Antioxidant and Anti-Diabetic Activities of the Methanolic Extract of *Premna integrifolia* Bark

Rajib Majumder, Shahina Akter, Zannatul Naim, Md. Al-Amin and Md. Badrul Alam

Abstract: *Premna integrifolia* Linn (Family: Verbenaceae) synonyms of *Premna serratifolia* has tremendous medicinal value. The methanolic extract of *Premna integrifolia* (MEPI) bark was subjected to investigate its antidiabetic activity along with its in vitro antioxidant activity. Oral glucose tolerance test (OGTT), normoglycemic test and alloxan induced diabetic test was consider for antidiabetic activity evaluation. In addition, total phenolic content, total antioxidant activity, scavenging of 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical as well as reducing power assessment was used to evaluate antioxidant potentiality of MEPI. The continuous post treatment for 120 min with the MEPI showed potential hypoglycemic activity in OGTT and normoglycemic rats. At a dose of 300 mg/kg the extract, considerable drop in elevated blood glucose level was observed in the alloxan induced diabetic (p<0.05) rat after 7 days. It revealed radical scavenging properties in the DPPH assay (IC₅₀ = 8.61 ± 0.16µg/ml). The finding of this study suggested that MEPI provide as a part of scientific support for the use of this species in traditional medicine for Diabetes mellitus.

Key words: *Premna Integrifolia* · Diabetes · Antioxidant · Reducing Power · Total Phenolic Content

INTRODUCTION

Diabetes mellitus (DM) is an endocrinal metabolic disorder characterized by chronic hyperglycemia, damaged pancreatic β cell with carbohydrate complication, metabolism of fat and protein occurred by abnormal secretion of insulin, defects in insulin action or both [1] additionally increased risk of complications of various vascular diseases. Specialist suggested that diabetes is the third leading cause of death due to the high percentage of morbidity and mortality after cancer and cardiovascular disorders. For DM various complications occurs in human body like renal failure, coronary artery disorders, blindness, neurological complications, cerebro-vascular disease, limb amputation, failure of function of different body parts with premature death etc [2]. Insulin therapy and life style modifications or long term use of oral hypoglycemic agents, physical exercises with dietary control can be the medication of Diabetes mellitus [3].

Plants play an important role in the treatment of Diabetes, especially who have limited resource and do not have any modern facilities, normally in developing countries. Report of ethnobotany revealed that about 800 medicinal plants have antidiabetic activity [4] and the bio active compounds like alkaloids, glycosides, terpenoids, carotenoids and floavonoids are very effective drugs both in preclinical and clinical studies [5, 6]. Various side effects by using insulin and oral hypoglycemic agent, modern countries and high-tech. pharmaceutical companies showed their interest to produce plant base medicine [7]. So, it is important to investigate different plants or plant base medicine for DM. On that point of view we try to figure out the hypoglycemic effect of *Premna integrifolia* on rats.

*Premna serratifolia* Linn (Family: Verbenaceae) synonyms of *Premna integrifolia*, It is known as “Agnimantha” in Ayurvedic system of medicine, is a small-sized tree or a large shrub, up to 9m in height with a comparatively short trunk and numerous branches.
The plants grow wild and planted in many districts of Bangladesh [8] and almost all parts of this plant i.e. root, leaf and bark have tremendous medicinal value. The roots are used in the treatment of diabetes, chyluria, inflammations, swellings, bronchitis, dyspepsia, liver disorders, piles, constipation and fever [9]. It is widely used by the traditional practitioners as cardiotonic, antibiotic, anti-coagulant, stomachic, carminative, hepatoprotective, antitumor etc [10]. Previous pharmacological studies include reports of hypolipidemic [11], anti-inflammatory [12], antidiabetic [13], hepatoprotective [10], anti-microbial [14] and antitumor activity [10].

Previous phytochemical investigations have revealed the presence of several glycosides including iridoid glycosides and phenylethanoids like premnethanoside A and B, Sudo, Takushi and Ide [15] some xanthones [16], steroids and saponins [17], flavonoids, triterpenoids and diterpenoids (including prennones A and C) in Premna serratifolia leaves [17]. Investigations on Premna serratifolia flower buds have revealed the presence of volatile oil comprising mainly 1-octen-3-0l, (Z)-n-hexanol, 2-phenyl ethyl alcohol, (E,Z)-2,4-nonadienal and linalool [19]. The roots has been found to contain the mixture of mono-, di-, tri-terpene hydrocarbons and oxygenated biological materials includes 1H-Cycloprop[e]azulen-7-ol, decahydro-1, 2-Furancarboxaldehyde, 5-(hydroxymethyl)tetramethyltricyclo[5.2.1](1,6]undecan-2-ol, Benzofuran, 2,3-dihydro, n-Hexadecanoic acid, 2-Propanoic acid [20].

Acute Toxicity Test: Test animals were divided into groups (n = 6 per group) which were administered different doses of the crude MEPI in Long-Evans rats (62.5, 125, 250, 500, 1000, 2000 and 4000 mg/kg p. o.) accordingly, while the control group received only the vehicle (1% Tween 80 in water, p. o.). The general signs and symptoms of toxicity were observed for 24 hr and mortality was recorded for each group at the end of this period [20].

In vitro Antioxidant Activity

The plants grow wild and planted in many districts of Bangladesh [8] and almost all parts of this plant i.e. root, leaf and bark has tremendous medicinal value. The roots are used in the treatment of diabetes, chyluria, inflammations, swellings, bronchitis, dyspepsia, liver disorders, piles, constipation and fever [9]. It is widely used by the traditional practitioners as cardiotonic, antibiotic, anti-coagulant, stomachic, carminative, hepatoprotective, antitumor etc [10]. Previous pharmacological studies include reports of hypolipidemic [11], anti-inflammatory [12], antidiabetic [13], hepatoprotective [10], anti-microbial [14] and antitumor activity [10].

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With a view to find the pharmacological rationale for some of the reported and traditional uses of the plant, the methanol extract of Premna serratifolia bark was evaluated for antidiabetic activity and for its effect on oral glucose tolerance as well as hypoglycemic activity in rats. It was also studied for its antioxidant potentiality in in vitro.

MATERIALS AND METHODS

Plant Materials and Extraction: The fresh barks of Premna integrifolia plant were collected from Sunderbans, Bagerhat in the month of September, 2009 and identified by DR. M.A. Razzaque Shah PhD, Tissue Culture Specialist, BRAC Plant Biotechnology Laboratory, Bangladesh. The dried and coarsely powdered leaves (400 g) were extracted with methanol at room temperature 25±1°C for 72 hr. The filtrate was evaporated to dry under reduced pressure (45°C) to afford the crude extract (yield ca. 6%) used in pharmacological screening.

Animals: Young Long-Evans rats of either sex aged 4 (four) months, weighing about 80-120gm were used for the experiment. The rats were purchased from the animal Research Branch of the International Centre for Diarrheal Disease and Research, Bangladesh (ICDDR, B) and is used for the evaluation of antidiabetic activity and for its effect on oral glucose tolerance as well as hypoglycemic activity. The animals were housed under standard laboratory conditions (relative humidity 55-65%, room temperature 23.0±2.0°C and 12-hr light: 12-hr dark cycle). The animals were fed with a standard diet and water ad libitum regularly in all animal experiments and all were monitored and maintained in accordance with CPCSEA guidelines on control and supervision. The guidelines of the Animal Experimentation Ethics Committee, ICDDR, B were followed here.

Chemicals and Reagents: Ammonium molybdate, Folin-Ciocalteu phenol reagent, were purchased from E. Merck (Germany). 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), ascorbic acid, quercetin and potassium ferric cyanide were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Normal saline solution was purchased from Beximco Infusion Ltd., Bangladesh. One Touch Glucometer (Accu-chek Sensor) and Diagnostic-kits were purchased from Roche Diagnostics GmbH, Mannheim, Germany. Metformin was obtained as a gift from Square Pharmaceuticals Ltd., Bangladesh. All chemicals were used of standard analytical reagent grade.

Acute Toxicity Test: Test animals were divided into groups (n = 6 per group) which were administered different doses of the crude MEPI in Long-Evans rats (62.5, 125, 250, 500, 1000, 2000 and 4000 mg/kg p. o.) accordingly, while the control group received only the vehicle (1% Tween 80 in water, p. o.). The general signs and symptoms of toxicity were observed for 24 hr and mortality was recorded for each group at the end of this period [20].
1601 spectrophotometer and the results were expressed as µ g/mg of gallic acid equivalent was determined. These data were used to estimate the phenolic contents using a standard curve obtained from various concentration of Gallic acid. Optical density of each sample was determined at 415 nm using an UV spectrophotometer. Total flavonoids content was calculated by interpolation on a standard curve established with a reference standard, Quercetin.

**Determination of Total Antioxidant Capacity:** The antioxidant activity of the MEPI was evaluated by the phosphomolybdendenum method according to the procedure of Prieto et al. [22]. The assay is based on the reduction of Mo (VI)-Mo (V) by the extract and subsequent formation of a green phosphate/Mo (V) complex at acid pH. Extract (0.3 ml) was combined with 3ml of reagent solution (0.6M sulfuric acid, 28mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then the absorbance of the solution was measured at 695 nm using a spectrophotometer (Shimadzu, UV-150-02) against blank after cooling at room temperature. Methanol (0.3 ml) is used as the blank experiment. The antioxidant activity is expressed as the number of equivalents of ascorbic acid using the following formula:

\[
C = \frac{(c \times V)}{m}
\]

Where: C—total antioxidant activity, mg/g plant extract, in Ascorbic acid; c—the concentration of ascorbic acid established from the calibration curve, mg/ml; V—the volume of extract, ml; m—the weight of pure plant extract, g.

**Free Radical Scavenging Activity Measured by 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH):** The free radical scavenging activity of MEPI based on the scavenging activity of the stable 1, 1-diphenyl-2-picryl-hydrazyl (DPPH) free radical was determined by the method described by Braca et al. [18]. MEPI (0.1 ml) was added to 3ml of a 0.004% MeOH solution of DPPH. Absorbance at 517nm was determined after 30 min and the percentage inhibition activity was calculated from

\[
\left[\frac{(A_c-A_i)}{A_c}\right] \times 100
\]

Where A<sub>c</sub> is the absorbance of the control and A<sub>i</sub> is the absorbance of the extract/ standard. IC<sub>50</sub> value was calculated from the equation of line obtained by plotting a graph of concentration (µ g/ml) versus % inhibition.

**Antidiabetic Activity**

**Study on Oral Glucose Tolerance Test (OGTT):** OGTT of plant extracts was carried out in overnight fasted normal rats, which were equally divided into four groups of six rats each. Group of normal control received only vehicle (1 ml of 0.3% CMC; p. o.) and standard group received 1 ml of reference drug Metformin suspended in the vehicle (150 mg/kg, p. o.), while group from third to fourth were administered with 1 ml of MEPI (150 and 300 mg/kg, p. o.) respectively. Thereafter, following 30 min post extract administration all the animals were fed with glucose (2 g/kg). Blood samples were collected from tail vein prior to dosing and then at 30, 60, 90 and 120 min after oral glucose administration [23]. The fasting blood glucose level was analyzed by using glucose-oxidase-peroxide reactive strips (Accu-chek, Roche Diagnostics, GmbH, Germany) [24].

**Study on Normoglycemic Rats:** Normoglycemic studies were carried out in overnight fasted normal rats, which were equally divided into five groups of six rats each. The normal control group received only vehicle (1 ml of 0.3% CMC; p. o.) and standard group received 1 ml of reference drug Metformin suspended in the vehicle (150 mg/kg, p. o.), while group from third to fourth were administered with 1 ml of MEPI (150 and 300 mg/kg, p. o.) respectively. Blood samples were collected from tail vein prior to dosing and then at 30, 60, 90 and 120 min after glucose administration [23]. The fasting blood glucose level was analyzed by using glucose-oxidase-peroxide reactive strips (Accu-chek, Roche Diagnostics, GmbH, Germany) [24].

**Study on Alloxan-induced Diabetic Rats:** Diabetes was produced by single intra-peritoneal injection (single dose) of alloxan monohydrate (120 mg/kg b. wt.) in 0.9% w/v NaCl solution (normal saline) to overnight fasted normal rats, aged 4(four) months with 80-120gm body weight. Blood glucose level was checked by using one-touch glucometer and hyperglycemia was confirmed after 72 hr of alloxan application. Rats have FBG>250 mg/dl were considered to be diabetic and were selected for studies. Animals selected were fasted over night and then divided into five groups (n=6) as follows: Group-I: Normal control rats (non-alloxanized) that was administered with vehicle (1 ml of 0.3% CMC; p. o.) only; Group-II: Diabetic control rats (Untreated, alloxanized); Group-III: Diabetic rats administered once with metformin (150 mg/kg b. wt.) as reference standard drug while; Group-III to V: Diabetic rats administered with MED (150 and 300 mg/kg/day) respectively. Treatment was continued for a period of
7 days following oral administration to the experimental animals by gastric intubation, using a force-feeding needle. Plasma glucose was estimated on withdrawing blood samples from tail vein prior to dosing (day 0) and then at regular intervals of day 3 and 7 respectively all groups of animals [24].

**Statistical Analysis:** All data were expressed as mean ± standard error of mean (S.E.M.). Statistical significance was analyzed by one-way analysis of variance (ANOVA) followed by Dunnett’s post hoc test of significance. All statistical analyses were performed with Prism 4.0 (GraphPad software Inc., San Diego, CA). \( P<0.05 \) was considered to be significant.

**RESULTS**

**Preliminary Phytochemical Analysis and Acute Toxicity:** The preliminary phytochemical analysis of the methanolic extract of *Premna integrifolia* indicated the presence of sterols, flavonoids, tannins and saponins. No lethal effects were observed within 24 hr after the administration of the extract at any of the doses used, even at the highest dose tested (4000 mg/kg). Therefore, the lethal dose \( (LD_{50}) \) of the extract in mice could not be determined.

**Antioxidant Activity**

**Total Phenolic Contents:** The total phenols content was found to be 31.92 ± 0.04 mg/g plant extract (in gallic acid equivalent), in crude extract of MEPI, presented in Table 1.

**Table 1:** Yield, total amount of plant phenolic compounds and total antioxidant capacity of MEPI

<table>
<thead>
<tr>
<th>Sample</th>
<th>Yield (%)</th>
<th>Total phenol mg/g plant extract (in GAE)*</th>
<th>Total antioxidant capacity mg/g extract (in ASC) b</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEPI</td>
<td>10%</td>
<td>31.92 ± 0.04</td>
<td>19.80 ± 0.02</td>
</tr>
</tbody>
</table>

*Gallic acid equivalents (GAE, mg/g of each extract) for the total phenolic content. Ascorbic acid equivalents (mg/g of each extract) for the total antioxidant capacity. The GAE and ASC values are expressed as Mean ± SEM of triplicate experiments.

**Table 2:** Hypoglycemic effect of MEPI in oral glucose tolerance test (OGTT)

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>5.64 ±1.22</td>
<td>10.00 ±3.06</td>
<td>11.12 ±0.82</td>
<td>9.1 ±2.19</td>
<td>7.78 ±4.37</td>
</tr>
<tr>
<td>Group II</td>
<td>5.32 ±1.32</td>
<td>8.26 ±3.74</td>
<td>7.28 ±3.21</td>
<td>6.14 ±3.21</td>
<td>4.94 ±3.22</td>
</tr>
<tr>
<td>Group III</td>
<td>5.54 ±1.26</td>
<td>9.28 ±3.19</td>
<td>9.04 ±3.25</td>
<td>7.24 ±4.26</td>
<td>6.26 ±1.75</td>
</tr>
<tr>
<td>Group IV</td>
<td>5.58 ±2.11</td>
<td>8.36 ±2.15</td>
<td>7.56 ±3.25</td>
<td>6.3 ±4.72</td>
<td>5.46 ±2.10</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, \( (n = 5) \); * \( p<0.05 \), One way ANOVA followed by Dunnet test as compared to vehicle control. Group I animals received vehicle (0.3% CMC), Group II received Metformin 150 mg/kg body weight, Group III and Group IV were treated with 150 and 300 mg/kg body weight (p. o.) of the MEPI.
Table 3: Hypoglycemic effect of MEPI in fasted normoglycemic rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
<th>90 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>5.56 ±1.12</td>
<td>5.46 ±1.06</td>
<td>5.48 ±2.82</td>
<td>5.02 ±3.11</td>
<td>5.06 ±2.31</td>
</tr>
<tr>
<td>Group II</td>
<td>5.48 ±1.42</td>
<td>4.10 ±2.24</td>
<td>3.28 ±1.27</td>
<td>2.60 ±1.01</td>
<td>1.74 ±1.22</td>
</tr>
<tr>
<td>Group III</td>
<td>5.44 ±1.22</td>
<td>4.70 ±1.19</td>
<td>3.88 ±2.05</td>
<td>2.86 ±2.20</td>
<td>2.04 ±1.15</td>
</tr>
<tr>
<td>Group IV</td>
<td>5.62 ±1.11</td>
<td>4.28 ±1.15</td>
<td>3.32 ±1.22</td>
<td>2.60 ±1.22</td>
<td>1.78 ±1.10</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, (n = 5); * p<0.05, Dunnet test as compared to vehicle control. Group I animals received vehicle (0.3% CMC), Group II received Metformin 150 mg/kg body weight, Group III and Group IV were treated with 150 and 300 mg/kg body weight (p. o.) of the MEPI.

Table 4: Hypoglycemic effect of MEPI in Alloxan induced diabetic animals

<table>
<thead>
<tr>
<th>Group</th>
<th>1st day</th>
<th>3rd day</th>
<th>7th day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>5.64±1.12</td>
<td>5.22 ±1.87</td>
<td>5.26 ±3.11</td>
</tr>
<tr>
<td>Group II</td>
<td>21.24±2.43</td>
<td>21.08 ±3.06</td>
<td>22.64 ±8.56</td>
</tr>
<tr>
<td>Group III</td>
<td>14.36±2.75</td>
<td>8.72 ±5.77</td>
<td>6.08 ±5.23</td>
</tr>
<tr>
<td>Group IV</td>
<td>16.24±1.22</td>
<td>12.02 ±1.15</td>
<td>8.22 ±3.51</td>
</tr>
<tr>
<td>Group V</td>
<td>14.72±3.41</td>
<td>9.58 ±5.24</td>
<td>6.48 ±3.23</td>
</tr>
</tbody>
</table>

Values are mean ± SEM, (n = 5); * p<0.05, Dunnet test as compared to vehicle control. Group I animals received vehicle (0.3% CMC), Group II received Alloxan 110 mg/kg body weight, Group III received Metformin 150 mg/kg body weight, Group IV and Group V were treated with 150 and 300 mg/kg body weight (p. o.) of the MEPI.

DISCUSSIONS

Determination of specific antioxidant species might be less useful than the knowledge of the total antioxidant capacity of a sample. The knowledge of total antioxidant activity can be useful in the analysis of changes in plasma antioxidant activity related to oxidative stress, or the understanding of structure-activity relationships of pure antioxidant species. Because of its simplicity and the cheap reagents it used, the Phosphomolybdenum method is an alternative in all methods those already available for the evaluation of total antioxidant capacity. The Phosphomolybdenum method was based on the reduction of Mo (VI) to Mo (V) by the compounds having antioxidant property and is successfully used to quantify vitamin E in seeds [22]. DPPH is a stable free radical that accepts an electron or hydrogen radical to become a stable diamagnetic molecule [25] and is usually used as a substrate to evaluate the antioxidant activity of a compound [26]. Based on the data obtained from this study, DPPH radical scavenging activity of MEPI (IC_{50} 8.61 ± 0.16µg/ml) was similar to the standard ascorbic acid (IC_{50} 10.29 ± 0.11µg/ml). These findings agree with previous reports on scavenging of free radicals with other parts of Premna integrifolia stem, bark, wood and roots [27, 28]. Moreover, it was revealed that MEPI did show the proton donating ability and could serve as free radical inhibitor or scavenger.
Present study indicates that MEPI (300mg/Kg b. wt.) significantly decreased serum glucose level in hyperglycemic rats. Alloxan is the most frequently employed agent for the induction of experimental diabetic animal models of human insulin-dependent diabetes mellitus. There is escalating evidence that alloxan caused diabetes by rapid exhaustion of a cells, by DNA alkylation and gathering of cytotoxic free radicals that is suggested to result from initial islet inflammation, followed by infiltration of activated macrophages and lymphocyte in the inflammatory focus. It leads to a fall in insulin release there by a drastic diminution in plasma insulin concentration leading to stable hyperglycemic states [29]. It induces diabetes by dose dependent destruction of ß-cells of islets of langerhans [30, 31]. So, in the present study alloxan was chosen to create diabetic condition in rat and significant hyperglycemia was achieved within 48 hours after alloxan (110g/kg b. wt. i. p.) injection. The results obtained (Table 2) showed that after a single administration of glucose 200 mg/kg in rat, there was a significant reduction (p < 0.05) of fasting blood glucose level during the 12 hr study period. The research on antidiabetic activity in alloxan diabetic rats, administration of MEPI of 300mg/kg body weight administered for 7 days was able to correct this anomaly and significantly (p<0.05 and p<0.001). Significant reduction of blood glucose was observed from the 7th day of the study. The comparable effect of the experimental extract with Metformin HCl may suggest similar mode of action since alloxan permanently destroys the pancreatic ß cells and the extract lowered blood sugar level in alloxan diabetic rats, indicating that the extract possesses extra pancreatic effect. On the progression of treatment with methanolic extract of *Premna integrifolia* (MEPI) (300 mg/kg b. wt.) produced maximum reduction to 6.48 ±3.23m mol/L on 7th day whereas reduction to 6.08 ±5.23m mol/L was found for metformin on 7th day (Table 4). These observations suggest that the experimental extract might acquire insulin like effect on peripheral tissues either by promoting glucose consumption metabolism or inhibiting hepatic gluconeogenesis since alloxan treatment causes permanent destruction of ß cells [32]. Changes in initial and final body weight in control and experimental groups are shown in Table 3 and 4. Significant weight loss was observed in diabetic rats compared to control non-diabetic rats. Treatment with *Premna integrifolia* bark extract or Metformin improved the body weight as compared to normal control rats.

The methanolic extract of *Premna integrifolia* may be considered to have good anti-hyperglycemic active principles. The phytochemical screening of *Premna integrifolia* bark revealed the presence alkaloids, glycosides, terpenoids, carotenoids and flavonoids known to be bioactive antidiabetic principles [5, 6]. Flavonoids are known to regenerate the damaged beta cells in the alloxan diabetic rats [33]. Phenolics are found to be effective anti-hyperglycemic agents [34]. In the present study, 31.92 ± 0.04 mg/g phenolic compounds were found to be present in the methanolic extract of *Premna integrifolia* bark (Table 1) so it may be one of the reasons that methanolic extract shows good hypoglycemic and anti-hyperglycemic activity. The antidiabetic effect of methanolic extract of *Premna integrifolia* may be due to the presence of more than one anti-hyperglycemic principle and their synergistic properties. In this study, the anti-hyperglycemic activity caused by metformin in alloxan-induced diabetic rats is an indication of the presence of some beta cells, as metformin is known to stimulate insulin secretion from beta cells.

**CONCLUSION**

Our preliminary pharmacological studies on the methanol extract of *Premna integrifolia* bark provide in a part of scientific support in traditional medicine, particularly in diabetes. However, further pharmacological investigations are required to understand its underlying mode of action on the antidiabetic activity. In addition, future bioactivity-guided phytochemical work should be carried out to identify any active constituent(s).

**REFERENCES**


