Assessment of phytochemical & pharmacological activities of the ethanol leaves extracts of Myrica esculenta BUCH. HAM.

Bipin Kumar Nayak1*, Purabi Deka1, N. Eloziia1
1Division of Pharmaceutical Sciences, Shri Guru Ram Rai Institute of Technology & Science, Patel Nagar, Dehradun 248001, Uttarakhand, India.

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ABSTRACT
OBJECTIVE: To elucidate potential antioxidant, antidiarrheal, anthelmintic and antibacterial activity of ethanol leaves extract of Myrica esculenta Buch. Hum. in different experimental models established in vitro and in vivo. METHODS: In vitro antioxidant activity was evaluated by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay. In vivo antidiarrheal studies was carried out in mice, and the activity was evaluated in castor oil induced diarrhea, anthelmintic activity on Paramphistomum cervi and Haemonchus contortus, and Disk diffusion assay was utilized to determine antibacterial activity against a number of pathogenic bacterial strains. RESULTS: In DPPH radical-scavenging assay, the extract exhibited strong radical-scavenging activity with the 50% inhibitory concentration value of 48.28 μg/ml. The extract significantly (p<0.001) enhanced the latent period and decreased the defecation in castor-oil induced diarrhea. The extract exhibited dose-dependent decrease in paralysis and death time of the helminthes. Potential antibacterial activity was exhibited by the extract against all the tested bacterial strains in disk diffusion assay. CONCLUSION: The result demonstrated that the ethanol leaves extract of M. esculenta has potential antioxidant, antidiarrheal, anthelmintic and antibacterial activity.

KEY WORDS: Myrica esculenta, antioxidant activity, antidiarrheal activity, anthelmintic activity, antibacterial activity, mice.

1. INTRODUCTION
The nature has provided the storehouse of remedies to cure all ailments of mankind. The traditional herbal medicines are still practiced in large part of our country mostly in tribal and rural areas. In many developing countries large section of population relies on traditional practioners, who are dependent on herbal folk medicine for their primary health care and has deep faith in it. Since the usages of these herbal medicines are increased, the issue regarding their safety, quality and efficacy in industrialized and developing countries are cropped up. Researcher always takes an attempt to perform pharmacological test to identify and isolate the bioactive compound. Modern medicines is developed gradually in this way[1,2].

Myrica esculenta (Myricaceae), commonly called ‘Katphala’ is an important medicinal tree distributed all along outer Himalaya region of Assam, in Khasia, Jaintia, Shimla, Bengal, Naga and Lushai hills extends to Singapore, China and Nepal. They are growing between 900-2100m above sea level[3,4]. The fruits of the tree are edible and are used in the preparation of a refreshing drink. The tree can grow up from 3m to 15m. The leaves of Myrica esculenta is said to many medicinal properties. It is stimulating, useful in cough, throat infection, asthma, bronchitis, urinary discharge, cholera, ulcers and is used in many other diseases[5,6].

*Corresponding author
Bipin Kumar Nayak
Division of Pharmaceutical Sciences, Shri Guru Ram Rai Institute of Technology & Science, Patel Nagar, Dehradun 248001, Uttarakhand, India.

Fig. 1: Myrica esculenta
The constituent of leaves are 4-hydroxy-1,8-cineole 4-O-β-Dapiofuranosyl-(1→6)-β-D-glucopyranoside, (1S,2S,4R)-2-hydroxy-1,8-cineoleβ-D-glucopyranoside, corchoionoside C, (6S,9R)-rososide, myricanol, 5-O-β-D-glucopyranosyl myricanol, arjunolic acid, arjunglucoside, 3-epi-ursonic acid, 3-O-(E)-caffeoylursonic acid, myricetin, myricitrin. On the spectrophotometric evidence flavone 4’-hydroxy-3’,5,5’-trimethoxy-7-O-β-D-glucopyranosyl (1→4)-α-L-rhamnopyranoside and 3’, 4’-dihydroxy-6-methoxy-7-O-α-L-rhamnopyranoside, β-Sitosterol, β-Sitosterol-β-glucopyranoside and quercetin were elucidated. The volatile oil was extracted by distillation and analyzed by gas chromatography-mass spectrometry. The major constituents were Nerolidol (13.46%), α-piene (13.46%), α-Selinene (12.28%), β-Caryophyllene (11.66%), β-Selinone (9.71%), α-Caryophyllene (8.94%), α-cadinol (5.32%), Linalool (4.06%) and β-cadinol (5.32%). The bark of M. esculenta have been reported to have a significant antibacterial, analgesic and anti-inflammatory properties. While the fruit of M. esculenta are known for their antioxidant and antibacterial in nature.

The present study was carried out to investigate antioxidant, analgesic, anti-diarrheal, anthelmintic and antibacterial activities of the ethanol leaves extract of M. esculenta.

2. MATERIALS AND METHOD

2.1. Collection of Plant Material
The leaves of M. esculenta were collected from the Gwldom, Dist Chamoli, (Uttarakhand). The plant was authenticated by Dr. Veena Chandra, Head “Botany Division” Forest research institute Dehradun State Uttarakhand (India).

2.2. Drying and Grinding
The fresh plant part subjected to shade drying to remove moisture. The plant materials were grinded into coarse powder using a mechanical grinder hammermill. The powder was kept in an airtight container to avoid any possible fungal attack and then stored in a dark, cool and dry place until the extraction was started.

2.3. Extraction
Powder plant material was subjected to cold extraction technique for the extraction. The powder was soaked in 95% ethanol for seven days with regular shaking and stirring. The extract was filtered off to separate the plant debris. Clean cotton plug and filter paper were used to get clear solution. Then filtrate was evaporated at 50°C with the help of rotary vacuum evaporator to yield crude extract. The crude extract was stored in refrigerator at 4°C (yield 1.73%).

2.4. Experimental Animals
In vivo pharmacological investigation was conducted on young Swiss albino mice of either sex (weight 18-25g, age 4-5 weeks) were used in present pharmacological investigation. They were obtained from the international Center for Diarrhoal Disease Research, Bangladesh (ICDDR, B). They were housed in the departmental animal house on standard laboratory diet and tap water ad libitum with a 12 hr dark cycle. Animals were fed with ICDDR, B formulated rodent food and allowed free access to water. The experiments using mice were carried out following the guidelines of the Animal ethics Committee.

2.5. Test Microorganisms
Bacterial strains including both gram-negative and gram-positive were collected from Microbiology Laboratory of ICDDR, B and they are stored in microbiology lab in Pharmacy Discipline, Khulna University. Bacterial strains were Staphylococcus aureus, Staphylococcus epidermis, Bacillus megaterium. Escherichia coli, Salmonella typhi and Vibrio cholera.

2.6. Chemicals and Reagents
Sodium monobasic phosphate, Ascorbic acid, sodium dibasic phosphate, trichloroacetic acid, sodium chloride, ferric chloride, acetic acid, magnesium sulfate, potassium ferricyanide and magnesium sulfate were obtained from Merck, Germany. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) and Folin-Ciocłeau’s reagent obtained from Sigma Chemical Co. Ltd., (St. Louis, MO, USA). Loba Chemie Pvt Ltd., India provided castor oil and Tween-80.

2.7. Reference Drugs
Loperamide and diclofenac sodium were purchased from square pharmaceuticals Ltd., Bangladesh. Standard ciprofloxacin disk was obtained from Oxoid Ltd., UK. Morphine was obtained from popular Popular Pharmaceuticals Ltd., Bangladesh.

2.8. In-vitro Antioxidant Assay
The ethanol leaves extract of M. esculenta was assessed for both quantitative and qualitative antioxidant activity. Quantitative assay was performed through DPPH free radical-scavenging assay and quantitative assay was performed through thin layer chromatographic technique followed by DPPH spray.

2.9. Quantitative Assay
This experiment was carried out according to the method of Sharma et al. DPPH scavenging activity of the M. esculenta leaves ethanol extract was estimated In vitro by 1, 1-diphenyl-2-picrylhydrazyl (stable DPPH free radical) assay. Sample was prepared by dissolving...
extract in ethanol at different concentration of 512, 256, 128, 64, 32, 16, 8, 4, 2, and 1 mg/ml. From each concentration 1 ml sample was taken in test tube, and 3 ml of 0.004 % DPPH solution in ethanol was added to each test tube and it was incubated at room temperature for 30 min. After incubation, absorbance was taken at 517 nm against blank. Control was prepared same as sample except addition of standard. Ascorbic acid was used as standard. % scavenging activity was calculated by using the following formula.

\[
\text{Percent scavenging activity} = \left( 1 - \frac{A_1}{A_0} \right) \times 100\% \text{, where } A_0 \text{ is the absorbance of control, and } A_1 \text{ is the absorbance of sample or standard.}
\]

2.10. Qualitative Assay

Thin Layer chromatogram (TLC) of the ethanol leaves extract was developed in polar, medium polar, and non-polar solvent system. Then 0.002% DPPH solution in ethanol was sprayed using spray gun. Compounds showed yellow spot on the purple back ground of the chromatogram[17].

2.11. Evaluation of Antidiarrheal Activity

2.11.1. Castor oil-induced diarrhea

Experimental animal were selected initially based on their sensitivity on their response to castor oil induced diarrhea and divided into four groups of six animals each. Group I received distilled water (10 ml/kg p.o.) served as control, group II received loperamide (3 mg/kg, p.o.) served as standard and group III and IV received M. esculenta leaves extract (250 and 500 mg/kg, p.o.) served as test group. After 60 min, all mice across the four groups received 0.5 ml castor oil orally to induce diarrhea. Then each animal was housed in separate plastic cage with white blotting paper at the base to count the number of faces. Observation period was 4 hr and blotting paper was changed every hour. Onset of diarrhea and total number of faces for each animal was recorded[18,19].

Percent inhibition of defection was calculated using the following equation: inhibition = \((D_0-D_1)/D_0 \times 100\%\); where \(D_0\) is the number of defection of control group, and \(D_1\) is the number of defection of test group.

2.11.2. Evaluation of Anthelmintic Activity

Anthelmintic activity of the extract was investigated on live parasites Paramphistomum cervi and Haemonchus contortus of cattle. The parasites were divided into different groups consisting of six parasites in each group. Extract at the concentrations of 25, 50, 100 and 200 mg/mL; 0.25, 0.5, 1 and 2 gm of extract were taken and triturated with 0.2% v/v of Tween 80 as a suspending agent and final volume was made to 10 ml for respective concentration with PBS. Live parasites P. cervi and H. contortus were collected from freshly slaughtered cattle at local abattoirs. After sanitation, parasites were stored in 0.9% phosphate-buffered saline (PBS) of pH 7.4 prepared with 8.01 g NaCl, 0.20 g KCl, 1.78 g Na2HPO4 and 0.27 g KH2PO4 in 1 litre of distilled water at 37±1°C. The reference standard albendazole (collected from Beximco pharmaceuticals Ltd., Bangladesh) at the concentrations of 15 mg/mL of 10 mL in PBS were prepared and transferred to Petri dishes. Control group was treated with 0.1% tween-80 in phosphate-buffered saline (PBS). Six parasites were placed in each Petri dish and observed. Time taken for paralysis and death was recorded for each group. Anthelmintic activity is expressed as the time required for paralysis and death of parasites as compared to control[20,21].

2.11.3. Evaluation of Antibacterial Activity

Antibacterial activity of the ethanol leaves extract of M.esculenta against a number of Gram- positive and Gram-negative bacterial strains was evaluated by discs diffusion assay[22,23]. Microorganisms used for this test were Staphylococcus aureus, Staphylococcus epidermis, Bacillus megaterium Escherichia coli, Salmonella typhi, and Vibrio cholera. These organisms collected from Microbiology Laboratory of ICDDR, B and they are stored in microbiology lab in Pharmacy Discipline, Khulna University, Khulna-9208. Standard antibiotics discs (Ciprofloxacin 5 µg/disk, Oxoid Ltd., UK) were impregnated with the test extract at the concentration (250 µg and 500 µg/discs), and control discs containing ethanol were placed along with test discs on nutrient agar medium inoculated with test organism. The Petri-dishes were than incubated at 37°C temperature for 18 hr. After incubation period, the zone of inhibition was measured using digital slide calipers.

2.12. STATISTICAL ANALYSIS

Result were expressed as mean ± standard error of mean (SEM). The difference between experimental and control group was determined by Student’s t-test. The result were considered statistically significant when \(p<0.001\).

3. RESULTS

3.1. Phytochemical Screening

In Phytochemical screening the ethanol leaves extract of M. esculenta showed the presence of reducing sugar, tannins, flavonoids, glycosides, gums and steroids.
3.3. Evaluation of Antidiarrheal Activity

3.3.1. Castor oil-induced diarrhea

The ethanol leaves extract of *M. esculenta* produced a significant (p<0.001) and dose-dependent prolongation in onset of diarrhea and reduction in frequency of defection in comparison to the control. The extract exhibited 63.63 and 78.40% inhibition of defection at the doses of 250 and 500 mg/kg, respectively, whereas the reference drug, loperamide exhibited 85.22% inhibition at the dose of 3 mg/kg.

### Table 2: Effect of ethanol leaves extract of *M. esculenta* on castor oil-induced diarrhea in

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Onset of diarrhoea (hr)</th>
<th>Number of stools after 4 hr</th>
<th>% Inhibition of defection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>32.2±3.59*</td>
<td>17.6±1.35*</td>
<td>-</td>
</tr>
<tr>
<td>Loperamide (3 mg/kg)</td>
<td>6</td>
<td>200.8±4.12*</td>
<td>2.6±0.50*</td>
<td>85.22</td>
</tr>
<tr>
<td>Extract (250 mg/kg)</td>
<td>6</td>
<td>94.8±3.70*</td>
<td>6.4±0.67*</td>
<td>63.63</td>
</tr>
<tr>
<td>Extract (500 mg/kg)</td>
<td>6</td>
<td>176.6±3.48*</td>
<td>3.8±0.58*</td>
<td>78.40</td>
</tr>
</tbody>
</table>

Result are expressed as mean ±SEM, SEM= Standard error for mean, *p<0.001 versus control, Student’s t-test.

3.3.3. Evaluation of Anthelmintic Activity

The ethanol leaves extract of *M. esculenta* was found to show anthelmintic activity which compared to standard drug albendazole. Paralysis occurred faster at higher concentrations of the extract (i.e. 5.1±0.24 minutes at 200 mg/ml) in *P. cervi*. A similar trend was observed for *H. contortus* (i.e. 9.5±0.11 minutes at 200 mg/ml). The relative index values of paralysis obtained were 3.53, 2.08, 1.13 and 0.68 for *P. cervi* and 2.01, 1.31, 0.75 and 0.54 for *H. contortus* at concentrations of 25, 50, 100 and 200 mg/ml *M. esculenta* extract, respectively, vs. albendazole (15 mg/ml). In contrast, the relative index for death in *P. cervi* was 0.92 (100 mg/ml) and in *H. contortus* was 0.84 (100 mg/ml) these suggests that the anthelmintic activity of *M. esculenta* extract at high concentration is comparable with albendazole. This result demonstrates that the *M. esculenta* extract at high concentration has a more potent wormicidal activity than albendazole against both of the parasites.

### Table 3: Effect of ethanolic leaves extract of *M. esculenta* in parasites

<table>
<thead>
<tr>
<th>Groups</th>
<th>Conc. (mg/ml)</th>
<th>Time (min) taken for paralysis (P) and death (D) * Relative index</th>
<th>Haemonchus contortus * Relative index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.10% Tween-80 in PBS</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Extract 25 mg/ml</td>
<td>25</td>
<td>26.5±0.69</td>
<td>3.53</td>
</tr>
<tr>
<td>Extract 50 mg/ml</td>
<td>50</td>
<td>15.6±0.30</td>
<td>2.08</td>
</tr>
<tr>
<td>Extract 100 mg/ml</td>
<td>100</td>
<td>8.5±0.14</td>
<td>1.13</td>
</tr>
<tr>
<td>Extract 200 mg/ml</td>
<td>200</td>
<td>5.1±0.24</td>
<td>0.68</td>
</tr>
<tr>
<td>Albendazole 15 mg/ml</td>
<td>15</td>
<td>7.5±0.18</td>
<td>1.00</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM, SEM= Standard error for mean, n= number of parasites (6), Relative index (P) denotes the time taken for paralysis to occur using *M. esculenta* extract/the time taken for paralysis to occur using the standard. Relative index (D) denotes the time taken for death to occur using *M. esculenta* extract/The time taken for death to occur using the standard.
3.3.4. Evaluation of Antibacterial by Discs Diffusion Assay

The ethanol leaves extract of *M. esculenta* showed moderate zone of inhibition against all the tested Gram-positive and Gram-negative bacterial strains. Zone of inhibition ranged between 7 to 8 and 10 to 12 mm, at the doses of 250 and 500 µg/discs.

**Table 4: Antibacterial activity of ethanol leaves extract of *M. esculenta* in disk diffusion assay**

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Type of bacteria</th>
<th>Diameter of zone of inhibition in mm</th>
<th>Blank 5 µg</th>
<th>Standard (Ciprofloxacin) 5 µg</th>
<th>Extract 250 µg</th>
<th>Extract 500 µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Staphylococcus aureus Gram(+)</td>
<td>0</td>
<td>30</td>
<td>7</td>
<td>10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Staphylococcus epidermidis Gram(+)</td>
<td>0</td>
<td>25</td>
<td>6</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacillus megaterium Gram(+)</td>
<td>0</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Escherichia coli Gram(-)</td>
<td>0</td>
<td>30</td>
<td>4</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salmonella typhi Gram(-)</td>
<td>0</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibrio cholerae Gram(-)</td>
<td>0</td>
<td>24</td>
<td>8</td>
<td>12</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

4. DISCUSSION

The preliminary Phytochemical screening showed the presence of various classes of constituents, such as reducing sugar, tannins, flavonoids, glycoside, gums and steroids might be responsible for its anti-inflammatory activity[24].

The ethanol leaves extract of *M. esculenta* was subjected to determine for its radical scavenging activity, a known mechanism by which antioxidant inhibit lipid oxidation. DPPH regularly demonstrate this radical scavenging activity. In this assay, compounds like flavonoids and phenols of the plant extract converts DPPH into stable non-reactive DPPH-II form by donating electron of hydrogen radicals. It was a concentration dependent manner and greatly comparable to well established antioxidant ascorbic acid. The ethanol extract exhibited a proportional to increase of absorbance along with the concentration which proves the presence of some active compounds those are capable of reacting with free radicals and converting them to stable non reactive form[25-27].

Antidiarrheal activity of the ethanol leaves extract of *M. esculenta* was tested by using the model of castor oil induced diarrhea in mice. Castor oil induce diarrhea by releasing its active metabolite ricinoleic acid that stimulate the peristaltic movement of small intestine and secretion of intestinal content due to the release of prostaglandins. The extract causes to increase in latent period and decreased the frequency of defecation as well as the number of stool. So, that it can be concluded that extract showed the antidiarrheal activity[28,29].

Anthelmintic activity of the ethanol leaves extract of *M. esculenta* was tested on both *Paramphistomum cervi* and *Haemonchus contortus* parasites model because structural and physiological resemblance with *Ascaris lumbricoids*. The extract showed dose dependent decrease in both paralysis time and death time of parasites[20,21].

In the antibacterial activity of the ethanol leaves extract of *M. esculenta* was explored disk diffusion assay method against all the tested Gram-positive and Gram-negative bacterial strains[18]. The extract showed activity only against *Staphylococcus aureus, Staphylococcus epidermis, Escherichia coli, Vibrio cholera* among six species of bacteria. On the basis of this result it can concluded that the ethanol leaves extract of *M. esculenta* possesses moderate antibacterial activity[23,30].

4. CONCLUSION

In the present study phytochemical and pharmacological investigation was carried out with the ethanol leaves extract of *M. esculenta*. The results of pharmacological activates suggest that the *M. esculenta* leaves extract has antioxidant potentiality and possesses Antidiarrheal, Anthelmintic and mild antimicrobial activities. Phytochemical analysis showed the presence of reducing sugar, tannin, flavonoid, saponin, glycoside and alkaloid which can show extensively pharmacological activity. These preliminary studies do not describe the actual mechanism of action of these activities. Further advanced investigations are required to identify the actual mechanism of action as well as to isolate bioactive compounds responsible for each pharmacological activity.

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REFERENCES


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