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Original Research Article

Assessment of analgesic and neuropharmacological activity of leaves of *Bixa orellana* (Family: Bixaceae)

Nahida Aktary[1], Sharmin Sultana[2]*, M. Lokman Hossain[1,2]

[1]Department of Pharmacy, Stamford University Bangladesh, Dhaka, Bangladesh
[2]Pharmacy Discipline, Khulna University, Khulna, Bangladesh

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***Correspondence:**
Sharmin Sultana,
E-mail: ssku3015@gmail.com

ABSTRACT

Background: *Bixa orellana* Linn, (Family: Bixaceae) commonly known as "Lipstick tree" have been extensively used traditional medicine in India and others part of the world to cure laxative, cardiotonic, hypotensive, expectorant, antibiotic, antipyretic, aphrodisaic etc. It has been found that *B. orellana* contains different phytochemical groups such as phenolic compounds, glycosides, tannins, steroids, alkaloids, saponins. The project work has been designed to investigate the phytochemical nature (group determination of plant constituents), analgesic and neuropharmacological activity of *B. orellana* leaves extract.
Methods: Analgesic activity of *B. orellana* was determined by acetic acid induced writhing, hot plate, tail immersion and paw tickling test/formalin induced nociceptor test. Open field, hole cross, tail suspension and light/dark box test were employed for the assessment of neuropharmacological activity of *B. orellana*.
Results: Phytochemical screening revealed the presence of reducing sugar, alkaloids, glycosides, gum, terpenoids, tannins, steroids and flavonoids. In analgesic activity test, the sample showed significant analgesic activity. The dose-dependent neuropharmacological activity is shown in neuropharmacological activity test.
Conclusions: Our exploration suggests that *B. orellana* contains bioactive compounds and it should be studied further for isolation and purification of such novel compounds.

Keywords: *Bixa orellana*, Bixaceae, Analgesic activity, Neuropharmacological activity

INTRODUCTION

Bixa orellana is a perennial, tall shrub that can reach 6-10 m (20-33 ft) high. It bears clusters of 5 cm (2 inch) bright white or pink flowers, resembling single wild roses that appear at the tips of the branches. The fruits of the *B. orellana* are globular, ovoid capsules arranged in clusters resembling spiky looking red-brown seed pods covered in soft spines. Each capsule, or pod, contains 30-45 cone shaped seeds covered in a thin waxy blood-red. When fully mature, the pod dries, hardens, and splits open, exposing the seeds. The shrub is most well known as the source of the red-orange, annatto pigment.[1] The pigment is derived from the pericarp (the waxy aril layer that covers the seeds) of the *B. orellana* fruit, the red-orange annatto dye is rich in the carotenoid pigment, 80% which consists of bixin (the red pigment) and norbixin or orelline (the yellow pigment). The main commercial producers of *B. orellana* are countries in Latin America (specifically Peru, Brazil and Mexico) which constitute 60% of total world production followed by Africa (27% of total world production) and Asia (12% of total world production).[2] Production statistics are not usually available, and would not provide a reliable guide to international trade since many of the producing countries use significant quantities domestically (e.g., Brazil is a large producer and consumer, needing additional imports). Traditionally, it is widely used in many

different countries for the treatment of a wide variety of health ailments such as kidney discomfort, diabetes mellitus, constipation, and hypertension, antibiotics, laxative, cardiotonic, anti-inflammatory, antipyretics, analgesics. It seems to possess high therapeutic potential for the treatment of various diseases making it a target for pharmacological studies aiming to validate and provide scientific evidence for the traditional claims of its efficacy.[3-6] As part of our ongoing research of traditional medicinal plant, this study evaluated the analgesic and neuropharmacological activity of methanol extract of *B. orellana* (MEBO) leaves.

METHODS

Collection of plant materials

The leaves of *B. orellana* were collected from Komolgonj, Moulobibazar, Sylhet, Bangladesh in May 25, 2016 at the daytime. Later on, the plant was identified and verified by the senior scientific officer of Bangladesh National herbarium, Mirpur, Dhaka and the given accession code was 43217 DACB.

Preparation of plant extract

About 300 gm of the powdered material was taken in a clean and flat-bottomed glass beaker and soaked in 2500 mL methanol (95%) (Merck, Germany) at 25±2 °C for 15 days associated regular shaking and stirring. The solvent mixture was filtrated by a piece of sterile and white cotton material and finally using Whatman No. 1 filter paper. The solvent was totally removed by air drying and obtained 3.5 gm extract. The obtained extract was used for the phytochemical screening as well as pharmacological studies.

Collection and maintenance of animals

Swiss-Albino mice of either sex having aged 4-5 weeks, purchased from the animal breeding house of Jahangirnagar University, Savar, Dhaka, Bangladesh were used for the experiment. They were retained in standard environmental condition and fed International Center for Diarrhoeal Disease Research; Bangladesh (ICDDR, B) formulated food and water. As these animals are very subtle to environmental changes, they were reserved before the test for at least 4-5 days in the laboratory. Animals were maintained under standard conditions (temperature: (24.0±1.0 °C), relative humidity: 55- 65% and 12 hrs light/12 hrs dark cycle) with proper cleaning of husk and excreta.

Drugs and chemicals

The drugs and chemicals involved in our study were distilled water, formalin, diclofenac sodium, acetic acid and diazepam. All the chemicals and solvent were analytical grade.

Phytochemical screening

The crude methanol extract of *B. orellena* was qualitatively tested for the revealing of different phytochemical groups like alkaloids, glycosides, flavonoids, tannins, reducing sugar, carbohydrates, steroids and saponins following standard procedures.[7]

Determination analgesic activity

The study of analgesic activity of the *B. orellana* was performed in animal models for both central and peripheral mechanism of pain.

Acetic acid induced writhing test

The antinociceptive activity of the samples was studied using acetic acid-induced writhing model in mice.[8] The animals were divided into control, positive control, and two test groups with five mice in each group. The animals of test groups were given samples at the doses of 200 and 400 mg/kg body weight. Positive control group was treated with standard drug, Diclofenac sodium at the dose of 75 mg/kg body weight and control group was treated with distilled water at the dose of 10 ml/kg body weight. After administration of sample, the mice were observed for specific contraction of body referred to as 'writhing' and compared with positive control group.

Hot plate test

Hot plate test was used to measure the response latencies based on the procedure describe by Basak et al.[9] In this experiment, hot plate was maintain at 50±5 °C. The reaction time was recorded for animals pre-treated with distilled water (10 ml/kg 30 min before orally) as control, extract at the doses of 200 and 400 mg/kg body weight (30 min before), diclofenac sodium (75 mg/kg body weight intraperitoneally, 15 min before) as positive control group. Animal were placed into the hot plate chamber and the time of latency was defined as the time period between the zero point, when the animal was placed on the hot plate surface and the time when animal licked its back paw or jumped off to avoid thermal pain. The latent period of response was taken as the index of antinociception.

Tail immersion test

The procedure is based on the observation that like morphine drugs selectively prolong the reaction time of the typical tail withdrawal reflex in mice.[10] The animals of the positive control, control and test groups were treated with diclofenac Na (75 mg/kg body weight), water (10 ml/kg body weight) and test samples at the doses of 200-400 mg/kg body weight, respectively. 1-2 cm of the tail of mice was immersed in warm water kept constant at 55 °C. The reaction time was the time taken by the mice to deflect their tails. The first reading was discarded and the reaction time was recorded as a mean

of the next three readings. A latency period of 20 sec was defined as complete analgesia and the measurement was stopped when the latency period exceeded to avoid injury to mice. The latent period of the tail-flick response was taken as the index of antinociception and was determined at 0, 30, 60, 90 and 120 min.

Paw tickling test/ formalin induced nociceptor

Animals were given 20 µl of a 2.5% formalin solution (0.92% formaldehyde) made up in saline and injected intraperitoneally in the ventral surface of the right hind paw. Animals were observed from 0 to 5 min. (neurogenic phase) and 15-30 min. (inflammatory phase) and the time spent licking the injected paw was recorded with a chronometer and considered as indicative the procedure.[10] The animals received MEBO (200 and 400 mg/kg b.w. orally) 30 min before, with basis of a previous time-response curve. Control animals were treated vehicle (10 ml/kg b.w. orally).

Determination of neuropharmacological activity

The purpose of this study was to examine neuropharmacological effect of methanol extract of leaves of *B. orellana* on mice in a peripheral model of CNS activity test.

Study design

Experimental animals were randomly selected and divided into four groups denoted as group-I, group-II, group-III, group-IV consisting of 5 mice in each group. Each group received a particular treatment i.e. control, positive control and the two doses of the extract. Each mouse was weighed properly and the doses of the samples and control materials were adjusted accordingly.

Open-field test

The open field test (OFT) is clearly the most frequently used method of all behavioural tests in pharmacology and neuroscience. Despite the simplicity of the apparatus, however, open field behaviour is complex. Consequently, it has been used to study a variety of behavioural traits, including general motor function, exploratory activity and anxiety related behaviours.[11]

Hole cross test

The most consistent behavioural change is a hyperemotional response to novel environmental stimuli. The aim of this study was to characterize the emotional behaviour of mice using the hole-board test. The number of head-dips in the hole-board test in single-housed mice was significantly greater. Spontaneous movement of the animals through the hole from one chamber to the other was counted for 5 minutes. The observations are made at 0, 30, 60, 90 and 120 minutes.[11]

Tail suspension

Tail suspension test is a common behavioural paradigm used to evaluate the antidepressant activity of experimental drugs. In this test, the mice were suspended by their tails. Every mouse remains suspended for 5 mins. The behaviour of the mouse to escape this aversive situation is recorded during this time Mice, suspended by their tails; intrinsically endeavour to get away from this aversive circumstance. However, as a result of the fizzled endeavour to get away, the mice experience despair and become immobile. The extent of immobility is thought to be associated with the depressive-like condition of the mice and is significantly diminished by antidepressant treatments.[12]

Light/dark box test

The light/dark test is based on the innate aversion of rodents to brightly illuminated areas and on the spontaneous exploratory behaviour of rodents in response to mild stressors, that is, novel environment and light. The test apparatus consists of a small dark safe compartment (one third) and a large illuminated aversive compartment (two thirds). The test was developed with male mice. The strain, weight and age may be crucial factors. The extent to which an anxiolytic compound can facilitate exploratory activity depends on the baseline level in the control group. Differences between the type and severity of external stressors might account for the variable results reported by different laboratories. The light/dark test may be useful to predict anxiolytic-like or anxiogenic-like activity in mice.[13]

Ethical approval

All the experimental mice were treated following the Ethical Principles and Guidelines for Scientific Experiments on Animals (1995) postulated by the Swiss Academy of Medical Sciences and the Swiss Academy of Sciences. The Institutional Animal Ethical Committee (SUB/IAEC/17.02) of Stamford University Bangladesh allowed all experimental rules.

Statistical analysis

Results were expressed as mean±S.E.M. Variance was analyzed using One-way Analysis Of Variance (ANOVA), followed by Newman-Keul's multiple comparisons test. $P<0.05$ was considered to be statistically significant.

RESULTS

Phytochemical screening

In the preliminary phytochemical screening the extract showed the presence of reducing sugar, alkaloids, glycosides, gum, terpenoids tannins, steroids and flavonoids.

Acetic acid induced writhing test

The extract significantly inhibited writhing response induced by acetic acid in a dose-dependent manner. The result was statistically significant and was comparable to the reference drug, diclofenac sodium. MEBO showed percent of inhibition of writhing 37.16 and 66.56 at the doses of 200 and 400 mg/kg body weight, respectively which was comparable to positive control.

Table 1: Result of phytochemical group test.

Phytochemical group	Results
Reducing sugar	+
Alkaloids	+
Glycosides	+
Gums	+
Combined reducing sugar	-
Terpenoids	+
Proteins	-
Tannins	+
Saponins	-
Carbohydrates	-
Steroids	+
Flavonoids	+

+: presence, -: absence.

Table 2: Analgesic effect of *B. orellana* by acetic acid-induced writhing in mice.

Treatment	Dose	Number of writhing±SEM	% of writhing inhibition
Control	10 ml/mice	42.60±0.78	-
Positive control	25 mg/kg b.w.	12.22±0.90	74.25
Group-I	200 mg/kg b.w.	34±1.04	37.16
Group-II	400 mg/kg b.w.	22±1.05	66.56

Table 3: Analgesic effect of *B. orellana* by acetic hot plate method in mice.

Test group	Dose	0 min	30 min	60 min	90 min	120 min
Control	10 ml/mice	12	8.18	5.82	5.78	6.14
Positive control	25 mg/kg b.w.	18.43	10.17	13.49	17.13	22.05
Group-I	200 mg/kg b.w.	12.76	10.21	12.34	13.70	8.37
Group-II	400 mg/kg b.w.	15.08	14.40	13.92	12.20	11.94

Table 4: Analgesic effect of *B. orellana* by tail withdrawal reflex in mice.

Treatment	Dose	Response time (in seconds)				
		0 min	30 min	60 min	90 min	120 min
Control	10 ml/mice	6.96	3.10	13.77	8.11	7.8
Positive control	25 mg/kg b.w.	15.41	5.19	5.46	3.66	2.68
Group-I	200 mg/kg b.w.	8.5	8.93	4.87	4.87	4.4
Group-II	400 mg/kg b.w.	37.09	14.65	2.70	3.14	4.19

Hot- plate test

The analgesic effects of the methanol extract of *B. orellana* at two different doses on the experimental mice was evaluated by Hot plate method. MEBO showed dose-dependent and comparable analgesic effect as shown in Table 3.

Tail immersion test

Table 4 shows the result of analgesic activity test that was carried out by tail-flick method. Time interval for the test was 30 minutes. The tail withdrawal reflex time after administration of the *B. orellana* was comparable to standard.

Paw tickling test/formalin induced nociceptor

The analgesic effects of the methanol extract of *B. orellana* at two different doses on the experimental mice evaluated by formalin induced nociceptor. MEBO showed comparable analgesic effect as shown in Table 5.

Open-field test

The neuropharmacological activity of MEBO by open field test is shown in Table 6. The number of squares travelled by the mice was suppressed significantly by MEBO from its initial score at the doses of 200 and 400 mg/kg body weight which is comparable to the reference drug, diazepam (Table 6).

Table 5: Analgesics effect of *B. orellana* by formalin induces nociceptor in mice.

Treatment	Dose	Response time(in seconds)		
		5 min	15 min	30 min
Control	10 ml/mice	19	11	21
Positive control	25 mg/kg b.w.	16	0	0
Group-I	200 mg/kg b.w.	5.7	1	2.47
Group-II	400 mg/kg b.w.	4	0	0

Table 6: Neuropharmacological effect of *B. orellana* by open field test.

Group	Dose	Observation				
		0 min	30 min	60 min	90 min	120 min
Control	10 ml/mice	54	22	24	21	21
Positive control	1 mg/kg b.w.	105	38	57	27	35
Group-I	200 mg/kg b.w.	60	33	25	23	29
Group-II	400 mg/kg b.w.	84	34	20	28	15

Hole cross test

In hole cross test, the sample significantly decreased the number of movement of mice compared to control group at the doses of 200 and 400 mg/kg body weight (Table 7).

Tail suspension test

The sample significantly decreased the number of movement of mice compared to control group at the doses of 200 and 400 mg/kg body weight in hole cross experiment (Table 8).

Lighter box test

The sample significantly decreased the number of movement of mice compared to control group at the doses of 200 and 400 mg/kg body weight in Lighter test experiment (Table 9).

Table 7: Neuropharmacological effect of *B. orellana* by hole cross test.

Group	Dose	Observation				
		0 min	30 min	60 min	90 min	120 min
Control	10 ml/mice	9	8	11	8	5
Positive control	1 mg/kg b.w.	24	4	10	4	12
Group-I	200 mg/kg b.w.	11	7	3	3	3
Group-II	400 mg/kg b.w.	7	3	4	3	4

Table 8: Neuropharmacological effect of *B. orellana* by tail suspension test.

Group	Dose	Observation	
		Movement (min)	Silent (min)
Control	10 ml/mice	3.68	1.32
Positive control	1 mg/kg b.w.	2.53	2.45
Group-I	200 mg/kg b.w.	3.41	1.59
Group-II	400 mg/kg b.w.	2.41	2.58

Table 9: Neuropharmacological effect of *B. orellana* by lighter box test.

Group	Dose	Observation			
		Movement light (min)	Movement dark (min)	Cross light	Cross dark
Group	10 ml/mice	5.54	4.46	3	4
Positive control	1 mg/kg b.w.	2.89	2.11	7	8
Group-I	200 mg/kg b.w.	1.78	3.22	5	4
Group-II	400 mg/kg b.w.	2.2	2.8	7	8

DISCUSSION

Pain is a disturbing feeling often triggered by intense or destructive stimuli. Nociceptive pain is the most accustomed because it arises from injured tissue, like a cut or a burn. Pain perception in the brain includes two altered pain systems: one of pain perception and one of pain modulation. Treating pain is one of the most important challenges world-wide. Currently, controlling of pain is mainly based on chemical medicine including non-steroidal anti-inflammatory drugs and gluco-corticoids, which possess various side effects such as cardiotoxicity, hepatotoxicity and immunological dysfunction. Thus, natural anti-inflammatory agents with their accurate understanding of synergistic action have attracted the consideration of many researchers for treatment of enteritis, arthritis, skin inflammation and so on.[14]

Anxiety disorders are common mental diseases of the central nervous system that include heterogeneous phenomena such as panic disorder, phobias, obsessive-convulsive disorders, and posttraumatic stress disorders.[15] Dysregulation of the GABAergic, serotoninergic, dopaminergic and adrenergic neuro systems have been associated in the pathophysiology of anxiety.[16]

The acetic acid-induced abdominal constriction test is used commonly for peripherally acting drugs. The pain induction happens by releasing endogenous substances as well as some other pain mediators such as arachidonic acid via cyclooxygenase, and prostaglandin biosynthesis.[17,19] Writhing is defined as constriction of the abdominal muscles accompanied by an extension of the forelimbs and elongation of the body. It was observed that MEBO significantly ($p<0.001$) reduced the abdominal constriction induced by acetic acid in a dose dependent manner. Therefore, it could be proposed that MEBO might contain pharmacologically active molecule(s) that impede with the blockade of the effect or the release of endogenous substances (arachidonic acid metabolites) that are responsible for the excitation of pain nerve endings.

The two extensively recognized methods for understanding the centrally acting antinociceptive activity are the hot plate and tail immersion tests. The hot plate test measures the response to a brief, noxious stimulus having a close resemblance to clinical pain. This test measures the complex feedback to a non-inflammatory, acute nociceptive input and is one of the models generally used for studying central nociceptive activity. The method is considered to be selective for the drugs acting centrally. Any agent that causes a prolongation of the hot plate latency using this test must be acting centrally. The methanol extract of *B. orellana* presented a longer latency time than the control group in the hot plate test in a dose related manner. Furthermore these test are distinguished by their tendency to respond the pain stimuli considering through neurological pathway. Opioid agents exhibit analgesic affects both via supra spinal records.[19]

In formalin test, MEBO showed significant antinociceptive activity in both neurologic (early phase) and inflammatory (late phase) pain response. Formalin induced pain is steadily inhibit by tropical analgesic and anti-inflammatory drugs including morphine, diclofenac sodium considering the inhibiting property of MEBO of second phase of morphine and might me suggested an anti-inflammatory action of the plant.

A key step in evaluating drug action on CNS is to observe its effect on locomotors activity of the animal. The activity is a measure of the level of excitability of the CNS and this decrease may be closely associated to sedation causing from depression of the central nervous system. We used open field, hole cross, tail suspension and lighter box tests as the methods of assessing sedative activity. The extract significantly decreased the locomotor activity as shown by the results in a dose-dependent manner.

In open field test, MEBO showed the significant sedative action in different time (0 min, 30 min, 60 min, 90 min, 120 min). Hole cross test measures the response of the sedative action of the MEBO on mice. The method is considered to be selective for the drugs acting centrally in different time (0 min, 30 min, 60 min, 90 min, 120 min). The tail suspension test showed that the depressing action of the extract was also evident from the movement and silent in the test animals at the doses of 200 and 400 mg/kg body weight (Table 8). MEBO gave significant result of sedative activity on mice. The lighter box test showed that the depressing action of the extract of *B. orellana* from the timing of the movement and the count of movement in the lighter box experiment (Table 9).

MEBO extract showed the significant result like the standard drug diazepam. In the light of the findings of the present study, it can be showed that the plant extract of *B. orellana* possesses remarkable analgesics and sedative activity test.

The biological effects of the MEBO was found in this study might be attributed to phytochemical groups in the plant. It has been reported that flavonoids bind with high affinity to the benzodiazepine site of the GABA receptor.[20, 21] Their common bioavailability and particularly their presence in the brain *in vivo* appear to play an vital role in the expression of their effects on the CNS.[22] Terpenoids and terpenoid related compounds have confirmed their success in animal models of anxiety.[23] MEBO was highly active as an adaptogen, it normalized acute, and chronic stress induced corticosterone changes in animals.[24] MEBO augmented the 5-hydroxytryptamine levels in hippocampus, hypothalamus, and cerebral cortex. The higher doses of MEBO produced significantly better anxiolytic effects compared to lorazepam.[25]

CONCLUSION

Our experimental findings characterise that numerous phytoconstituents existing in MEBO demonstrate analgesic and anxiolytic activity. These results support that the MEBO has analgesic and anxiolytic properties like diazepam which act through binding to benzodiazepines site on GABA-BDZ receptor complex. Supplementary advance studies are required to identify and isolate the active phytoconstituents associated with observed bioactivities in animal behavioural models.

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