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RESEARCH PAPER

Analgesic Effect of Methanolic Extracts of Leaf, Bark and Fruit of *Averrhoa bilimbi* Linn

Md. Abdul Matin Sarker, A. Y. Sk Feroz Uddin Ahmed Chowdhury*

Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh

Abstract

Background: *Averrhoa bilimbi* Linn. (Oxalidiaceae) is very popular in folk remedy because the plant has high medicinal value and majority parts of the plant such as leaf, fruit and flower are used to treat fever, mumps, pimple, inflammation, rheumatism, itches, boils, bilious colic, stomach ache, aphthous ulcer, cough, cold, syphilis, hypertension, diabetes etc. However, so far no scientific work has been performed which may support its use in pain.

Objective: The present study was undertaken to evaluate possible analgesic actions of methanol extracts of *Averrhoa bilimbi* Linn. (Oxalidiaceae) leaf, bark and fruits in animal models to support its traditional use for first time.

Methods: The crude methanolic extracts of *Averrhoa bilimbi* Linn. (Oxalidiaceae) leaf, bark and fruits were investigated for the evaluation of analgesic potential in mice. Analgesic activity was assessed by using acetic acid induced writhing method, formaldehyde induced paw licking method and tail immersion method.

Results: Methanolic extract (250 and 500 mg/kg) of leaf demonstrated maximum analgesic effect in tail immersion suggesting it to be a centrally acting analgesic. On the other hand leaf extract (250 and 500 mg/kg) reduced acetic acid induced pain significantly ($p<0.05$) and bark extract (500 mg/kg) also reduced acetic acid induced pain significantly ($p<0.05$) but the effect is less than standard Diclofenac (100 mg/kg, 61.29% inhibition) ($p<0.001$). In the formalin induced paw licking time test, methanolic leaf extracts reduced nociception induced by formalin injection in both phases significantly ($p<0.05$-0.001) among which at higher dose (500 mg/kg) was most effective in later phase (41.81% inhibition) ($p<0.001$).The results demonstrate the analgesic properties of extracts.

Conclusion: The study indicates that methanolic extracts of *Averrhoa bilimbi* has significant analgesic action which support the traditional use of this plant.

Keywords: Analgesic; *Averrhoa bilimbi*; Diclofenac sodium; Methanolic extracts.

Introduction

Averrhoa bilimbi Linn. is a medicinal plant of Oxalidaceae family and commonly known as Billimbi in Bangla and the plant is native to Moluccas (Indonesia) and Malaysia, it is indigenous to many other areas in the tropics, especially Asia and American regions. *Averrhoa bilimbi* is cultivated throughout the Bangladesh but it is chiefly found in Brahmanbaria, Cumilla, Chattogram and Dhaka. The plant is small tree with small red flowers coming out on the main branches and sour oblong-angular fruits. It is basically a tree of tropical climate, lesser resistant to cold, grows excellent in wealthy and well-drained soil. *Averrhoa bilimbi* is evergreen and perennial tree; 3-10 m in height; flower started around February and blooms and fruits more or less continuing till December. Bilimbi fruit contains few (2-5) flat disc -like, about 6-8 mm wide, sleek and hazel seeds without aril.

In Philippines, paste of leaves is used when itching and on pimples, swellings of mumps and rheumatism, and on eruptions of skin; leaf is used on bites of toxic organism; in Malaysia, fresh or fermented leaf is used for therapy of Syphilis; infusion of leaves is used for treatment of cough, in rectal inflammations and also used as a tonic after childbirth; rectal inflammation is alleviated by decoction of leaves.[1-3] Fruit conserve is used in cough, beri-beri and biliousness; fruit syrup is used for cure of pyrexia and inflammation and as haemostatic of rectal bleeding and to relieve internal hemorrhoids; in Java, people eaten bilimbi fruits mixed

***Correspondence:** A.Y. Sk Feroz Uddin Ahmed Chowdhury, Professor, Department of Pharmacy, Jahangirnagar University, Savar, Dhaka-1342, Bangladesh.*
Email: ferozahmed1954@yahoo.com
ORCID: 0000-0003-4577-4205

with pepper to make sweating when they are feeling "under the weather"; in India, bilimbi fruit is utilized to control obesity; in French Guyana, decoction or syrup of bilimbi fruit is used to treat inflammation of liver, diarrhoea, pyrexia and other conditions of inflammation; flower infusions are effective against cough and thrush.[4-6]

Bilimbi is used as a folk remedy for therapy of numerous illnesses. Bilimbi fruits are ediable. Fruits are helpful in piles, scurvy, febrile excitement and haemorrhages. Oxalic acid, ascorbic acid, tannins and minerals are abundantly found in bilimbi fruits; whitlows are treated with young fruits.[7] Fruit curry is useful in piles and scurvy.[8]

Averrhoa bilimbi fruits juice is useful for washing spots of hands and dirtiness of cloth, and also tarnishes of brass due to its high amount of oxalic acid.[9] Literature survey revealed that, the different parts of *Averrhoa bilimbi* indicated the presence of alkaloids, flavonoids, steroids, saponins and tannins; and possesses antioxidant; antimicrobial; anticoagulant; anti-fertility; antimalarial; anthelmintics; cytotoxic; hepato-protective; hypoglycaemic; hypolipidemic; hypotensive; nephrotoxic; pediculicidal; thrombolytic and wound healing actions.[10-24] A literature survey of this plant did not retrieve any information regarding the analgesic activity of the bilimbi plant. This research aimed to evaluate analgesic activity of methanol extract of extracts of *Averrhoa bilimbi* leaf, bark and fruits in animal models.

Materials and Methods

Collection and Extraction of Plant material: The plant was collected from Central Medical Stores Depot (CMSD) campus, Tejgaon, Dhaka in March 2017 and identified as *Averrhoa bilimbi* Linn. by Taxonomist of the Bangladesh National Herbarium. Collected leaves, fruits and stem barks were washed and dried. The dried plant substances were pulverized and extracted with methanol (95%) by maceration. The solvent extracts were concentrated and preserved for the further work.

Experimental Animals: Swiss albino mice (25-30g) of either sex were procured from the animal research lab in the Department of Pharmacy Jahangirnagar University, Savar, Dhaka and kept in standard environment (Temp: 27.0±1.0^{o}, RH: 55-65% and 12 hrs light/12 hrs dark cycle) and fed with an ideal diet and aqua supplied *ad libitum*.

Acute Toxicity Test: The test samples were administered to experimental animals at various doses (0.1, 0.25, 0.5, 1.0, 2.0, 3.0 and 4.0 gm/kg body wt., p.o). After administration, toxicity test parameters were observed for 1 hr. Thereafter the animals were observed each 1 hr for subsequent 5-6 hr. Again the experimental animals were observed for one week.[25]

Experimental Design: The experimental animals were separated into eight categories each containing five animals. Category ordination and chemical administered with dose was as follows:

Category 1: Control (1% Tween 80, 10ml per kg body wt.)

Category 2: Standard (Model specific, mg per kg body wt.)

Category 3: ALM (250 mg per kg body wt.)

Category 4: ALM (500 mg per kg body wt.)

Category 5: ABM (250 mg per kg body wt.)

Category 6: ABM (500 mg per kg body wt.)

Category 7: AFM (250 mg per kg body wt.)

Category 8: AFM (500 mg per kg body wt.)

[Here, ALM= *Averrhoa bilimbi* leaf methanolic extract, ABM=*Averrhoa bilimbi* bark methanolic extract, AFM=*Averrhoa bilimbi* fruit methanolic extract]

Oral route is chosen for route of administration of all doses.

Formalin Induced Paw Licking Test: The method of Hunskaar and Hole was used for the study.[26] Mice were categorized and treated with chemical and dose as per experimental design and standard Diclofenac (100 mg/kg, p.o.). 0.1 ml formalin (2.7%) was injected subcutaneously into the tergal surface of the back left-foot of the mice after 1 hour of administration of drug. Injected paw licking period was recorded. Mice were watched for periods of 5min following injection of formalin (acute phase) and for 5min periods starting at 20th min later injection of formalin (delayed phase). The percentage (%) inhibition of paw licking was estimated.

Acetic Acid-Induced Writhing Test: The method of Koster *et al.*(1959) was used.[27] Mice were categorized and treated with chemical and dose as per experimental design and standard Diclofenac (100

mg/kg, p.o.). After one hour of treatment, every pretreated test animal was injected intraperitoneally with 0.7% acetic acid (10 ml/kg). The responses of writhing were calculated for every mouse for a period of 5 minutes following 15 minutes of intraperitoneal injection of acetic acid and the mean of writhing for every category was calculated. The percentage (%) inhibition of writhing was estimated.

Tail Immersion Test: The method of D'Amour and Smith was used.[28] Mice were categorized and treated with chemical and dose as per experimental design and reference drug Tramadol (100 mg/kg, p.o.). The mice were fasted for 16 hours. After treatment, the time of basal reaction was measured by dipping the lower 5 cm of the tail of mouse in hot aqua exactly 55 °C. The mice react by withdrawing the tail within a few seconds. Tail withdrawal period (in sec.) from hot aqua was calculated. Mice reaction time exceeding 10 second was removed from the test. The break off time of immersion is 15 second. The reaction time (latent period) of tail flick response was measured at 0, 30, 60, 120 and 180 minute following the treatment.

Statistical Analysis: Animal tests were completed by Independent-Sample T Test. Results were compared with control. *p* values < 0.05 and 0.001 were considered as statistically significant.

Result

Acute Toxicity: From this toxicity test no lethality or toxic sign was found until 4000 mg/kg dose of all extracts during the period of the study (observation 7 days). In addition, no weight-loss, change of pellet consumption or visceral change (macroscopic) of treated animals were detected. The result of toxicity test parameters such as general appearance, activity and movement and stimulus response (sound, touch, light) were normal; and lacrimation, salivation, piloerection, irritability, sedation, paw licking and convulsions were absent. The acute toxicity results were same for all extracts and doses of *Averrhoa bilimbi* leaf, bark and fruit. So, no LC_{50} could be obtained and extracts were considered to be safer with broad therapeutic range. Therefore, two comparatively high dose (250 and 500 mg/kg) for all extract was taken for all the *in-vivo* models.

Formalin Induced Paw Licking: The *Averrhoa bilimbi* leaf and bark methanolic extracts inhibited the licking response significantly. The methanolic extracts of *Averrhoa bilimbi* leaf (500 mg/kg, p.o.) demonstrated very significantly (*P*<0.001) decrease in the second or late phase of formalin induced paw licking, which was less than the reference drug diclofenac sodium. Paw licking inhibition activity decreased in the following order: Diclofenac sodium >ALM>ABM>AFM (Table I).

Acetic Acid Induced Writhing: The *Averrhoa bilimbi* methanolic extracts inhibited writhes in a manner which dependent on dose. The greatest percentage of writhing inhibition (44.08%) was presented by a dose of 500 mg/kg of bark extract of *Averrhoa bilimbi* Linn. Writhing inhibition activity decreased in the following order: Diclofenac sodium>ABM> ALM>AFM (Table II).

Table I: Results of various extracts of *A. Bilimbi* in formalin induced paw licking test.

Group	Dose (mg/kg)	First 5 min	Inhibition (%)	Second 5 min	Inhibition (%)
Control	-	53.40 ± 2.92	-	46.40 ± 3.03	-
Diclofenac Na	100	26.20 ± 2.57**	50.94	16.92 ± 2.61**	63.53
ALM	250	47.60 ± 2.38	10.86	33.00 ± 1.70*	28.88
	500	40.80 ± 3.93*	23.59	27.00 ± 3.03**	41.81
ABM	250	52.40 ± 3.33	1.87	40.80 ± 1.82	12.07
	500	46.80 ± 2.75	12.36	33.60 ± 2.01*	27.58
AFM	250	51.20 ±2.03	4.12	42.80 ± 2.10	7.76
	500	48.80 ± 2.15	8.61	39.00 ± 2.21	15.95

Results are expressed as mean ± SEM (n=5). *P<0.05, **P<0.001, significant compared to control.

Table II: Result of various extracts of *A. bilimbi* in acetic acid-induced writhing test.

Group	Dose (mg/kg)	Number of writhing responses	Inhibition (%)
Control	-	18.60 ± 2.25	-
Diclofenac Na	100	7.20 ± 1.39 **	61.29
ALM	250	11.80 ± 1.90 *	36.55
	500	10.60 ± 1.43 *	43.01
ABM	250	13.00 ± 1.70	30.11
	500	10.40 ± 1.07 *	44.08
AFM	250	15.40 ± 2.08	17.20
	500	14.80 ± 0.80	20.40

Results are expressed as mean ± SEM (n=5). *P<0.05, **P<0.001, significant compared to control.

Table III: Results of various extracts of *A. bilimbi* on latency time in tail immersion test.

Group	Dose (mg/kg)	Latency Time (s)				
		0 min	30 min	60 min	120 min	180 min
Control	-	2.51±0.34	2.45±0.39	3.08±0.07	3.53±0.33	3.43±0.41
Tramadol	100	2.84±0.50	4.77±0.35**	5.19±0.36**	5.87±0.30**	6.29±0.72**
ALM	250	2.59±0.31	3.19±0.21	4.03±0.13*	3.83±0.38	4.99±0.31*
	500	2.92±0.26	4.25±0.44*	4.59±0.35*	5.42±0.38**	5.69±0.25**
ABM	250	2.78±0.27	2.95±0.25	3.32±0.23	4.13±0.07	4.06±0.08
	500	2.72±0.26	3.45±0.29	4.12±0.19*	5.04±0.16*	4.64±0.13
AFM	250	2.95±0.08	3.24±0.28	3.98±0.20	3.94±0.34	4.13±0.10
	500	2.84±0.14	3.33±0.17	3.72±0.21	3.68±0.20	4.11±0.07

Results are expressed as mean ± SEM (n=5). *P<0.05, **P<0.001, significant compared to control.

Tail Immersion: The methanolic extracts of the *Averrhoa bilimbi* were also tested to estimate anodyne action by immersing tail and using Tramadol as reference standard. The methanolic extracts of *Averrhoa bilimbi* have exhibited excellent latency time in this model in dose dependent manner. The highest latency time was exhibited by 500 mg/kg dose of leaves methanolic extract of *Averrhoa bilimbi* Linn at 3 hours (5.69±0.25 s) with significant value *P*<0.001. The latency time (s) of the methanolic extract of *Averrhoa bilimbi* Linn were of following order: Tramadol>ALM> ABM>AFM (Table III).

Discussion

In acute toxicity test study, the methanolic extracts of *Averrhoa bilimbi* did not show any toxicity in mice up to 4000 mg/kg hence doses of 250 mg/kg and 500 mg/kg were selected.

The formalin model is very helpful to measure pain and it has two phases, the early phase (lasting the first 5 min,) and the late phase (lasting 20-30 min. after injection). Non-inflammatory pain (neurogenic pain) is reflected by the early phase whereas inflammatory pain reflected by the late phase.[26, 29] The *Averrhoa bilimbi* extracts reduced the licking time of first phases (analgesic phase) and second phase (inflammatory phase) (Table I). All extracts reduced the pain in a concentration dependent manner. In first phases, leaf extract showed higher percentage of inhibition (23.59%) significantly (p<0.05) with a dose of (500 mg/kg p.o.). In the second phase, leaf extract at higher dose exhibited maximum inhibition (41.81%) (p<0.001). The early phase is due to direct activation of nociceptors; bradykinin releases in this phase. Opioids analgesics inhibit the first phase.[30-32] The late phase is owing to a reaction of inflammation; histamine, serotonin, PG and excitatory amino acids release in this phase.[33-34] NSAIDs and opioids analgesics inhibit the second phase. Both early and late phases are inhibited by central acting drugs whilst only late phase inhibited by peripheral acting drugs like NSAIDs.[35] The methanolic extracts of *Averrhoa bilimbi* significantly diminish the paw licking time in the late phase, so it exhibits NSAIDs like action.

Pain sensation is represented by writhing model of acetic acid-induced through initiating locally inflaming response. It is thought that mast cells of peritoneum, acid-sensing ion channels and paths of prostaglandin may mediate the response.[36-37] Current research reports show that huge PGE2 and PGF2á liberated within the first 30 min after acetic acid injection. The resultant pain is represented by constriction of muscle of the abdomen shown by an expansion of front paws and prolongation of body. The methanolic extracts of *Averrhoa bilimbi* inhibited writhes inducing by acetic acid (Table II). The *Averrhoa bilimbi* leaf and bark methanolic extracts exhibited significant result ($p<0.05$). Hence, the noted results indicate that prostaglandins may be related in the activity of the *Averrhoa bilimbi* extracts.

The analgesic action of narcotic analgesics was also estimated by tail immersion test. Table III exhibits results of different extracts of *Averrhoa bilimbi* on latency period in tail immersion. The *Averrhoa bilimbi* leaf extract showed highest efficacy and the test again justified the effectiveness as centrally acting analgesic. Both peripheral and central mechanism of pain is inhibited by opioid analgesics whereas only peripheral pain is inhibited by non-opioid analgesics or NSAIDs.[38] The *Averrhoa bilimbi* leaf extract inhibits both peripheral and central mechanism of pain, proposing that the anodyne action of the leaf methanolic extract perhaps for their interaction with opioid receptors.

Conclusion

In the present study, methanolic extracts showed significant analgesic potential demonstrating that the samples are biologically active. Analgesic capacity of the methanolic extracts of various parts of *Averrhoa bilimbi* was estimated for both centrally acting analgesic property applying tail immersion method and peripheral analgesic action applying acetic acid-induced writhing model. In addition, antinociceptive potential was tested using formalin induced licking time test. The *Averrhoa bilimbi* leaf methanolic extract (250 and 500 mg/kg) showed maximum analgesic potential in tail immersion suggesting it to be centrally acting anodyne. On the other hand, leaf extract (250 mg/kg and 500 mg/kg) and bark extract (500 mg/kg) reduced acetic acid induced pain significantly ($p<0.05$). In the formalin induced licking time test, methanolic leaf extract reduced nociception induced by formalin injection in both phases significantly ($p<0.05$-0.001) among which 500 mg/kg dose was most effective in later phase ($p<0.001$). The results of present research suggest that the methanolic extracts of *Averrhoa bilimbi* possess significant analgesic activity and *Averrhoa bilimbi* leaf, bark and fruits may be source for analgesic agent. Further studies are necessary for identification of active principle(s) and exact mechanism(s) of analgesic activity.

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