



EVALUATION OF ANTIOXIDANT, ANALGESIC AND ANTIDIARRHOEAL ACTIVITY OF *FLACOURTIA JANGOMAS* (LOUR.) RAEUSCH. LEAVES

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Summary

In the present study, ethanol extract of leaves of *Flacourtia jangomas* was pharmacologically investigated to explore and evaluate the analgesic, antioxidant and antidiarrhoeal activities to provide a suitable lead, which may be utilized in future to pursue a new line of investigation. The extract exhibited a significant inhibition of DPPH radical scavenging activity in concentration dependent manner with IC₅₀ value of 11 µg/mL whereas the IC₅₀ value for the standard ascorbic acid was 5 µg/mL. Total phenol content of the extract was 601.03 mg GAE/100 g of dried plant material measured by Folin Ciocalteu's reagent. In reducing power assay, the extract revealed strong ferric reducing power in concentration dependent manner as compared with standard ascorbic acid; which was substantiated by high absorbance increased with the concentration. In acetic acid-induced writhing test, the extract (250 and 500 mg/kg) exhibited significant ($p < 0.001$) inhibition of writhing reflex in dose dependent manner; which was strongly comparable to the standard drug diclofenac sodium. Pain threshold level was appreciably raised in dose dependent manner by the extract in hot-plate test and results were statistically significant ($p < 0.001$). In *in vivo* antidiarrhoeal test, castor oil induced diarrhoeal model was utilized in which the extract (250 and 500 mg/kg) increased onset of diarrhoea and decreased the total number of stools as compared with the control in significant ($p < 0.001$) and dose dependent manner. Above results of the present study explored that the extract has some potential bioactivities which demand further investigations like LC-MS to isolate bioactive compounds and to identify underlying mechanisms.

Keywords: *Flacourtia jangomas*, 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin Ciocalteu's reagent, reducing power assay, analgesic activity, antidiarrhoeal activity

Introduction

Medicinal plants are most extensive source of indigenous medicines. From the ancient era medicinal plants are being used as indigenous medicines in the treatment of different ailments in different parts of the world. Based on the traditional uses lots of Bangladeshi plants have already been investigated for bioactivities as well as many bioactive compounds have already been isolated in different natural products laboratories.

Bioactive compounds like alkaloids, flavonoids, phenolics, tannins, glycosides, volatile oils possess a great potential for pharmacological activity and can be used in therapeutic purposes. Extensive varieties of these bioactive compounds are being isolated from medicinal plants day by day. Hence medicinal plants serve as an important source of raw materials for drug discovery.

Flacourtia jangomas (Lour.) Raeusch. (Family-Flacourtiaceae) is a lowland tree usually cultivated in Southeast and East Asia, grows to a height of 10 m. In Bangladesh, it is commonly known as Lukluki, Tokroi, Paniamala, Paniala and widely found in Sylhet, Chittagong Hill-Tracts and Cox's Bazar. It is a small tree often armed with heavy thorns and branches with simple spines. Leaves are 5-10 cm long, oblong or ovate in shape and flowers are small, varies in colors from white to green, 7.5-25 mm long. Fruits are 18-23 mm long and when ripe look brownish purple and taste sweet (1).

Fruits of *F. jangomas* are traditionally used in biliousness, fever and digestive disorders (1,2). Leaves and barks are used in the treatment of diarrhoea, bleeding gums, toothache, piles and weakness of limbs (3,4).

In previous study, anti-diabetic potential of leaves and stem of *F. jangomas* was evaluated in streptozotocin and alloxan-induced diabetic rats (5,6). Antimicrobial and cytotoxic properties of roots were also investigated and reported (7). Phytochemical investigation of stem and bark revealed two limonoids: limolin and jangomolide (8).

Upon literature survey and based on traditional uses leaves of *F. jangomas* were subjected to several pharmacological investigations to ascertain antioxidant, analgesic and antidiarrhoeal activities and thereby to substantiate the claim about the plant to be used in folklore medicine.

Materials and Methods

Plant Materials Collection

The leaves of *Flacourtia jangomas* were collected from Sylhet, Bangladesh in November' 2011 and identified by the experts at Bangladesh National Herbarium, Mirpur, Dhaka (Accession no: DCAB-37520). A voucher specimen was also deposited there for future reference and further study.

Drying and Grinding

Leaves were separated from all sorts of unwanted materials and undergone shade drying. A suitable commercial grinder was used to crush the leaves into coarse powder. Then it was stored in air tight container to avoid fungal growth until the extraction was started.

Extraction

For extraction, cold extraction method was utilized in which 100 g of grinded powder was soaked in 500 mL of ethanol in a glass container for seven days accompanying regular shaking and stirring. Plant debris was removed from the extract by filtration using clean, white cotton plug and it was done twice to get clear solution. Rotary evaporator was used to evaporate ethanol from the extract. After drying, deep purple gummy crude extract was obtained. The yield was 7.35% of dried plant material. Extract was stored in refrigerator at 4 °C until experiment was conducted.

Test Animals

For the present study, Swiss-Albino mice of either

sex (3-4 weeks of age and 20-25 g of weight) were collected from International Centre for Diarrheal Disease and Research, Bangladesh (ICDDR, B). The mice were kept in well ventilated cages in standard laboratory environment at the temperature of 25 ± 0.5 °C and relative humidity of 56-60% and supplied with formulated rodent pellet food and water *ad libitum*. The present investigations in mice were performed according to the guidelines provided by Institutional Animal Ethics Committee (9) and ethical guidelines of ICDDR, B.

Chemicals, Reagents and Drugs

Ascorbic acid, gallic acid, potassium ferricyanide, trichloroacetic acid, sodium carbonate, ferric chloride and acetic acid were purchased from Merck, Germany. Folin-Ciocalteu's reagent and free radical 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich, USA. Tween-80 and castor oil were purchased from Loba Chemie Pvt Ltd, India. Solvents and all other chemicals were of analytical grade. Standard drug diclofenac sodium and loperamide were obtained from Beximco Pharmaceuticals Ltd, Bangladesh and morphine was obtained from Popular Pharmaceuticals Ltd, Bangladesh.

In Vitro Antioxidant Activity Test

Antioxidant activity of the extract was determined using stable free radical DPPH (1,1-Diphenyl-2-picrylhydrazyl) both qualitatively and quantitatively (10, 11).

Qualitative Analysis

TLC plates were developed with solvent systems of different polarities to resolve non-polar, medium polar and polar compounds. The plates were sprayed with 0.02% DPPH in ethanol. Bleaching of DPPH (yellow on purple background) by the resolved bands was observed for 30 min and was noted.

Quantitative Analysis

For quantitative assessment using the stable free radical DPPH, stock solution of the leaves extract was prepared in ethanol at the concentration of 1024 µg/mL. Serial dilution of the sample was carried out with the ethanol to acquire the desired final concentrations of 512, 256, 128, 64, 32, 16, 8, 4, 2, 1 µg/mL. Then 3 mL of 0.004% DPPH solution was added to 1 mL of each diluted sample and incubated for 30 minutes at room temperature to complete the reaction before taking absorbance at 517 nm. Ascorbic acid was used as standard antioxidant. Activity of the extract was calculated using the formula: percent inhibition = $(1 - A_s/A_c) \times 100$, where A_c is the absorbance of control and A_s is the absorbance of standard or sample. IC_{50} value was determined from the graph (% inhibition vs concentration).

Total Phenolic Content Determination

Total phenol content of leaves of *F. jangomas* was determined by Folin Ciocalteu's reagent (12). Ethanol leaves extract of 0.5 g was dissolved in 50 mL of 80% aqueous methanol and sonicated for 20 min. Then sonicated 2 mL mixture was centrifuged for 15 min at 14000 rpm to collect 1 mL supernatant. Gallic acid was used as standard and result was expressed in terms of gallic acid equivalent (GAE). Different concentrations (15.62, 31.25, 62.5, 125, 250 and 500 mg/L) of gallic acid were prepared in methanol with serial dilution.

Extract and gallic acid at all concentrations of 1 mL was taken in 25 mL of volumetric flask followed by addition of 9 mL of distilled water and 1 mL of diluted (1 mL reagent in 9 mL distilled water) Folin-Ciocalteu's reagent. After 5 minutes, 10 mL of 7% Na_2CO_3 was added and adjusted to 25 mL with distilled water. Before taking absorbance at 750 nm, mixture was incubated at room temperature for 30 minutes. Blank was prepared same as above except addition of standard and extract.

Reducing Power Assay

Method previously described by Oyaizu, 1986 was followed for the determination of ferric reducing power of the ethanol leaves extract (13). Various concentrations (15.62, 31.25, 62.5, 125, 250 and 500 µg/mL) of ethanol extract (1 mL) were mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1% potassium ferricyanide (K_3FeCN_6) and then incubated at 50 °C for 20 min. After addition of 2.5 mL of trichloroacetic acid (10%), mixture was centrifuged at 3000 rpm for 10 min. Then 2.5 mL from the supernatant was mixed with 2.5 mL of distilled water and 0.50 mL of $FeCl_3$ (0.1%, w/v) with shaking. Five minutes later, absorbance was measured at 700 nm against blank. Blank was prepared without addition of sample and standard. Ascorbic acid was used as standard and results were compared with it.

Screening of Analgesic Activity

Analgesic activity of the ethanol leaves extract of *F. jangomas* was screened by two widely used models: acetic acid induced writhing and hot-plate test.

Acetic Acid Induced Writhing Test

Randomly selected experimental animals of either sex were divided into four groups symbolized as group-I, group-II, group-III and group-IV consisting of 5 mice in each group and received a particular treatment i.e. control, positive control and the two doses of the ethanol leaves extract.

Extract (250 and 500 mg/kg), standard drug diclofenac sodium (25 mg/kg) and control as 1% tween-80 in distilled water (10 mL/kg) were administered orally through feeding needle followed by a thirty minutes interval to ensure proper absorption of the administered treatments. Then 0.7% acetic acid solution was administered intraperitoneally to each animal as writhing inducing agent. After an interval of 5 min, number of squirms (writhing) was counted for 10 min (14,15). Percent inhibition of

writhing was compared with control and standard to screen analgesic activity.

Hot-Plate Test

Experimental animals were screened based on their response at 3-5 sec when placed in hot-plate maintained at the temperature of $55 \pm 0.5^\circ C$. Screened mice were divided into four groups consisting of 5 mice in each group and received a particular treatment.

Animals of test groups were orally treated with the extract at the doses of 250 and 500 mg/kg. Positive control group was treated with morphine (5 mg/kg, i.p.) control group was treated with 1% tween-80 in distilled water (10 mL/kg, p.o.). Animals were placed on hot-plate to measure reaction time. Reaction time was recorded at before (0) and after the administration of treatments at 30, 60, 90 and 120 min when animals licked their paws or jumped from the plate (16). To avoid paw damage 15 sec was used as cut-off point in the present investigation.

Evaluation of *In Vivo* Antidiarrhoeal Activity

The method previously described by Uddin *et al.*, 2005, was followed for the present study with slight modification (17). Experimental animals were screened based on their response to castor oil induced diarrhoea and divided into four groups having five mice in each group. The ethanol leaves extract of *F. jangomas* at the doses of 250 and 500 mg/kg was administered to the test groups. Control group was supplied with 1% tween-80 in distilled water (10 mL/kg) while positive control group received standard anti-motility drug loperamide (3 mg/kg). All the treatments were administered orally with feeding needle. After 60 min, castor oil of 0.5 mL was administered to each animal. Individual animals of each group were placed in separate cages having adsorbent paper beneath and examined for the presence of diarrhoea in every hour during the observation period of 4 h after the castor

oil administration. Number of stools was counted at each successive hour throughout the whole observation period for each animal. The onset of diarrhoea was also counted for each animal and compared with the control.

Statistical Analysis

All experimental results were expressed as mean \pm SEM ($n = 5$) and statistical analysis was performed by Student's t-test with Prism 5.0 (GraphPad software Inc., San Diego, CA). Results of the present investigations were considered as statistically significant when $P < 0.05$.

Results

DPPH Scavenging Activity

The extract of *F. jangomas* leaves revealed IC_{50} value of 11 μ g/mL while standard ascorbic acid showed IC_{50} value of 5 μ g/mL. DPPH radical scavenging activity of both extract and standard ascorbic acid was increased linearly with the increase of concentration at lower concentration level and saturation was occurred at higher concentration level (Figure 1). From the graph it was clear that, scavenging activity of the extract was strongly comparable to the standard antioxidant ascorbic acid at all concentrations and the activity was in concentration dependent manner.

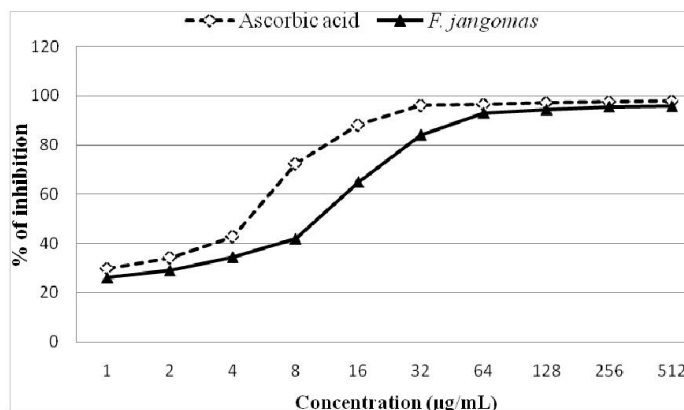


Figure 1: DPPH scavenging activity of *F. jangomas* leaves

Phenolic Content Determination

The trend line equation of standard calibration curve of gallic acid was $y = 0.097x + 0.007$ ($R^2 = 0.990$) in which y is the absorbance and x is the phenolic content expressed as gallic acid equivalent (GAE). The total phenolic content of *F. jangomas* leaves extract was found to be 601.03 mg GAE/100 g of dried plant material (Figure 2).

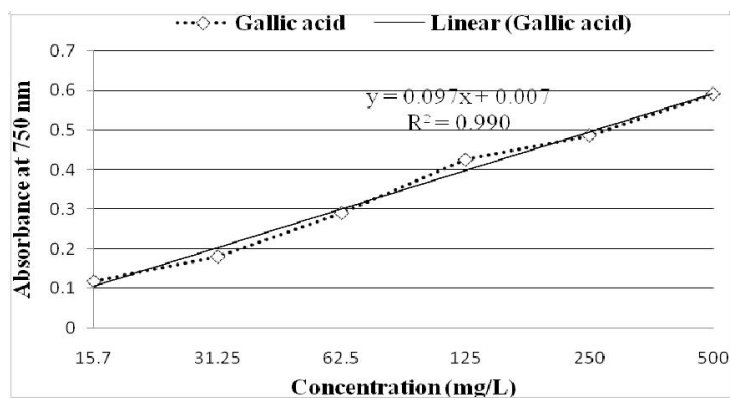


Figure 2: Standard calibration curve for Gallic acid

Reducing Power Assay

Reducing power of the ethanol leaves extract of *F. jangomas* was increased with the concentration of sample characterized by the increase of absorbance in concentration dependent manner. Ferric ion reducing ability of the extract was strongly comparable with the standard ascorbic acid (Figure 3). The extract showed absorbance of 0.517, 0.597, 0.723, 0.923, 1.346 and 1.592 at the concentrations of 15.62, 31.25, 62.5, 125, 250 and 500 μ g/mL, respectively while standard ascorbic acid showed absorbance of 0.712, 0.883, 1.136, 1.541, 2.421 and 3.165 at the same concentrations, respectively.

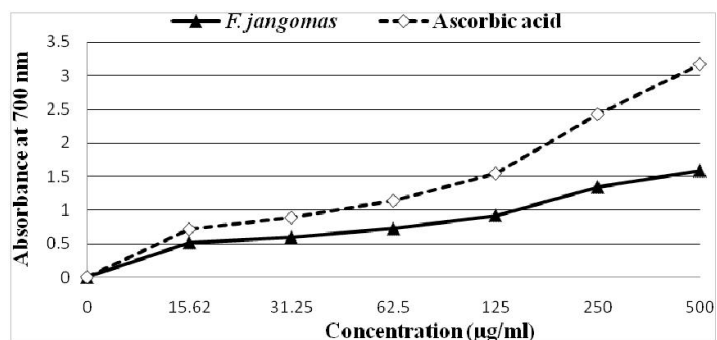


Figure 3: Reducing power of *F. jangomas* leaves

Acetic Acid-Induced Writhing Test

In acetic acid induced writhing test, the ethanol leaves extract of *F. jangomas* showed significant ($P < 0.001$) inhibition of writhing in dose dependent manner as compared with control. The extract exhibited 45.45 and 67.05% inhibition of writhing at the doses of 250 and 500 mg/kg, respectively while standard diclofenac sodium exhibited 76.14% inhibition of writhing in experimental mice (Table 1).

see Table 1.

Hot-Plate Test

The leaves extract of *F. jangomas* (250 and 500 mg/kg) and standard morphine (5 mg/kg) significantly ($P < 0.001$) increased pain threshold level as compared with control in dose dependent manner. Reaction time was increased from 30 min and persisted throughout the observation period of 120 min with gradual decrease of activity with the passage of time. The extract showed maximum reaction time at 60 min at both doses and standard morphine showed at 90 min.

see Table 2.

In Vivo Antidiarrhoeal Test

The leaves extract of *F. jangomas* produced statistically significant ($P < 0.001$) increase in onset of diarrhoea and reduction in frequency of defecation as compared with control in dose dependent manner. The extract exhibited 74.05 and 85.50% inhibition of defecation at the doses of 250 and 500 mg/kg, respectively while standard loperamide showed 88.00% inhibition of defecation at the dose of 3 mg/kg. Table 3 clearly showed that the extract produced strongly comparable antidiarrhoeal activity which was also substantiated by the prolongation of latent period as compared with control and standard.

see Table 3.

Discussion

Free radicals are harmful as they can participate in superfluous side reactions due to their chain reaction properties which resulting in cell damage, blemishing of food, degradation of various materials like rubber, gasoline etc. Life threatening diseases for example cancer, stroke, diabetes may occur as a result of the consequences of free radicals. Antioxidants are that molecules which can halt these chain reactions by removing free radical intermediates.

Phenolic compounds are responsible for antioxidant properties due to their ability to donate electron resulting the conversion of highly reactive free radicals to nonreactive stable molecules. Phenolic compounds may be of three types- non flavonoids like hydroxybenzoic acid, flavonoids like flavones, flavonols, flavanones etc and type three is tannins. The ethanol extract of *F. jangomas* showed strong antioxidant activity in DPPH free radical scavenging assay and it may be due to the presence of flavonoids (18). It has been established through various *in vitro* studies that flavonoids have the ability to scavenge superoxide, hydroxyl and peroxyl radicals; thereby affecting various steps in the arachidonate cascade via lipoyxygenase cyclooxygenase-2 (19).

Like flavonoids, tannins carry a high degree of free radical scavenging property which may also be the prime reason behind antioxidant activity (20). That is why total phenolic content determination was important for making correlation between antioxidant property and amount of phenolics. From the present study we obtained a notable amount of phenolics which eventually supports its strong antioxidant activity. Reducing power assay is another established method for antioxidant activity assessment (21). Ethanol extract exhibited a proportional 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 as well as terminating chain reactions.

Acetic acid induced abdominal contraction so called writhing was utilized to assess peripherally acting analgesic activity in mice. Intraperitoneal administration of acetic acid (0.7%) causes localized inflammation through the release of endogenous pain mediators. Such pain stimulus causes the release of free arachidonic acid (AA) from tissue phospholipid by the action of phospholipase A₂ and other acyl hydrolases. All the eicosanoids for example prostaglandins, thromboxanes and prostacyclines are synthesized via the cyclooxygenase pathway from arachidonic acid (22). The released prostaglandins, mainly prostacyclines (PGI₂) and prostaglandin-E have been reported to be responsible for pain sensation by exciting the Ad-fibres. Activity in the Ad-fibres causes sensation of sharp localized pain (23). Any agent that lowers the number of writhing will demonstrate analgesia by inhibition of prostaglandin synthesis, a peripheral mechanism of pain inhibition. Ethanol extract of *F. jangomas* considerably lowered the number of writhing in a dose dependant manner which was strongly comparable to the standard drug diclofenac sodium.

Phenolic acids and flavonoids like rutin, quercetin and luteolin are responsible for significant analgesic activity (24,25). Presence of high amount of phenolic content in the extract also rationales its strong analgesic activity.

Centrally acting analgesic activity of the leaves of *F. jangomas* was investigated in mice by the most widely used hot-plate test. Drugs having central analgesia acts in spinal cord level to inhibit transmission of pain sensation. Opioid analgesics like morphine acts in spinal cord level by binding with μ , δ , and κ receptors, present in pre and post synaptic membrane, was used in the present investigation as standard drug. It is well established that non-steroidal drugs do not increase pain threshold level while steroidal drugs like narcotics and local anesthetics are of strong potential analgesia (26). The extract showed significant increase in pain threshold which substantiated centrally acting analgesia in dose dependent manner and results were strongly comparable to standard morphine.

Diarrhoea is considered as the passage of watery stools or unformed bowel contents three or more times in a day (27). Castor oil liberates ricinoleic acid from the corresponding triglyceride that causes irritation and inflammation of intestinal mucosa which in turn triggers prostaglandins release resulting the stimulation of gastrointestinal motility to cause secretion (28). Most of the commonly applied antidiarrhoeal therapies act through reduction in propulsion and peristalsis, increase in mucosal absorption or reduction in secretion via antisecretory mechanism (29,30). Antidiarrhoeal activity of ethanol extract was found highly significant in comparison to standard drug loperamide which may be due to its anti-secretory effect.

Conclusion

From these investigations, it may be concluded that the ethanol leaves extract of *F. jangomas* showed potential antioxidant, analgesic and anti-diarrhoeal activities in different established models. These results also justify the use of leaves in traditional medicines. It is important to isolate and characterize the active compounds responsible for these activities through LC-MS and other advanced techniques.

Acknowledgement

We are especially gratified to the authority of International Centre for Diarrheal Disease and Research, Bangladesh (ICDDR, B) for providing experimental mice. We are also grateful to the authorities of Pharmacy Discipline, Khulna University for providing laboratory facilities to carry out the present pharmacological studies.

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Table 1: Effect of *F. jangomas* leaves on acetic acid induced writhing in mice

Treatment <i>n</i> = 5	Dose (mg/kg)	No. of writhes	% inhibition
Control	---	17.6±0.927	---
Diclofenac sodium	25	4.2±0.581*	76.14
Extract 250	250	9.6±0.678*	45.45
Extract 500	500	5.8±1.623*	67.05

Values are mean ± SEM, SEM= Standard error for mean. *P < 0.001 in comparison to control (Student's t-test).

Table 2: Effect of *F. jangomas* leaves in hot-plate test in mice

Treatment <i>n</i> = 5 Dose (mg/kg)	Reaction time (sec)				
	0 min	30 min	60 min	90 min	120 min
Control ---	3.14±0.05	3.20±0.04	3.26±0.05	3.22±0.06	3.31±0.08
Morphine 5	3.54±0.09*	6.36±0.11*	7.48±0.12*	9.44±0.11*	8.02±0.13*
Extract 250	3.41±0.05*	4.33±0.07*	5.82±0.09*	4.95±0.08*	3.99±0.10*
Extract 500	3.21±0.04*	5.21±0.09*	6.31±0.07*	6.87±0.13*	5.05±0.11*

Values are mean ± SEM, SEM= Standard error for mean. *P < 0.001 in comparison to control (Student's t-test).

Table 3: Effect of *F. jangomas* leaves on castor oil induced diarrhea in mice

Treatment <i>n</i> = 5	Dose (mg/kg)	Onset of diarrhea (min)	No. of stools after 4 hours	% Inhibition of defecation
Control	---	34±3.15*	23±1.58*	---
Loperamide	3	198.6±2.34*	2.4±0.51*	89.56
Extract 250	250	101.4±3.39*	7.8±0.37*	66.56
Extract 500	500	179.4±4.83*	3.8±0.49*	83.39

Values are mean ± SEM, SEM= Standard error for mean. *P < 0.001 in comparison to control (Student's t-test).