hence can be helpful in comparing the reduction potential of unknown compounds. A key mediator released by activated macrophages that has been

Table 2: Effect of plant extracts on hepatic antioxidative stress markers in streptozotocin-induced diabetic rats after 30 days of treatment

Treatment	MDA (nmol/mg protein)	GSH (µg/g liver)	GR (nmol/min/mg of protein)	GPx (nmol/min/mg of protein)	GST (nmol/min/mg of protein)
Healthy untreated	12.15±0.30	719.80±7.45	9.12±1.06	10.58±0.98	10.92±1.47
Diabetic untreated	36.38±1.01	419.65±8.77	5.33±0.54	4.61±1.03	4.75±0.15
G. arborea (1.00 g/kg)	26.50±0.25*	606.47±8.04*	7.92±0.81*	8.56±1.00*	7.44±1.42*
Glibenclamide (0.50 mg/kg)	22.09±0.25*	564.71±9.86*	6.39±0.14*	6.38±0.18*	5.34±0.20*

The values are expressed as mean±SEM (n=6/group). *Statistically significant from streptozotocin induced diabetic control rats at P<0.05 (ANOVA followed by Dunnett's test). MDA: Malondialdehyde, GSH: Reduced glutathione, GR: Glutathione reductase, GPx: Glutathione peroxidase, GST: Glutathione S-transferase

implicated in toxicity is nitric oxide. It has been pointed out that modulating nitric oxide production can modify tissue injury.[26] Thus, development of specific nitric oxide scavengers is considered important due to lack of endogenous enzymes responsible for the inactivation of nitric oxide.[27]

Diabetogenic action of streptozotocin occurs due to synergistic actions of DNA alkylation followed by fragmentation of DNA (deoxyribonucleic acid), activation of poly ADP (adenosine diphosphate)-ribose polymerase result in the inhibition of synthesis and secretion of insulin.[28] However, STZ-diabetic rats serve as an excellent model to study molecular, cellular and morphological changes of oxidative stress in diabetes.[29] The estimation of glycosylated hemoglobin is considered as a reliable marker of glycemic control and a well-accepted parameter used in the diagnosis and predicting the prognosis of the diabetic state.[30] The extract produced a significant reduction in the percentage of $HbA_{1C,}$ implying a considerable glycemic control after 30 days of treatment. However, the effect is less than the effect of standard drug glibenclamide.

The AST and ALT are found in large quantities in the liver where they play an important role in the metabolism of amino acids. However, as a result of cellular damage caused by reactive oxygen species or toxicity to the liver, these enzymes may leak from the hepatocytes into the circulation where their levels become elevated. Therefore, the elevated levels of AST and ALT are indicators of functional disturbance of liver cell membranes and cellular infiltrations. In addition, ALP is membrane bound, and its alteration is likely to affect the membrane permeability and produce derangements in the transport of metabolites. Significant increase in serum activities of AST, ALT, and ALP in STZ-diabetic rats is also consistent with published data.[31] However, the values were significantly decreased in plant extract-treated diabetic rats. Accordingly, results revealed the extract of G. *arborea*-accelerated regeneration in hepatocytes, thus decreased the leakage of ALT, AST, and ALP into systemic circulation. Further, the liver histopathology matches with biochemical results.

Reduction of activities of hepatic antioxidant enzymes such as GR, GPx, and GST were observed in diabetic rats indicating impaired liver function. GR is a secondary antioxidant enzyme used for the regeneration of GSH from oxidized glutathione, particularly susceptible to oxidative damage from peroxynitrite. GR activity is specifically inhibited by oxidative stress,[32] which was also proved by a significant reduction of GR in untreated diabetic rats ($P < 0.05$). GPx is predominantly distributed in the cytosol and mitochondria of hepatocytes, and the activity depends on the concentration of GSH. Further, GST work with antioxidant systems and involved in the defense mechanisms in response to the oxidative stress. The present study provoked a reduction in the activity of GST in untreated diabetic rats. Return of the above antioxidant enzymes to near normalcy may be due to the presence of active phytochemicals as polyphenols and flavonoids in the G. *arborea* extract. This may be due to the protection of cellular proteins against oxidation through glutathione redox cycle, prevention of intracellular enzyme leakage resulting from cell membrane stability, hepatocellular regeneration, direct detoxification of reactive oxygen and nitrogen species.[33] Oxygen-free radicals exert their cytotoxic effects on membrane phospholipids resulting in the formation of MDA. As a secondary product of LPO, MDA would reflect the degree of oxidation in tissues. The elevation of extent of LPO in liver tissue of diabetic rats is mainly due to the substantial reduction in hepatic GSH and depletion of antioxidant scavenger systems.[34] The findings of the present study showed the protective effects of the extract of G. *arborea* on lipid peroxides.

Even though there was a pronounced antihyperglycemic effect in diabetic rats treated with glibenclamide, we report for the first time that the extract of G. *arborea* appeared to be more effective than glibenclamide in ameliorating the oxidative stress in diabetic rats. The antihyperglycemic action of glibenclamide is mediated through stimulating insulin secretion via β-cells in the pancreatic tissue,[35] and the results suggest that there is no direct effect on oxidative stress in diabetic rats. However, the results are in accordance with the findings of several other authors

who also observed more powerful antioxidant activities in herbal extracts than that of glibenclamide.[36] In fact, this may further strengthen the direct *in vivo* antioxidant activity of the plant extract.

Conclusion

The data revealed that the extract of G. *arborea* markedly restored liver enzymes, improved antioxidant status of the liver tissues in STZ-diabetic rats. The *in vivo* antioxidant activities of the G. *arborea* extract may be ascribed to the attenuation of free radical-mediated oxidative damage in diabetes mellitus. The long-term remedy with the G. *arborea* bark may also be useful for the prevention/attenuation of associated complications of diabetes mellitus. Secondary metabolites mainly as polyphenol compounds, flavonoids present in the plant extract may attribute to the antioxidative effects in diabetic rats.

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