Anti-diabetic Activity of Rhizophora mucronata Leaves in Streptozotocin-Nicotinamide Induced Animal Model

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ABSTRACT

Background: Diabetes mellitus is a metabolic disorder, where it is necessary to achieve and maintain good glycemic control. Though various achievements have been made in conventional and herbal medicines as anti-diabetic, there is a need to develop a well-defined treatment to manage the disease along with its multiple complications. *Rhizophora mucronata*, a true mangrove, abundant in the Sundarban region is traditionally used for its anti-diabetic properties but has very few scientific validations till date. The present study aimed to investigate the anti-hyperglycemic efficacy of the hydro-alcoholic extract of *Rhizophora mucronata* Lam leaves on Streptozotocin-Nicotinamide induced Type 2 diabetic rats.

Methods: The mangrove leaves were collected, identified and extracted with ethanol (50%) in the Soxhlet apparatus. Extract treatment was done for 28 consecutive days in Streptozotocin-Nicotinamide induced Type 2 diabetic rats. Body weight, blood glucose profiles were measured along with serum lipid profile and serum insulin. Histological studies of the pancreas were also observed.

Findings: The hydro-alcoholic extract of *Rhizophora mucronata* Lam leaves significantly reduced the blood glucose level. It also reduced the serum lipids in a dose-independent manner. Regular administration of extract maintained the normal serum insulin level. The study revealed *Rhizophora mucronata* leaves are a rich source of magnesium content and the Gas chromatographic study showed the presence of Squalene.

Conclusion: Present study revealed the beneficial potential of a hydro-alcoholic extract of *Rhizophora mucronata* Lam leaves as an anti-hyperglycemic agent having lipid-lowering action along with insulin mimicking property. Further research is required to identify the bioactive component responsible for its potent anti-diabetic action.

To cite this article

Keywords: Rhizophora mucronata, Mangrove, Diabetes Mellitus, Ethanol, Lipid, Insulin.

1. Introduction:
Type 2 diabetes mellitus (T2DM) is a major prevalent endocrine disorder and has become an epidemic globally. International Diabetes Federation stated that in 2015, 415 million people were detected as diabetic globally. It was projected that the diabetic population will increase to approximately 642 million by 2040 (Ogurtsova et al., 2017). Type 2 diabetes is characterized by tissue insulin resistance combined with a relative deficiency in insulin secretion. Chronic hyperglycemia leads to insulin resistance, hyperlipidemia, and several long-term complications like heart attack, nephropathy, retinopathy and neuropathy (Filla & Edwards, 2016). The drugs currently available as anti-diabetic have been used singly and also in combination, even with the addition of insulin, but achieving the sustained glycemic control is still challenging. The World Health Organization is also promoting traditional medicine to develop cost-effective,
widespread medical care, particularly in developing countries (Schuster, 2001).

In the present era, the mangrove ecosystem has opened a new avenue in research in the field of drug development from natural sources due to their unique bioactive secondary metabolites. They grow in the coastal intertidal region between land and sea in inhospitable environmental condition and have an effective defense system to overcome different biotic and abiotic stresses. Therefore they produce a wide array of novel natural products which are generally absent in the terrestrial plants and can be explored for their potent biological and medicinal values (Simlai & Roy, 2013).

*Rhizophora mucronata* is a widespread, diverse true mangrove, has ethnomedicinal use in various ailments including diabetes (Nour et al., 2016; Sur et al., 2015; Alikunhi et al., 2012). Though several types of research have been done to elucidate the pharmacological and chemical properties of this mangrove, more extensive research is needed to substantiate its folkloric use for therapeutic purpose, identification of potent bioactive compound and mechanism responsible for its action. The present study investigated the anti-diabetic potential of the hydro-ethanolic extract of *Rhizophora mucronata* Lam leaves on Streptozotocin-Nicotinamide (STZ-NA) induced Type 2 diabetic rats.

### 2. Methodology:

**2.1. Collection and Identification of Plant Material:**

*Rhizophora mucronata* leaves were collected from Sundarban, West Bengal, India, and were authenticated from Botanical Survey of India, Howrah, West Bengal (CNH/55/2013/Tech. II/19 dated 02.12.2013). The fresh leaves were washed with distilled water.

**2.2. Extraction of Plant Material:**

Fresh *Rhizophora mucronata* Lam. leaves were shade-dried, pulverized into coarse powder and extracted with hydro-ethanol (50%) in soxhlet apparatus. Thereafter, the solvent was removed under reduced pressure and the extract was dried. The hydro-alcoholic extract of *Rhizophora mucronata* Lam. leaves was designated as RME.

**2.3. Animals:**

Adult Wistar albino rats both male and female, 150-200gm body weight were used for this study. Animals were kept in the Institutional animal house, maintaining proper housing condition, diet and water *ad libitum*. The animal experiments were conducted in accordance with the accepted principles for laboratory animal use and care (CPCSEA). The study was approved by the Institutional Animal Ethics Committee, R. G. Kar Medical College, Kolkata.

**2.4. Induction of Type 2 Diabetes:**

Experimental induction of Type 2 diabetes mellitus in adult male Wistar rats was done by using Streptozotocin-Nicotinamide (STZ-NA) induced diabetic model (Masiello et al., 1998; Pierre et al., 2012). The rats fasted overnight and Nicotinamide (NA) 110 mg/kg b.w. (dissolved in normal saline) was administered intraperitoneally to each rat. After 15 mins, Streptozotocin (STZ) 60 mg/kg b.w. (dissolved in citrate buffer pH 4.5) was injected intravenously into those rats.

**2.5. Experimental Design:**

Development of diabetes was confirmed by the evaluation of fasting blood glucose status initially and 72 hrs after STZ injection. Only those animals with blood glucose levels above 250 mg/dl, considered as diabetic and used for the study (Masiello et al., 1998; Pierre et al., 2012). The diabetic rats were randomly selected for six groups - normal control, diabetic control, diabetic rats treated with standard drug Glibenclamide and diabetic rats treated with *Rhizophora mucronata* leaf extract (RME) in three different concentrations. The experimental design became as follows (n=6):

- **Group I:** Normal control (without STZ-NA, distilled water 0.1ml/kg, orally).
- **Group II:** Diabetic control (STZ-NA induced, distilled water 0.1ml/kg, orally).
- **Group III:** Diabetic rats treated with Glibenclamide (10 mg/kg b.w., orally).
- **Group IV:** Diabetic rats treated with RME (100 mg/kg, orally).
- **Group VII:** Diabetic rats treated with RME (200 mg/kg, orally).
- **Group VI:** Diabetic rats treated with RME (400 mg/kg, orally).

The treatments were continued once daily for 28 consecutive days. Body weight and the fasting blood sugar profile of all the rats were measured on day 0, 7, 14, 21 and 28. On day 28th blood collections were done from retro-orbital plexus of all the rats and then sacrificed. The serum was isolated and lipid profile including triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), high-density lipoprotein (HDL) of all groups of rats were estimated by using standard diagnostic kits. The serum insulin levels in the rats were evaluated using a rat insulin ELISA kit (Ray biotech). The levels of insulin in different groups were compared. At the end of the experiment, all the rats were euthanized. The pancreas tissues of different groups of rats were excised immediately, washed in normal saline and fixed in 10% formalin. The histological examination of rat pancreas was done with hematoxylin-eosin staining and examined under light microscopy.
2.6. Microelements Analysis by Atomic Absorption Spectrophotometer:

Atomic absorption spectroscopic study was used to detect the presence of minerals and microelements (Abbas et al., 2015) Calcium, magnesium, copper, chromium, and zinc are such five essential minerals of plants, required for their biological systems and also essential components of many enzymes that play important role in the physiological and biochemical processes. Estimation of these microelements in Rhizophora mucronata leaves was done. 5gms of dry powdered Rhizophora mucronata leaves were taken in a porcelain crucible and heated at 550ºC in a muffle furnace for 2hrs. The ash was collected, 10ml nitric acid (conc.) was added and gently heated for 2-3hrs until the sample preparation (also called digestion) was completed. Thereafter, 5ml of 70% perchloric acid was mixed and heated for 1hr. Then few drops of hydrogen peroxide were added to discolor the digested material. It was then dissolved inadequate water, filtered and the filtrate was collected as a sample. Three replicate samples were made and a blank was prepared without the extract.

The analysis was done using Varian Spectra AA140 (Netherlands) Flame Atomic Absorption Spectrophotometer (AAS) compared to the standard plot. The detection limit of this instrument was 0.0001 ppm.

2.7. Gas Chromatography Electrochemical Analysis (GC-ECD):

Gas chromatography with electrochemical detection (GC-ECD) analysis was performed using Agilent Technologies (USA) gas chromatograph and equipped with MSD 5977A electrochemical detector. An Agilent Wax capillary column, 30 m long, 0.25 mm internal diameter, and 0.25 μm thickness was used. The column initial temperature was programmed at 50°C (5 min) with an increase of 5°C/min up to 250°C (10 min). Helium was used as carrier gas. The temperature of the injector and detector was 250°C. An injector volume of 1μl was used. The leaf sample of Rhizophora mucronata was prepared according to a standard method (Joel & Bhimba, 2010). The components were identified by matching their mass and chemical structure with those recorded in an instrumental standard spectral library.

2.8. Statistical Analysis:

All the study data were expressed as mean ± SEM. Statistical analysis was done by one-way ANOVA (analysis of variance), followed by Dunnet test for post-hoc analysis using a 5% level of significance (p<0.05). The statistical software package used for analysis was a statistical package for the social sciences (SPSS 15).

3. Results:

It was revealed from Figure 1 that after induction of diabetes in Wistar rats, blood glucose concentrations increased significantly (compared with the diabetic control group). On repeated administration of the hydroalcoholic extract of the leaves of Rhizophora mucronata (RME) for 28 days, a significant decrease in the fasting blood sugar level was observed in the treated diabetic rats as compared to the diabetic control. The doses of 200 mg/kg and 400mg/kg body weight of the extract were able to reduce the blood glucose level in comparison with the diabetic control group (p<0.05). Inhibition of fasting blood glucose level was 70.22%, 76.67% and 76.92% in RME treated (100mg/kg, 200mg/kg and 400mg/kg orally respectively) rats compared with diabetic control group at 28th day of the study. Whereas reduction in elevated blood glucose for standard drug Glibenclamide (10mg/kg body weight orally) was 59.06%. Induction of diabetes resulted in a significant decrease in body weight of diabetic control rats compared with normal control group at the end of the experiment. Administration of RME to diabetic rats improved the body weight of animals at the end of 4 weeks which was found to be significant in 200 mg/kg b.w. (p<0.05).

![Blood glucose profile](image)

Figure 1. Blood glucose profile of all group of rats in Streptozotocin-Nicotinamide induced diabetic model. Data was interpreted as Mean ± S.E.M. Statistical analysis was done by one-way ANOVA followed by Dunnet test (p<0.05)
Figure 2A, 2B & 2C represent the pancreas tissues of normal control, diabetic control and Glibenclamide (10mg/kg) treated rats respectively. The respective tissue sections of the diabetic rats treated with RME 100mg/kg, 200mg/kg & 400mg/kg are shown in Figure 2D, 2E & 2F respectively. The histology of pancreas of the normal control rats showed the normal arrangement of the islets (IL) of various sizes scattered throughout the exocrine tissue with no visible lesion. The islets appeared lightly stained than the surrounding acinar cells. The pancreas of STZ-NA induced diabetic rats showed pathological changes in both exocrine and endocrine components. The acinar cells were swollen and decreased number of islets was present. The islets degenerated in the diabetic control, standard drug Glibenclamide 10mg/kg and RME 100mg/kg treated groups. Treatment with the hydro-alcoholic extract of Rhizophora mucronata leaves in both 200mg/kg and 400mg/kg showed near normal acini and regenerated islets in the section of the pancreas of the treated rats. 200mg/kg dose showed more effectiveness in regenerating the islets in the pancreatic tissue.

| Table 1. Estimation of lipid profile of different group of rats in Streptozotocin-Nicotinamide induced diabetic model |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
| **Biochemical Parameters (mg/dL)** | Normal Control | STZ-NA Diabetic Control | STZ-NA + Glibenclamide (10mg/kg) | STZ-NA + RME (100mg/kg) | STZ-NA + RME (200mg/kg) | STZ-NA + RME (400mg/kg) |
| Triglycerides | 69.66 ± 3.6 | 107.39 ± 5.65 | 39.66 ± 9.58* | 49.99 ± 0.545* | 52.96 ± 4.32* | 59.8 ± 6.34* |
| Cholesterol | 35.4 ± 3.3 | 150 ± 3.69 | 95.48 ± 8.06* | 87.04 ± 1.22* | 38.19 ± 1.45* | 33.33 ± 1.77* |
| HDL | 18.6 ± 1.34 | 11.2 ± 3.54 | 12.2 ± 1.5* | 14.5 ± 1.79* | 15.6 ± 2.48* | 18.76 ± 6.88* |
| LDL | 2.86 ± 1.30 | 117.3 ± 4.56 | 75.35 ± 2.77* | 62.54 ± 1.39* | 11.99 ± 1.54* | 2.61 ± 0.84* |
| VLDL | 13.93 ± 0.15 | 21.48 ± 0.76 | 7.93 ± 0.64* | 9.99 ± 0.49* | 10.59 ± 0.26* | 11.96 ± 1.02* |
| AI | 0.15 ± 0.15 | 10.47 ± 0.26 | 6.18 ± 0.55* | 4.31 ± 0.52* | 0.77 ± 0.05* | 0.14 ± 0.36* |
| CRI | 1.9 ± 0.69 | 13.39 ± 0.26 | 7.83 ± 0.59* | 6 ± 0.45* | 2.45 ± 0.03* | 1.78 ± 1.16* |

Values were mean ± SEM (n=6). Statistical analysis was done using one-way ANOVA followed by Dunnet test; * denotes level of significance p<0.05.

| Table 2. Effect of the hydro-alcoholic extract of Rhizophora mucronata leaves on serum insulin level in Streptozotocin-Nicotinamide diabetic model |
|---------------------------------|----------------|----------------|----------------|----------------|----------------|
| GROUPS | NORMAL CONTROL | DIABETIC CONTROL | GLIBENCLAMIDE (10MG/KG) | RME 100MG/KG | RME 200MG/KG | RME 400MG/KG |
| INSULIN (µIU/ML) | 42.75 ± 2.21 | 30 ± 2 | 31 ± 1.91 | 32.33 ± 1.05 | 35 ± 1.15 | 38.83 ± 1.33 |

Values were mean ± SEM (n=6). Statistical analysis was done using one-way ANOVA followed by Dunnet test; p<0.05.

Table 3. Atomic absorption spectrophotometric analysis with Rhizophora mucronata leaves

<table>
<thead>
<tr>
<th>AAS ANALYSIS OF RM</th>
<th>MG/KG RM LEAVES</th>
</tr>
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<tbody>
<tr>
<td>CALCIUM</td>
<td>6412.8 ± 24.09</td>
</tr>
<tr>
<td>MAGNESIUM</td>
<td>8640 ± 32.48</td>
</tr>
<tr>
<td>ZINC</td>
<td>10.49 ± 1.96</td>
</tr>
<tr>
<td>COPPER</td>
<td>3.02 ± 0.84</td>
</tr>
<tr>
<td>CHROMIUM</td>
<td>BDL (&lt;0.2)</td>
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Atomic absorption spectrophotometric analysis showed the presence of different micronutrients calcium, magnesium, zinc, copper in the hydro-alcoholic extract of
**Rhizophora mucronata** leaves (RME) in different concentrations. Among them, calcium and magnesium were found mostly and in high amount i.e. 6412.8mg/kg and 8640mg/kg of leaves respectively (Table 3).

![Figure 2](image1.png)

**Figure 2.** Histological sections of the pancreas tissues of different groups of rats stained by hematoxylin and eosin [Magnification 40x]. The Islets are marked with arrows.

The **Rhizophora mucronata** leaves showed different peaks in the gas chromatography electrochemical analysis chromatogram. Compounds were identified through electrochemical detector attached with gas chromatography apparatus.

![Figure 3](image2.png)

**Figure 3.** GC-ECD Chromatogram of **Rhizophora mucronata** leaves.

The chromatogram revealed various peaks for respective components present in the leaf of **Rhizophora mucronata** Lam that was detected by the GC-ECD are shown in (Figure 3). The chemical characterization of **Rhizophora mucronata** leaves by GC-ECD analysis revealed 14 different polar compounds including some unsaturated fatty acid, phenolic acid compounds. The most prevailing compound was Squalene (23.6%), a 30-carbon triterpene with the retention time (R.T.) 31.045 min. Presence of aR-Turmerone (16.5%, R.T. 11.367 min), Benzene propanoic acid (15.41%, R.T. 16.785 min), Estra-1,3,5(10)-tri-en-17β-ol (9.19%, R.T. 17.117 min) are the other important compounds, which are very useful information for structural elucidation and identification of the bioactive compounds.

4. Discussions:

The secondary metabolites produced by the plants mainly the polyphenols are considered as an effective antioxidant and anti-diabetic components (Habtemariam& Varghese, 2014; Umeno et al., 2016). **Rhizophora mucronata** leaf extract contains polyphenols and flavonoids as the major phytoconstituents (Joel & Bhimba, 2010; Arumugam et al., 2014; Ray et al., 2014). In the present study also, rich amount of phenolic acids and flavonoids were present in the **Rhizophora mucronata** leaf extract, which might be the major phytoconstituents responsible for its anti-diabetic activity. An earlier study revealed the anti-hyperglycemic activity of the fresh leaves of **Rhizophora mucronata** in Alloxan-induced and Streptozotocin induced Type 1 diabetic models, also revealing insulin-mimic action (Ray et al., 2014). However, the hydroalcoholic extract of the leaves was found to be decreasing the elevated blood sugar in Streptozotocin-induced Type 1 diabetic model distinctly in 100mg/kg and 200mg/kg doses (Adhikari et al., 2017; Pandey et al., 2014).

In the present study, the anti-hyperglycemic activity of the hydro-alcoholic extract of **Rhizophora mucronata** leaves was evaluated in Streptozotocin-Nicotinamide induced Type 2 diabetic rats and it showed a significant reduction in the blood glucose level in a dose-independent manner (Figure 1). The fasting blood glucose levels in the treated rats were significantly decreased about 70-76% compared to the untreated diabetic control rats and the extract treatment also maintained a healthy bodyweight throughout the study period.

In another study Sur et al. (2015) reported the anti-diabetic action of the hydro-methanolic extract of **Rhizophora mucronata** leaves at 50mg/kg and 100mg/kg doses in neonatal Type2 diabetes model. Type 2 diabetes results from impaired insulin action, which is also associated with abnormal lipid metabolism. These lead to hypercholesterolemia and the risk of myocardial infarction is also increased (Pierre et al., 2012; Mediene-Benchekor et al., 2001). In the present study, the hydro-alcoholic extract of **Rhizophora mucronata** leaves showed hypolipidemic action by significant attenuation in the lipid profiles of all the treated rats. It decreased the serum cholesterol, triglyceride, LDL-cholesterol levels to near normal level than the diabetic control rats and raised the HDL-cholesterol (Table 1). In another research
Gurudeeban et al. (2016) similarly reported that the dichloromethane fraction of *Rhizophora mucronata* leaves in 100mg/kg body weight dose showed potent anti-diabetic action in Type 2 diabetic rats along with significant reduction in total cholesterol, triglyceride levels, whereas increased HDL-cholesterol. The polyphenols are suggested as having a beneficial action in the pancreatic β-cells, peripheral tissues.

Thus, they can increase the insulin sensitivity, insulin action and possess glucose-lowering potentials (Umno et al., 2016; Dominguez Avila et al., 2017). In this study, the slightly decreased levels of serum insulin were observed in the diabetic control rats. The test extract demonstrated distinct action on serum insulin levels in the treated rats by maintaining it like normal levels and thereby indicating the insulin-mimic action (Table 2). The histological study of the pancreas also revealed the damaged and reduced number of islets in the diabetic control rats. The extract, particularly in 200mg/kg and 400mg/kg doses, showed regeneration of pancreatic islets and a sufficient number of islets were present. Alkhuni et al. (2012) reported that *Rhizophora* species revealed the presence of insulin-like protein and the anti-diabetic activity was exerted through regulation of insulin action.

The presence of different essential elements like calcium, magnesium, zinc, copper were detected in the *Rhizophora mucronata* leaves extract (RME) by the atomic absorption spectrophotometric analysis. Among them, magnesium and calcium were found in high amount (Table 3). This was also in support with the study result from Al Hagibi et al. (2018), where the rich content of magnesium was found in the mangrove leaves from Yemen, followed by iron, copper, zinc. Medina et al. (2015) reported the mangrove plants *Rhizophora mangle* have salt tolerance capacity due to their efficient salt exclusion mechanism.

Thus, they can exclude sodium and accumulate potassium, magnesium in their biomass. Recently it has been proven that dietary intake of calcium and magnesium plays an inevitable role in the prevention of Type 2 diabetes (Barbagallo et al., 2015; Ahn et al., 2017). Therefore, the high magnesium and calcium content in the *Rhizophora mucronata* mangrove leaves may be beneficial as a mineral supplement in the prevention and treatment of diabetes. Chemical characterization of *Rhizophora mucronata* leaves by GC-ECD analysis revealed 14 different polar compounds including some unsaturated fatty acid, phenolic acid compounds (Figure 3). Squalene, a 30-carbon triterpene was the predominant compound (23%) among them. Squalene is reported as a natural antioxidant and also has anti-hyperglycemic action by acting as an α-glucosidase inhibitor and may also increase insulin secretion (Nazaruk et al., 2015). Some other researchers also supported the presence of a rich amount of Squalene (19.19%) in the *Rhizophora mucronata* leaf (Joel & Bhimba, 2010). The use of triterpenes as the inhibitors of advanced glycation end products (AGEs) may be a potentially effective strategy for the prevention and treatment of diabetes and its related complications. This may lead to the development of new multitarget bioactive drugs (Nazaruk et al., 2015; Hou et al., 2009).

5. Conclusion:

It can be concluded that Sundarban mangrove *Rhizophora mucronata* Lam. leaves have beneficial anti-hyperglycemic effect with insulin-mimetic actions and also have lipid-lowering efficacy in Streptozotocin-Nicotinamide induced Type 2 diabetic rats, The active phytoconstituents of the *Rhizophora mucronata* Lam leaves mostly the triterpene, phenolic acid, and its derivatives must be a promising source of therapeutic approach for the multifaceted disease like diabetes and its associated complications.

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