Antiradical and antidiabetic properties of standardized extract of Sunderban mangrove *Rhizophora mucronata*

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Submitted: 25-02-2014 Revised: 02-05-2014 Published: 12-03-2015

ABSTRACT

Background: Mangroves have the ability to grow where no other vascular plants survive. *Rhizophora mucronata* is a true mangrove and traditionally used to treat diabetes and its allied complications. **Objectives:** In the present study, we standardized the 80% methanolic standardized extract of *R. mucronata* leaves (RH) and found out its antiradical and antidiabetic activities. **Materials and Methods:** The methanolic extract of *R. mucronata* leaves (RH) was standardized and quantified for phenolics, flavonoinds, gallic acid, quercetin, and coumarin. The reducing abilities and antiradical activities of RH were performed *in vitro* methods like, 1,1-diphenyl-2-picrylhydrazyl, nitric oxides, superoxides, hydroxyl, and ABTS (2,2′-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid). Thereafter, RH was evaluated for it antidiabetic potentialities on streptozotocin (STZ)-induced type-2 diabetes. STZ (90 mg/kg, intraperitoneal) was administered to 2 days old pups to induce diabetes. RH was fed at doses of 50 and 100 mg/kg and glibenclamide (positive control) at 5 mg/kg, when the rats were 6 weeks old and continued for 10 weeks. Fasting glucose was monitored before and after the treatment. Further, lipid peroxides and reduced glutathione level were estimated on rat liver. **Results:** The results obtained from this study revealed RH possesses flavonoinds and also gallic acid, quercetin, and coumarin. Further, it has antiradical activities. It has also reduced blood glucose level in type-2 diabetic rats and reduced the formation of lipid peroxidation in liver. RH enhanced the level of glutathione in liver tissue. **Conclusion:** RH exhibits source of natural antioxidants and great potentialities as an antidiabetic agent by improving the hyperglycemia through its antiradical action.

Key words: Antioxidant, diabetes, free radicals, mangrove, pups, streptozotocin

Access this article online

Website: www.phcog.com

DOI: 10.4103/0973-1296.153094

Quick Response Code:

INTRODUCTION

Rhizophora mucronata L.(Rhizophoraceae), a true mangrove is widely distributed along the delta of Sunderbans (21°32' and 22°40'N and between 88°05' and 80°00'E) – the largest mangrove belt (4200 km^2) in India.[1,2] One of the principal uses of this plant is the extraction of tannins.[3] The bark, root, leaves, fruit, and flowers of *R. mucronata* have been used in traditional medicine in South Asian countries including India for treating diabetes, diarrhea, hepatitis, inflammation, wounds and ulcers, etc.[4-6] So far, identified chemical constituents of *R. mucronata* are alkaloids, hydrolysable tannins, polyphenols, flavonoids, triterpenes, inositols, polysachharides, saponins, anthrocyanidins, etc.[4-10] It has bactericidal and antiviral properties.[11] Ehanolic extract of *Rhizophora* has shown antidiabetic antihyperglycemic activity, but its underlying mechanism till to explore.[12] Oxidative stress may be a common pathway relating diverse seemingly distinct mechanisms proposed for the pathogenesis of complications in diabetes.[13,14] Natural products classified into terpenoids, alkaloids, flavonoids, phenolics, and some other categories have shown antidiabetic as well as antioxidant potential.[15-17] The aim of this study was to assess the antiradical and antidiabetic potentialities of *R. mucronata* and to elicit the underlying mechanisms. For this purpose, we studied the role of *R. mucronata* in oxidative stress by radical scavenging activity and focus on its active ingredients. We also evaluated antidiabetic activity of *R. mucronata* in streptozotocin (STZ) induced type-2 diabetic rats.

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MATERIALS AND METHODS

Animals

Inbred, Wistar strain albino rats were selected for this study. Principles of laboratory animal care guidelines (NIH publication number 85–23, revised 1985) were strictly followed. The animals were maintained under 12:12 h dark: Light cycle and controlled temperature (25 ± 3°C); fed with food (Provimi, Bengaluru, India) and water *ad libitum*. Institutional Animal Ethical Committee approved the experimental protocol. All the experiments were performed between 08:00 and 16:00 h.

Test drug preparation (DST/537/5G-4/09-03)

The leaves of *R. mucronata* was collected from the mangrove forest area of Sunderban, West Bengal and identified by Botanical Survey of India, West Bengal. A voucher specimen (DST/537/5G-4/09-03) was deposited in the departmental herbarium. The shed-dried powdered leaves of *R. mucronata* were extracted with hydro-methanol (20–80%) in soxhlet apparatus and concentrated by distilling the solvents and dried under reduced pressure. The extractive value was determined by gravimetric method and expressed as percentage yield. The bioactive phytoconstituents presence in hydro-methanolic extract of *R. mucronata* (RH) was evaluated as described by Harbone.[18] In brief, presence of a*lkaloids* in PE mangrove extracts were find out by Dragendroff's test, sterols and triterpenoids by Liberman Buchard test, saponins by Froth test, tannins by lead acetate test, carbohydrates by Fehling's test, reducing sugars by Benedict test, p*roteins* by Million's test, phenolics by ferric chloride test, flavonoids by aluminum chloride test, and anthraquinone g*lycoside* by Borntrager's test.

Standardization of test drug

Gallic acid (3,4,5-trihydroxybenzoic acid), quercetin (5,7,3',4'-tetrahydroxy flavonol) and coumarin (1,2-benzopyrone) – three known biomarker were used to standardized the extract.[19,20] RH was diluted into 1 mg/ml in methanol and spotted in the form of bands with on a precoated silica gel plates (Merck, $60F_{254}$, 20 cm × 20 cm) using Camag Linomat five applicator. The plates developed in a solvent system (toluene: Ethyl acetate: Formic acid = 4.5:3:0.2) in for 30 min. The densitometric scanning was performed on Camag TLC Scanner3 at absorbance 280 nm. The amount of gallic acid, quercetin, and coumarin present in RH was calculated against respective markers and expressed per mg of extract.

Estimation of phenolic compounds

The amount of total phenolics in RH was determined as described earlier.[21] To 0.2 ml of RH solution in methanol (1 mg/ml), 1.0 ml of Folin-Ciocalteu reagent (1:2 with deionized water) was added, mixed, and incubated in the dark for 5 min. Thereafter, 0.8 ml of 7.5% sodium carbonate solution was added, mixed, and further incubated in the dark for 30 min at room temperature. Finally, 3 ml of deionized water was added, and the absorbance at 765 nm was measured (Ultrospec 2000 Ultraviolet-visible spectrophotometer, Pharmacia Biotech, USA). The total phenolic content was expressed as gallic acid equivalent (GAE) in μg/mg of extract.

Estimation of flavonoids

Aluminum chloride colorimetric technique was used for estimation of flavonoids content present in the mangrove extract.[22] 0.2 ml of RH (1 mg/ml) was mixed with 0.8 ml methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate, and 2.8 ml of distilled water. It was left at room temperature for 30 min after which the absorbance of the reaction mixture was measured at 415 nm. The calibration curve was plotted by preparing the quercetin solutions at concentrations 12.5–100 μg/ml in methanol.

Estimation of reducing power

The reducing power of RH was evaluated as described earlier.[21] 0.1 ml of RH was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6), and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. Then, 2.5 ml of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with distilled water (2.5 ml) and ferric chloride solution (0.5 ml, 0.1%). The absorbance was measured at 700 nm. Reducing power was given in ascorbic acid equivalent in μg/mg of extract.

Estimation of 1,1-diphenyl-2-picrylhydrazyl radical scavenging

The radical scavenging activity of RH was determined using the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH).[21,23] 0.1 ml of RH in methanol at different known concentrations was mixed with 3.9 ml of 0.135 mM DPPH solution and was allowed to stand in the dark for 30 min. Butylated hydroxytoluene was used as a positive control. The absorbance values were measured at 517 nm. The sample concentration required to scavenge 50% of DPPH (IC_{50}) was determined.

Estimation of superoxide anion radical scavenging

Superoxide anion scavenging activity of RH was determined following the method of Liu *et al.*[24] The superoxide radicals were generated in 3 ml of tris-HCl buffer (0.1M, pH 7.4) containing 0.75 ml of nitro blue tetrazolium (300 μm) solution, 0.75 ml of NADH (936 μm) solution and 0.3 ml of RH at different concentrations (10–100 μl in methanol). The reaction started by adding 0.75 ml of phenazine methosulfate (120 μm) to the mixture. The reaction mixture

was exposed to light at room temperature for 5 min. Thereafter, the absorbance was read at 560 nm. L-ascorbic acid was used as a positive control. The percent inhibition of superoxide anion generation of RH was calculated and was expressed as IC_{50}.

Estimation of nitric oxide radical scavenging

RH in different concentration was mixed in 0.5 ml of 1 M phosphate buffer saline, 2 ml of 10 mM sodium nitroprusside and incubated at 25°C for 150 min. Potassium nitrite solution was used as a standard. After incubation, 0.5 ml of the reaction mixture containing nitrite was pipetted and mixed with 1 ml of 0.33%sulphanilic acid reagent and allowed to stand for 5 min for completing diazotization. Then, 1 ml of 1% naphthylethylenediamine dihydrochloride was added, mixed, and allowed to stand for another 30 min.[25] The absorbance of pink-colored chromophore was measured at 540 nm.

Estimation of hydroxyl radical scavenging

The extent of hydroxyl radical ($HO^{\bullet}$) scavenging of RH from Fenton reaction was quantified using 2'-deoxyribose oxidative degradation as described by Elizabeth and Rao.[26] The reaction mixture contained 0.1 ml of 2.8 mM deoxyribose, 0.1 ml of 0.1 mM $FeCl_3$, 0.1 ml of 0.1 mM EDTA, 0.1 ml of 1 mM H_2O_2, 0.1 ml of 0.1 mM ascorbate, 0.1 ml of 20 mM phosphate buffer (pH 7.4) and 1 ml of RH at different concentrations. The mixture was incubated at 37°C for 1 h. At the end of the incubation period, 1.0 ml of 1% thiobarbituric acid (TBA) was added and heated at 95°C for 20 min to develop the color. After cooling, the TBA reacting substances (TBARS) formation was measured at 532 nm. The percent TBARS production for positive control (H_2O_2) was fixed at 100%, and the relative percent TBARS was calculated for the extract treated groups.

Estimation of ABTS radical scavenging

ABTS (2,2'-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid) radical cation decolorization assay was determined according to the method of Re *et al.* (1999).[27] The stock solution included $ABTS^+$ solution (7 mM) and ammonium persulfate (2.45 mM). The working $ABTS^+$ solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12–16 h at room temperature in the dark. The solution was then diluted by mixing 1 ml of $ABTS^+$ solution with methanol to obtain an absorbance of 0.7 (±0.01) at 734 nm. Different concentration of RH was added to 1 ml of ABTS solution and after 7 min the absorbance was read at 734 nm and the percent inhibition was calculated and expressed as IC_{50}.

Antidiabetic activity in Type-2 rats

Type-2 diabetes was induced in 2 days old pups by a single intraperitoneal injection of STZ or STZ (Sigma, St. Louis, MO, USA), at a dose of 90 mg/kg body weight in 0.1 M citrate buffer (pH 4.5) as described by Wang *et al.*[28] At 3 weeks and 6 weeks, fasting blood glucose was monitored by Glucometer (Accu-Chek Active, Roche, Mannheim, Germany)[12] and the rats were divided into four groups ($n = 6$) as follows: Group I: Normal water control group fed daily with deionized water (2 ml/kg); Group II: Glibenclamide (GB) positive control group fed daily with GB (5 mg/kg); Group III and Group IV: Fed daily with RH at a dose of 50 mg/kg and 100 mg/kg respectively. The treatment was continued for 28 consecutive days and finally, fasting blood glucose was monitored. Thereafter, all rats were sacrificed under deep anesthesia, and the liver was dissected out. The liver homogenate was prepared to estimate lipid peroxidation,[29] glutathione,[30] and protein[29,30] spectrophotometrically.

Statistical analysis

Data have been summarized by routine descriptive statistics. All statistical analyses were performed using the SPSS software, version 17.0. Differences between and within groups have been assessed for statistical significance by standard parametric and nonparametric tests, as appropriate. $P < 0.05$ was taken as the level of statistical significance in all tests.

RESULTS

Chemical standardization

RH showed the presence of phytoconstituents such as, alkaloids, phenolics, flavonoids, triterpenoids, steroids, glycosides, saponins, and tannins in group tests. The eluted materials were nearly 9.54%. In Table 1, RH showed rich in phenolics (1.03 μg GAE/mg extract) and flavonoids (2.84 μg QE/mg extract). Moreover, high-performance thin layer chromatography (HPTLC) densiometric analysis confirmed the presence of gallic acid (19.317 μg/mg extract), quercetin (10.451 μg/mg extract), and coumarin (21.847 μg/mg extract) in RH [Table 1 and Figure 1].

Antiradical activities

In vitro antiradical properties of RH were exhibited in Table 2. The 50% inhibitory reducing capacity of RH was 4.50 ± 0.190 μg/mg, and that may serve as a significant indicator of its antioxidant potential. Moreover, IC_{50} values of RH on DPPH radical scavenging activity were 0.551 μg/mg, nitric oxide (NO) radical inhibitory activity was 0.360 μg/mg, superoxide scavenging activity was 0.634 μg/mg, $HO^{\bullet}$ was 0.525 μg/mg, and $ABTS^+$ scavenging action was 0.149 μg/mg.

Antidiabetic activity

As summarized in Table 3, all animals showed significant elevation of fasting blood glucose level at weeks 6 as

Table 1: Chemical constituents in hydro-methanolic leaves extract of *Rhizophora mucronata*

	Phenolics (µg GAE/mg extract)	Flavonoids (µg QE/mg extract)	HPTLC densitometric quantification (µg/mg extract)		
			Gallic acid	Quercetin	Coumarin
RH	9.82±0.057	27.09±0.122	19.317±0.324	10.451±0.129	21.847±0.221

All tests were performed in triplicate and results represented as mean±SEM RH means hydro-methanolic extract (20:80) of *Rhizophora mucronata* leaves. GAE r^2=0.9980 and QE r=0.9981. In HPTLC analyses, R_f value of gallic acid, quercetin and coumarin were 0.22, 0.38 and 0.66 respectively. Correlation coefficient or r of gallic acid was 0.99681, quercetin was 0.99662 and coumarin was 0.99164. The densitometric scanning was performed on Camag TLC scanner 3 at absorbance 280 nm and operated by multilevel winCATS planar chromatography manager. RH: Relative humidity; HPTLC: High-performance thin layer chromatography; GAE: Gallic acid equivalent; QE: Quercetin equivalent; SEM: Standard error of the mean

Table 2: *In vitro* antioxidant activities of *Rhizophora mucronata* leaves

	Radical scavenging activities of RH	
	Positive control	50% inhibitory concentration µg/mg extract
Reducing power	L-ascorbic acid	42.93±0.190
$DPPH^+$ scavenging	Butylated hydroxyl toluene	5.25±0.039
NO radical scavenging	Potassium nitrite	3.44±0.038
$O_2^•$ scavenging	L-ascorbic acid	6.04±0.012
$HO^•$ scavenging	Hydrogen peroxide	5.01±0.072
$ABTS^+$ scavenging	L-ascorbic acid	1.42±0.009

All tests were performed in triplicate and results represented as mean±SEM. RH means hydro-methanolic extract (20:80) of *Rhizophora mucronata* leaves. RH: Relative humidity; SEM: Standard error of the mean; DPPH: 1,1-diphenyl-2-picrylhydrazyl; ABTS: 2,2′-azino-bis-3-ethyl benzthiazoline-6-sulphonic acid; NO: Nitric acid; HO: Hydroxyl radical; O_2: Oxygen

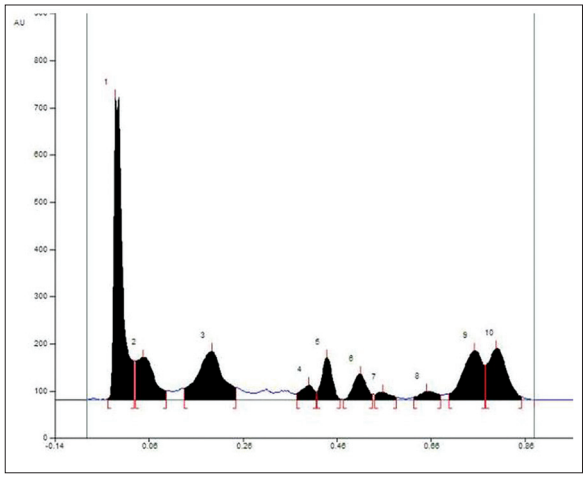

Figure 1: High-performance thin layer chromatography densitometric chromatogram of hydro-methanolic leaves extract of *Rhizophora mucronata*. Pick three identified as gallic acid, pick four as quercetin, and pick eight as coumarin

compared with basal (weeks 3) level. There were significant differences in fasting blood sugar activity among the STZ control, GB (5 mg/kg) and RH (50 and 100 mg/kg) treated groups ($P < 0.05$). When compared with the STZ group, RH treatment produced a dynamic dose-dependent decrease in fasting blood glucose concentration (−11.69% and −21.38%) after consecutive 4 weeks treatment, in similar to GB (−19.66%). Moreover, Table 4 exhibits, RH treatment significantly and dose-dependently reduced the elevation of lipid peroxides (−27.83% and −41.14%), while enhanced the concentration of glutathione (81.86% and 105.58%) in liver in respect to STZ-induced control. But, GB did not alter the lipid peroxides levels but significantly enhanced the glutathione concentration (34.41).

DISCUSSION

Substantial scientific evidence suggested that under situations of oxidative stress reactive oxygen species (ROS) such as superoxide, hydroxyl, peroxyl radicals, and reactive nitrogen species (RNS) such as NOs and peroxynitrite are generated and the balance between antioxidation and oxidation is believed to play an important role in pathophysiology of human diseases such as diabetes, cardiovascular diseases, neurodegenerative disorders, inflammation, arthritis, and cancer.[31] Recent investigations have shown that the antioxidant properties of plants could be correlated with oxidative stress defense.[13,32] Plant polyphenols are antioxidants with redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers.[33] As many studies indicate mangrove may be a rich source of novel compounds along with providing a new source of many already known biologically active compounds with antioxidant properties.[4,10,12,21,34] In the present study, hydro-methanolic extract of leaves of *R. mucronata* exhibited all major phytoconstituents like alkaloids, phenolics, flavonoids, triterpenoids, steroids, glycosides, saponins, and tannins. It has been recognized that phenolics and flavonoids play a significant role in the treatment of diabetes.[15,17,35] The densitometric HPTLC analytical results illustrated the presence of gallic acid, quercetin, and coumarin in the leaves of *R. mucronata*. There are considerable pharmacological data to support the antioxidant and radical scavenging properties of these three compounds.[19,20,35]

The antiradical properties of RH were carried out in different *in vitro* models. The extract showed strong reducing capability and DPPH radical scavenging activity. The DPPH radical is considered to be a model for a lipophilic radical. A chain in lipophilic radicals is initiated by the lipid autoxidation.[21,34] Further, the extract inhibited

Table 3: Effect of 4 weeks treatment of RH on FBG in STZ-induced type 2 rats

Group	Dose (mg/kg)	FBG (mg/100 ml blood)			
		Week 3	Week 6	Week 10	Percentage change
STZ	-	96.6±3.78	174±6.53***	188±8.11	8.04
GB	5	92.4±5.69	178±5.34***	143±7.01***	−19.66 (−23.93)
RH	50	90.8±6.03	171±4.29***	151±3.26**	−11.69 (−19.68)
RH	100	99.3±4.15	173±6.27***	136±5.11***	−21.38 (−27.65)

FBG levels of rats on week 3 were compared with week 6 within the groups. Rats in STZ group were treated with distilled water orally (2 ml/kg). Values were represented mean±SEM and were analyzed by paired *t*-test within groups. The percentage changes were prepared within groups (before and after treatment) and percentage changes in parentheses indicate the changes of blood sugar in week 10 (between groups) when compared with STZ treated control. **P<0.01 and **P<0.001 compared with week 6. STZ, GB, and RH means hydro-methanolic extract (20:80) of *Rhizophora mucronata* leaves. FBG: Fasting blood glucose; RH: Relative humidity; STZ: Streptozotocin; GB: Glibenclamide; SEM: Standard error of the mean

Table 4: Effect of 4 week treatment of RH on lipid peroxides and glutathione in the liver in STZ-induced type 2 rats

Group	Dose (mg/kg)	Lipid peroxides (nM MDA/mg protein)	Percentage change	Glutathione (nM/mg protein)	Percentage change
STZ	-	12.54±1.52	-	2.15±0.071	-
GB	5	11.71±1.36	−6.61	2.89±0.054***	34.41
RH	50	9.05±1.12*	−27.83	3.91±0.063***	81.86
RH	100	7.38±0.97**	−41.14	4.42±0.082***	105.58

Rats in STZ group were treated with distilled water orally (2 ml/kg). Values were represented mean±SEM and were analyzed by *t*-test compared to STZ. The percentage changes were compared with STZ. **P<0.01 and **P<0.001 compared with STZ. STZ, GB, and RH means hydro-methanolic extract (20:80) of *Rhizophora mucronata* leaves. RH: Relative humidity; STZ: Streptozotocin; GB: Glibenclamide; SEM: Standard error of the mean; MDA: Malondialdehyde

the formation of $ABTS^+$ radicals. The discoloration of $ABTS^+$ cation reflects the capacity of an antioxidant species to donate electrons or hydrogen atoms to deactivate these radicals.[27] Recently, NO has emerged as an important mediator of cellular and molecular events that impact the pathophysiology of vascular disorders and inflammatory processes, especially during the complication of diabetes.[25,33] NO may cause cytotoxicity through formation of the potent oxidant peroxynitrite, the reaction product of NO with $O_2^{\bullet}$.[36] The RH extract exhibited strong NO radicals and also superoxide radicals scavenging activity. $HO^{\bullet}$ is an extremely reactive-free radical in biological systems and has been implicated as a highly damaging species in free radical pathology.[26,37] This radical has the capacity to join nucleotides in DNA and cause strand breakage.[37] $HO^{\bullet}$ scavenging capacity of the extract is directly related to its antioxidant activity. In the present study, RH extract showed strong $HO^{\bullet}$ scavenging abilities. Therefore, it may be concluded that RH extract has the ability to scavenging free radicals due to its antioxidant properties.

Chronic oxidative stress due to hyperglycemia may play an important role in progressive of β-cell dysfunction.[12,13,37,38] ROS increases the generation of tumor necrosis factor-alpha expression and aggravates insulin resistance.[39] Studies show that the majority of the plasma antioxidants are depleted in type-2 diabetes patients while antioxidant therapy showed helpful in relieving symptoms and complications in the diabetic patients.[33,40] In this study, polyphenol-rich RH extract showed potential antidiabetic action in STZ induced type-2 diabetic rats. Further, it restricts the oxidative stress in liver tissues as marked by reduced level of lipid peroxides and higher level of glutathione. Glutathione is one of the essential compounds for maintaining cell integrity because of its reducing properties and participation in the cell metabolism. The neonatal STZ rat model is very much resembles human noninsulin dependent diabetes and effective in search of antidiabetic principles.[41] Previous studies reported the plant has antihyperglycemic action in rat.[12] Therefore, it assumed that the hydro-methanolic extract of *R. mucronata* leaves may lower the generation of ROS and RNS through its radical scavenging properties and thus protect the β-cell function from oxidative stress. Eventually, this study may lead to the development of effective compounds from *R. mucronata* for complementing therapeutic approaches to the treatment of complications in diabetes.

ACKNOWLEDGMENTS

This work was supported by Department of Science and Technology, West Bengal, India (537/5G-4/09).

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Cite this article as: Sur TK, Hazra AK, Bhattacharyya D, Hazra A. Antiradical and antidiabetic properties of standardized extract of Sunderban mangrove *Rhizophora mucronata*. Phcog Mag 2015;11:389-94.

Source of Support: This work was supported by Department of Science and Technology, West Bengal, India (537/5G-4/09). **Conflict of Interest:** None declared.