Identification of bioactive glucose-lowering compounds of methanolic extract of *Hodgsonia heteroclita* fruit pulp

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1. ABSTRACT

We have investigated the anti-oxidative and glucose-lowering effects of 70% methanolic extract of *H. heteroclita* fruit pulp (MHE). Anti-oxidative property of MHE was assessed by free radical scavenging assays and compound level screening by LC-MS profiling. *In silico* analysis and *in vivo* preclinical validation were also performed using molecular docking and alloxan-induced diabetic model, respectively. MHE showed high anti-oxidant activity in DPPH radical scavenging assay with an IC₅₀ of 0.37 μg/mL. The LC-MS profile of MHE substantiated the presence of p-hydroxy acids including benzoic, salicylic, p-coumaric, o-coumaric, caffeic, protocatechuic, gentisic, 2,4-dihydroxybenzoic, gallic, vanillic, syringic, and ferulic acids. Among these, caffeic acid was the most likely compound to interact with 1R0E at Val⁷⁰, Ala⁸³, Lys⁹⁷, Asp¹³³, Val¹³⁵, Leu¹⁸⁸, Cys¹⁹⁹, and Asp²⁰⁰, and with 1Q4L at Ile⁶², Ala⁸³, Asp¹³³, Tyr¹³⁴, Val¹³⁵, Arg¹⁴¹, and Val¹⁷⁰. Treatment of rats with MHE showed significant reduction in serum glucose levels as compared to control rats. Taken together, the results show that MHE has compounds...
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with anti-diabetic effect, which could be partially due to the anti-oxidant effects of the active components in MHE.

2. INTRODUCTION

Glycogen synthase kinase-3β (GSK-3β), a well-known modulator of glycogen metabolism, is now established as a Ser/Thr protein kinase having been playing a major role in several pathways including apoptosis, initiation of protein synthesis, cell proliferation and differentiation (1–3). Numerous studies have reported various inhibitors of GSK-3β (3–5). Henriksen and Dokken (2006) indicated that an over-expression of GSK-3 in skeletal muscle of rodent models of obesity and type 2 diabetic humans are allied with an impaired capability of insulin to trigger glucose clearance and glycogen synthase (6). This makes GSK-3β a relevant target for diabetes.

Popularly celebrated as Chinese lard plant, Hodgsonia heteroclita (Roxb.) Hook. f. & Thomson (Cucurbitaceae) is found in India, China, Bangladesh, Malaysia and Nepal (7–9). In India, H. heteroclita is found in the hills of the entire North-Eastern province and is an important folkloric medicinal plant indispensable for the life of the tribes of this region (10). The seed oil (62–71%) is commonly used in cooking and beverages by the tribes of Assam (Karbis and Dimasa) (10). The tribes of Arunachal Pradesh (Nishi), Meghalaya (Jaintia, Garo and Khasi) and Mizoram (Chakma, Hajong, Tongbe and Riang) use the roasted seeds of H. heteroclita to garnish food items. The crushed seeds of H. heteroclita are reported, though not scientifically validated, for the treatment of intestinal worms by Manipuri tribes (Naga and Kuki) and Nagaland tribes (Angamis and Rengma) (11). In Nagaland, Angamis and Rengma tribes apply the fruit pulp of H. heteroclita to cure bacterial infections of feet. Seed powder is given in digestion and stomach pain. The Bhil, Chaimal, Mizel and Orang tribes of Tripura apply leaf juice on fresh cuts and wounds to stop bleeding and also on ulcers (12). The oil is also used as a base for medicines in North-Eastern India. Additionally, the roasted endosperm of H. macrocarpa, another species of the Hodgsonia genus, is given to the women and children as energetic food by mixing with other food items (13,14).

H. heteroclita (HH) is locally known as Hagrani jwgwnar among the Bodos and known for its ethno-botanical values (15). In their letter to editor, Usha et al. (2017) indicated HH for its anti-diabetic activity (16). Swargiary and his coworkers (2013) recorded the uses of H. heteroclita fruit pulp by Bodo tribes to control diabetes (17). Though different parts of the plant were recorded for its myriad of applications as traditional medicine, there has been no scientific evidence that validate its conventional values till date. Therefore, this plant virtually remains unexplored on scientific grounds. The current study is an attempt to identify the bioactive compounds in the methanolic extract of H. heteroclita fruit pulp (MHE) using liquid chromatography-mass spectroscopy (LC-MS) and the probable mechanism using In silico tools by identifying the correct binding mode of the compounds investigated in this study using computer-aided molecular modeling techniques.

3. MATERIALS AND METHODS

3.1. Plant material collection and extraction, and in-vitro studies

H. heteroclita fruits were collected from the forest of Kokrajhar District, Bodoland Territorial Area Districts (BTAD), Assam, India, during November-December, 2013. After confirmation by a plant taxonomist a voucher specimen (Voucher No. DBT/BU/001) was deposited at the Botany Department, Bodoland University, Assam, India.

The collected fruits were thoroughly washed with distilled water, air dried and stored in a cool and dark place. Then, the dried pulps were sliced, and powdered using mechanical grinder. 10 g powdered sample was subjected to Soxhlet apparatus using 70% methanol (v/v) in 1:15 m/v ratio (18). The extraction was carried out at boiling temperature for 6 h for three cycles. The extract was evaporated under pressure at 50°C to obtained constant weight and stored at 4°C until required. Before use, MHE was dissolved in double-distilled water (DDW) at desired concentrations.

3.2. In vitro antioxidant activity and free radical scavenging activity (DPPH method)

The anti-oxidant activity of MHE along with ascorbic acid reference standard was assessed for free radical scavenging effect of the stable DPPH free radical as previously reported (19). The discoloration was measured at 517 nm using Thermo UV1 spectrophotometer. In case of control, the solvent methanol was taken instead of the plant extract sample. Percentage scavenging of the DPPH free radical was measured using the following equation:

\[
\text{scavenging effect (\%)} = \frac{(A_0 - A_1)}{A_0} \times 100
\]

where \(A_0\) was the absorbance of the control and \(A_1\) was the absorbance in the presence of the sample.

3.3. Ferric reducing power assay

The method of Oyaizu (1986) was used to determine the reducing power of MHE. Different
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concentrations of MHE extract (0.2–2.0 mg/mL) in phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [K₃Fe(CN)₆] (2.5 mL, 1%) was mixed (20). The mixture was incubated at 50°C for 20 min. A portion (2.5 mL) of TCA (10%) was added to the mixture, which was then centrifuged at 3,000 rpm for 10 min. The upper layer of the solution (2.5 mL) was mixed with DDW (2.5 mL) and FeCl₃ (0.5 mL, 0.1%), and the absorbance was recorded at 700 nm. Butyl hydroxyl toluene (BHT) and phosphate buffer (pH 6.6) were used as a reference standard and blank, respectively.

3.4. Liquid chromatography-mass spectroscopy (LC-MS) analysis

Derivatization procedure: A small amount of concentrated crude methanol extract was taken in a separating funnel and shaken by adding water and ethyl acetate in the ratio of 1:4. The upper layer was collected and concentrated in a rotary evaporator to about 1.5 mL Added 100 μL of N, O-Bis(trimethylsilyl) trifluoroacetamide and trimethyl chlorosilane (BSTFA+TMCS) and 20 μL pyridine, and heated at 60°C for 30 min. From the layers which are separated from the crude extracts, a small amount of the extract was taken and evaporated out totally. To this, acetonitrile was added and the filtrate was added to 50 μL BSTFA+TMCS solution. Solution was heated at 60°C at water bath for 30 min. The cooled solution was filtered using 0.45 μ membrane filter to a vial (21).

LC-MS Analysis: LC-MS analysis was carried out on a PerkinElmer TurboMass Spectrophotometer (Norwalk, CTO6859, USA) which includes a PerkinElmer Auto sampler XLGC. The column used was Perkin-Elmer Elite - 5 capillary column measuring 30 m × 0.25 mm with a film thickness of 0.25 mm composed of 95% dimethyl polysiloxane. The carrier gas used was Helium at a flow rate of 0.5 mL/min. 1μL sample injection volume was utilized. The inlet temperature was maintained as 250°C. The oven temperature was programmed initially at 110°C for 4 min, then an increase to 240°C, and then programmed to increase to 280°C at a rate of 20°C ending with a 5 min. Total run time was 90 min. The MS transfer line was maintained at a temperature of 200°C. The source temperature was maintained at 180°C. LC-MS was analyzed using electron impact ionization at 70eV and data was evaluated using total ion count (TIC) for compound identification and quantification. The spectrums of the components were compared with the database of spectrum of known components stored in the LC-MS library. Measurement of peak areas and data processing were carried out by TurboMass-OCPTVS-Demo SPL software. The compounds detected in MHE using LC-MS were further followed with In silico studies to know the interaction between GSK-3β and the best possible target.

3.5. In silico studies and molecular docking for the binding mode of the inhibitors

The investigation for the possible binding approach of the natural compounds to GSK-3β is an effort to develop a potent GSK-3β inhibitor. Initially, after assessing X-ray crystal structures of GSK-3β, there are roughly two types of structures available in protein data bank (PDB), based on the side chain conformation of residue Phe85 and Arg135 as previously reported by Kim et al. (2009). The possible reason of these conformation changes is because of Gly-rich loop present in GSK-3β structures (22). The author had grouped PDB structures as 1R0E like (in pink) and 1Q4L like (in green) as shown in Figure 1. The active site was considered on the basis of previous literature (23).

The binding mode of PDB structures (1R0E and 1Q4L) were considered for docking with predefined binding site amino acid residues like Asp130, Val135, Leu132, Lys65 and Gly87. The LC-MS generated compounds (ligands) were docked into the binding site of GSK-3β (1R0E and 1Q4L). Docking was conducted using CDOCKER algorithm with a set of virtually screened ligands from ADMET as listed in Table 1. The receptors and ligands were prepared as per the prescribed protocol of CDOCKER in Discovery Studio 3.5. and reported previously (21). Additionally, simulation annealing was introduced to simulate the complex and the system was heated at 500k target temperature, followed by cooling at 500k. Finally, the interaction between the active site's amino acid and the compound's non-bond interactions was analyzed using the view interaction tool in Discovery Studio 4.5 Visualizer.

3.6. Molecular dynamics simulation

Finally, the best screened pose of the docked complex from docking study and apoprotein was subjected to nanoscale simulation process using Discovery Studio 3.5 with the integration of leap-frog Verlet and SHAKE constraint. The simulation was executed in five different steps for apoprotein and protein-ligand complex by applying CHARMM force field followed by minimization with 1000 steps using steepest descent and conjugate gradient, respectively. Each individual system was heated from 50k to 350k with 4ps of simulation time. Consequently, the system was equilibrated for 40ps of simulation time to achieve even distribution system with degrees of freedom. Finally, production in the canonical (NVT) ensemble was subjected with equal Tmass and Pmass at 300k for 1600ps. Additionally, spherical-cutoff method of electrostatics was implemented to study the all non-bonded energy without using periodic boundary condition. Periodic boundary conditions enable a simulation to be performed using a relatively small
Table 1. ADME and toxicity descriptors of the compounds

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<th>Solubility</th>
<th>BBB</th>
<th>Absorption</th>
<th>CYD2D6</th>
<th>Hepatotoxic</th>
<th>CPR TD50 (mg/kg body weight/day)</th>
<th>AM</th>
<th>Rat oral LD50</th>
<th>RIT LC50 (mg/m3/h)</th>
<th>Daphnia EC50 (mg/l)</th>
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<td>NM</td>
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</table>

AM= Ames mutagenicity, NM= Non-Mutagen, CPR= Carcinogenicity Potency, Rat RIT= Rat inhalation toxicity, Absorption level 0 as Good Absorption, BBB (Blood Brain barrier) level 3 shows low to Medium, Solubility level 4 as optimal
number of particles in such a way that the particles experience forces as though they were in a bulk solution.

3.7. In vivo animal studies

The male Wistar albino rats weighing 140–160 g were housed under laboratory conditions of light and dark cycles of 7:00 am to 7:00 pm, temperature (25°C ± 2°C), relative humidity (68% ± 1%) and provided standard rat pellet (Lipton India Ltd., Bangalore, India) and tap water ad libitum. The study protocol was approved and cleared by internal ethical approval committee (IEAC) of Maharani Lakshmi Ammanni College (1368/ac/10/CPCSEA), Bangalore, India.

3.7.1. Experimental design for deciphering anti-diabetic activity

After several days of acclimatization, alloxan diluted in citrate buffer (150 mg/mL, 0.1 M, pH 4.0) was injected intraperitoneally to the experimental animals, following overnight fasting. Two days later, the diabetic state was confirmed by blood samples collected from tail vein method and the fasting blood glucose level was measured by Accu-Chek (Roche, USA) once a week. Diabetic rats were divided into 6 groups (n = 8 per group), avoiding any inter-group differences in blood glucose levels, including a normal group (NL), a positive control (diabetic glibenclamide; DG), negative control (not treated; DC), animals fed orally using 16g ball point needle with low (20 mg/kg bw/mL) and high dosage (40 mg/kg bw/mL) of extract (LH & HH; suspended in 0.1% di-methyl sulfoxide (DMSO)). The normal and control diabetic group was given 0.1% of DMSO. Diabetic rats treated with standard drug insulin (DI) (2 units/kg of bodyweight) (Insugen, Biocon, India) daily intraperitoneally for 45 days.

3.8. Statistical analysis

The three times repeated results were expressed as mean ± standard error of the mean (SEM) and analyzed statistically using two-way analysis of variance (ANOVA) using GraphPad Prism software and p<0.0.5 was considered to be statistically significant.

4. RESULTS AND DISCUSSIONS

Plants have been a source of medicine since the time immemorial. Different plants are key ingredients of many herbal formulations that fight against various human related health ailments. Therefore, detailed study of plant materials is of prime focus for the advancement and quality control of folk formulations. Keeping this in mind, the present study was undertaken to evaluate the anti-oxidant activity and identify the bioactive compounds present in the methanolic extract of H. heteroclitafruit pulp (MHE) native to Bodoland, India using LC-MS, to find out probable inhibitors such as salicylic acid, ferulic acid, vanillic acid, caffeic acid (Table 3) of the GSK-3β and their mechanistic approach to treat diabetic conditions followed by in vivo validation using alloxan-induced diabetic animals.

4.1. Anti-oxidant activity

The anti-oxidant effect of MHE as assessed by its ability to scavenge DPPH radical was found to be with EC<sub>50</sub> of 6.03 μg/mL as compared to the reference anti-oxidant ascorbic acid (EC<sub>50</sub>=3.75 μg/mL). The radical scavenging pattern of MHE was dose dependent (Table 2). The reducing power of MHE showed better reducing effect as compared to the standard BHT (Figure 2). The observed effect could be due to the presence of the mixture of compounds present in MHE. Moreover, the extract demonstrated strong radical scavenging activity comparable to the synthetic anti-oxidant BHT, which could be possibly due to the presence of phenolic compounds in the extract.

4.2. LC-MS analysis

The phyto-constituents identified from MHE with their IUPAC name, molecular formula, common name, chemical structure, retention time (RT), and concentration (peak area %) using LC-MS are represented in Table 3. Among the
identified phyto-compounds, p-hydroxybenzoic acid (24), salicylic acid (25), protocatechuic acid (26), p-coumaric acid (25), caffeic acid (27), vanillic acid (28), ferulic acid (28), and gallic acid (29, 30) have proven anti-diabetic activity. In addition to anti-diabetic activity, p-hydroxybenzoic acid is also reported for its anti-fungal, anti-mutagenic, estrogenic, and anti-microbial activities (31). Salicylic acid is known for its keratolytic, anti-inflammatory, anti-pyretic, analgesic, anti-septic and anti-fungal properties (31). Used as a flavoring ingredient in foods and drugs, p-coumaric acid and gentisic acid have noted anti-cancerous properties (31–33). Magnani et al. (2014) indicated the antioxidant and prevention of premature aging activities of caffeic acid (34). Prior studies have shown protocatechuic acid as a promising compound imparting various pharmacological activities like anti-oxidant, analgesic, anti-inflammatory, anti-rheumatic and anti-arthritic activity (35). Gentisic acid inhibits low-density lipoprotein oxidation in human plasma (36). 2,4-dihydroxy benzoic acid has thyroid peroxidase inhibitory effect and snake venom 5'-nucleotidase inhibitor vanillic acid has proven astringent, antineoplastic and bacteriostatic activities (31). Gallic acid possesses anti-melanogenic (37) and antioxidant properties (30). Ferulic acid has therapeutic potentials against various diseases like cancer, diabetes, cardiovascular dysfunction, neurodegenerative and inflammatory diseases, and in aging. It has a strong anti-oxidant property (38). Syringic acid has anti-oxidant, anti-bacterial and hepatoprotective activities. (31). All identified compounds in MHE are known for its pharmacological activities and shown in Table 3.

4.3. Virtual screening and docking

The receptor influenced to bind compounds with highest dock score and favorable bonding is considered as the best receptor. The compounds caffeic acid, vanillic acid, ferulic acid and p-coumaric acid satisfies ADMET property, and found to be inside 95% and 99% confidence ellipsoids for absorption and blood brain barrier (BBB) (Figure 3). ADMET plot was found to be non-hepatotoxic and non-inhibitors of metabolizing enzyme (CYP2D6, a class of Cytochrome 450 class of enzyme). This specifies that these compounds found to be well-metabolized in Phase-I metabolism. Furthermore, these screened compounds show good absorption (Table 1), has the potential to cross the BBB with optimal solubility. Perhaps, all the compounds have shown a non-mutagenic effect when subjected to Ames test in addition to satisfying RO5 violations and Veber’s rules of drug-likeness property but are ruled out in ADMET screening.

Validation of docking conformation with default bound ligand of 1R0E compared with docked poses of caffeic acid (31,37) forms 11 favorable non-bond interaction with least RMSD deviation of
### Table 3. Phytochemical constituents of *Hodgsonia heteroclita* fruit pulp identified using LC-MS analysis

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>IUPAC Name</th>
<th>Molecular Formula</th>
<th>Common Name</th>
<th>Chemical Structure</th>
<th>RT (min)</th>
<th>Height</th>
<th>Area</th>
<th>Peak Area (%)</th>
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<tbody>
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<td>(E)-3-(4-hydroxy-3-methoxy-phenyl)prop-2-enoic acid</td>
<td>C₇H₆O₅</td>
<td>Ferulic acid</td>
<td><img src="image" alt="Structure" /></td>
<td>5.69</td>
<td>128</td>
<td>23.34</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>4-hydroxy-3,5-dimethoxybenzoic acid</td>
<td>C₈H₈O₅</td>
<td>Syringic acid</td>
<td><img src="image" alt="Structure" /></td>
<td>6.23</td>
<td>89</td>
<td>36.79</td>
<td>100</td>
</tr>
</tbody>
</table>
0.037Å for pose number 10, but docking of caffeic acid (28.04) with 1Q4L forms 10 favorable interaction of RMSD deviation of >0.05Å for pose number 7. So, we have considered caffeic acid which shows the least deviation with 1R0E as a better target for GSK-3β inhibitor. RMSD deviation of other compounds like vanillic acid, ferulic acid and p-coumaric acid was >0.0.90Å (Figure 1). Bond-forming residues in case of the compound caffeic acid with 1R0E are Val\textsuperscript{70}, Ala\textsuperscript{83}, Lys\textsuperscript{85}, Gly\textsuperscript{97}, Asp\textsuperscript{133}, Val\textsuperscript{135}, Leu\textsuperscript{188}, Cys\textsuperscript{199}, Asp\textsuperscript{200} and caffeic acid with 1Q4L are Ile\textsuperscript{62}, Ala\textsuperscript{83}, Asp\textsuperscript{133}, Tyr\textsuperscript{134}, Val\textsuperscript{135}, Arg\textsuperscript{141}, Val\textsuperscript{170} as shown in Figure 4 (A & B) and (C & D), respectively.

4.4. Dynamics and simulation

The best pose of 1R0E–caffeic acid complex from docking and its apo-form of protein (1R0E) were subjected to 1.6 ns MD simulations using Discovery Studio 3.5. The stability of the protein complex and its folding pattern was studied using root-mean-square deviation (RMSD), root-mean-square fluctuation (RMSF), and Radius of gyration ($R_g$). RMSD of the 1R0E–caffeic acid complex and apoprotein were observed to oscillate steadily within the range about 2.2Å - 2.6Å along the simulation. After an initial rise in the RMSD, the system converges around 750 ps, indicating the stability and equilibration state of the proteins. Backbone deviation of apoprotein was much observed at a time interval of 400 to 600 ps, whereas, in a case of the bound complex there is no much deviation noticed (Figure 5). RMSD results indicated that the complex did not experience large conformational and structural changes, and it was stable due to the RMSD evolved within the acceptable range (39). RMSF studies are constructive in the measurement of Cα average atomic mobility in dynamics process, In agreement with the studies of the RMSF, much deviation was observed for apoprotein than ligand-protein complex (40) (Figure 6). The Rg was also calculated to analyze the folding pattern of the protein during simulation and it is defined as the root mean square distance of the collection of atoms from their common center of gravity. The results of gyration of apoprotein shows that the change in pattern at time interval of 50 to 350 ps and not much deviation was observed in ligand -protein folding pattern (Figure 7). Overall simulation of 1R0E–caffeic acid complex had shown more stability (~19827.50 kcal/mol) than an apo-form of protein (~19592.21 kcal/mol).

4.5. In vivo animal studies

Figure 8 depicted the fasting blood glucose (FBG) in different experimental groups. Prior to alloxan administration, blood glucose levels of all the experimental rats were checked and the animals were selected based on FBG that was not significantly different. However, there was a significant increase (more than 220 mg/dL) in FBG level was noticed after 72 h administration of alloxan and rats were considered...
Bioactive glucose lowering compounds of Hodgsonia

Figure 4. (A&B) 1R0E docked with Caffeic acid, (C&D) 1Q4L docked with Caffeic acid.

Figure 5. RMSD plot of 1R0E-Caffeic acid complexes and 1R-apo protein, during 1600 ps simulations.
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**Figure 6.** RMSF plot of 1R0E-Caffeic acid complexes and 1R-apo protein, during 1600 ps simulations.

**Figure 7.** Radius of gyration plot of 1R0E-Caffeic acid complexes and 1R-apo protein, during 1600 ps simulations.
diabetic (29). Although a significant reduction in glucose level was marked from the second week onward, a decrease in FBG was more evident on the third week in the group receiving a high dose of MHE (HH) and found significant ($p < 0.05$) as compared with DC rats. Administration of LH, HH and DG led to significant decrease in blood glucose levels by 45, 52 and 47% on the 42nd day when compared to diabetic animals (Figure 8). The anti-diabetic action could be due to the presence of polyphenolic compounds present in MHE. Additionally, the pronounced effect could be due to the presence of caffeic acid as indicated by In silico study demonstrated before.

5. CONCLUSION

In conclusion, the results of the anti-oxidant effect of methanolic extract of *H. heteroclita* fruit pulp point out to a strong protective activity against free radicals and oxidative agents *in vitro*. Thus, it might be possible that the anti-hyperglycaemic property of MHE could be partially contributed by the anti-oxidant effects of the active components present in the fruit pulp of *H. heteroclita* and caffeic acid could be responsible for the anti-diabetic activity. The current study is the first scientific report with compound detection and anti-oxidant activity of methanolic extract of *H. heteroclita* fruit pulp as well as mechanistic evidence for its anti-diabetic property followed by in-vivo validation.

6. ACKNOWLEDGMENTS

TU, AKG, DS and DN performed the experiments, prepared the manuscript, and analyzed the data. LP, GW and DB performed some of the experiments and wrote a part of the manuscript. SKM conceived the idea, designed and coordinated the experiments, improved the manuscript and generated funding. The infrastructure support from the DBT-BIF Facility and BT-Finishing School to Maharani Lakshmi Ammanni College for Women (MLACW) is gratefully acknowledged. The authors are thankful to the management for providing animal house facility, MLACW, Bangalore, India. The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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