

ANTINOCICEPTIVE ACTIVITY OF METHANOLIC EXTRACT OF *ACANTHUS ILICIFOLIUS* LINN. LEAVES

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Abstract

The methanolic extract of *Acanthus ilicifolius* Linn. (Acanthaceae) leaves was assessed for its possible antinociceptive activity by using acetic acid-induced writhing, formalin and hot plate test. The oral administration of the MeOH extract at the doses of 250 and 500 mg/kg of body weight showed significant and dose dependent antinociceptive activity in all the three models used. This indicates the existence of both central and peripheral mechanism of antinociceptive action of the extract. All the experimental results suggest that methanolic extract of *A. ilicifolius* leaves contain constituents having antinociceptive properties and thus support its popular folkloric uses in the management of pain.

Key words: *Acanthus ilicifolius*, Acanthaceae, Antinociceptive activity, Acetic acid-induced writhing test, Formalin test, Hot plate test.

Acanthus ilicifolius Linn. Yapraklarının Metanollü Ekstresinin Antinosiseptif Aktivitesi

Acanthus ilicifolius Linn. (Acanthaceae) yapraklarının metanollü ekstresi olası antinosiseptif aktivite yönünden, asetik asitle indüklenen kıvranma testi, formalin ve hot plate testleri yardımıyla değerlendirilmiştir. Metanollü ekstrenin 250 ve 500 mg/kg dozlarında oral uygulanması sonucunda, kullanılan her 3 modelde de anlamlı ve doza bağımlı antinosiseptif aktivite tespit edilmiştir. Bu bulgu, ekstrenin antinosiseptif etkisinde santral ve periferik mekanizmaların varlığını göstermektedir. Deneysel verilere dayanarak, *A. ilicifolius* yapraklarının metanollü ekstresinin antinosiseptif özelliklere sahip bileşikler içerdiği ve ağrı tedavisinde halk ilacı olarak kullanımını destekleyici bulgu olduğu düşünülmektedir.

Anahtar kelimeler: *Acanthus ilicifolius*, Acanthaceae, Antinosiseptif aktivite, Asetik asitle indüklenen kıvranma testi, Formalin testi, Hot plate testi.

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INTRODUCTION

Acanthus ilicifolius Linn. (Acanthaceae), locally known as ‘Hargoza’ is a spiny shrub found in the low laying coastal areas of southern districts in Bangladesh and the vast area of mangrove forest, the Sunderbans. It is also widely distributed in India and other tropical regions of Asia. The leaves of *A. ilicifolius* are used to treat rheumatism, neuralgia, snake bite, paralysis and asthma (1). Tea brewed from the leaves relieves pain and purifies the blood (2). Alcoholic extract of *A. ilicifolius* was found to be effective against tumour progression and carcinogen induced skin papilloma formation in mice (3). Biological study of methanolic extract of *A. ilicifolius* leaves showed that it has inhibitory effect on carrageenan induced rat paw edema indicating to possess the anti-inflammatory effect of the plant (4). Aqueous leave extract of *A. ilicifolius* is beneficial in restoring haematological and hepatic histological profiles and in lengthening the survival of the animals against the proliferation of ascites tumour *in vivo* (5). Literature review revealed that the plant is rich with enormous bioactive compounds. Acancifolioside, acetoside, isoacetoside, acanthaminoside, (+)-lyoniresinol 3a-O-beta-glucopyranoside, (-)-lyoniresinol, alpha-amyrin, ilicifolioside A and ilicifolioside B have been isolated from *A. ilicifolius* (6, 7). Among these isolated compounds, acetoside, isoacetoside and (+)-lyoniresinol 3a-O-beta-glucopyranoside have been reported to have effect on growth and differentiation of osteoblasts (8). Leishmanicidal activity of 2-benzoxazolinone, isolated from the leaves of this plant has also been documented (9). From the aerial parts of *A. ilicifolius*, a new aliphatic alcohol glycoside (ilicifolioside C) and two new (Z)-4-coumaric acid glycosides, (Z)-4-coumaric acid 4-O-beta-D-glucopyranoside and (Z)-4-coumaric acid 4-O-beta-D-apiofuranosyl-(1^{''}->2['])-O-beta-D-glucopyranoside were reported (10). It is evident from the existing information that the plant is rich in biologically active compounds but the basis for the traditional uses of this plant in painful condition has not yet been scientifically reported. The present study therefore, is intended to investigate the antinociceptive activity of the methanol extract from the leaves of *A. ilicifolius*.

MATERIALS AND METHODS

Plant material collection and extraction

The leaves of *Acanthus ilicifolius* were collected from the Sundarbans (Mangrove Forest) and were identified by the experts of Bangladesh National Herbarium, Mirpur, Dhaka, Bangladesh (Herbarium number- 31382). A voucher specimen is deposited in Pharmacy Discipline, Khulna University, Khulna, Bangladesh. About 400 g of dried powdered material was taken in a clean, flat-bottomed glass container and soaked in 1000 ml of 80% MeOH for 7 days accompanying occasional shaking and stirring. The whole mixture then underwent a coarse filtration by a piece of clean, white cotton material. Then it was filtered through filter paper. The filtrate thus obtained was evaporated by using a rotary evaporator to get a viscous mass. The viscous mass was then dried to get the extract (yield: 7.5% w/w). The extract thus obtained was used for phytochemical and pharmacological screening.

Animals

The experimental animals, Swiss-albino mice of either sex (weighing 20-25 g) were purchased from the Animal Research Branch of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B). The animals were kept at animal house (Pharmacy Discipline, Khulna University) for adaptation after their purchase under standard laboratory conditions (relative humidity 55- 65%, room temperature 21.0 ± 2.0 °C and 12 h light/dark cycle) and fed with standard diets and had free access to tap water.

Preliminary phytochemical screening

The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents, such as alkaloids, flavonoids, saponins, steroids, glycosides, gums and tannins. In each test 10% (w/v) solution of the extract in methanol was taken unless otherwise mentioned in individual test (11, 12).

Test for alkaloids

Mayer's test: 2 ml solution of the extract and 0.2ml of dilute hydrochloric acid were taken in a test tube. Then 1ml of Mayer's reagent was added. Yellow color precipitate was not formed that indicated the absence of alkaloids.

Dragendroff's test: 2 ml solution of the extract and 0.2 ml of dilute Hydrochloric acid were taken in a test tube. After adding 1 ml of Dragendroff's reagent, orange brown precipitate was not formed which indicated the absence of alkaloids.

Tests for flavonoids

A few drops of concentrated hydrochloric acid were added to a small amount of an alcoholic extract of the plant material. Immediate development of a red colour was taken as an indication of the presence of flavonoids.

Test for saponins

1 ml solution of the extract was diluted to 20 ml with distilled water and shaken in a graduated cylinder for 15 minutes. 1 cm layer of foam indicated the presence of saponins.

Test for steroids

Liebermann-Burchard test: 1 ml solution of the extract was taken and then 2 ml Liebermann-Burchard reagent was added. The appearance of reddish purple color indicated the presence of steroid.

Sulphuric acid test: After taking 1 ml solution of the extract in a test tube, 1 ml sulphuric acid was added. The presence of red color indicated the existence of steroids.

Tests for glycosides

A small amount of alcoholic extract was dissolved in 1 ml of water and a few drops of aqueous sodium hydroxide solution were added. A yellow colour was taken to signify the presence of glycosides.

Test for gums

Molisch test was performed for the existence of gum in the sample. 5 ml solution of the extract was taken and then Molisch's reagent and Sulphuric acid were added. Absence of red violet ring at the junction of two liquids indicated the absence of gums.

Test for tannins

Ferric chloride test: About 0.5 g of extract was dissolved in 5 to 10 ml of distilled water and filtered. A few drops of 5% ferric chloride solution were added to the filtrate. A greenish black precipitate was formed which confirmed the presence of tannins.

Potassium dichromate test: 5ml solution of the extract was taken in a test tube. Then 1ml of 10% Potassium dichromate solution was added. A yellow precipitate was formed in the presence of tannins.

Antinociceptive Activity Tests

Acetic acid-induced writhing test

The acetic acid-induced writhing test was evaluated according to procedures reported previously (13 – 15). The experimental animals were randomly divided into control, positive control and test groups with six mice in each group. The animals of test groups received test substance at the doses of 250 and 500 mg/kg body weight, positive control group was administered diclofenac sodium at the dose of 25 mg/kg of body weight and vehicle control group was treated with 1% Tween 80 in water at the dose of 10 ml/kg body weight orally 30 min before intraperitoneal administration of 0.6% acetic acid. After an interval of five minutes, the mice were observed for specific contraction of body referred as ‘writhing’ for 30 min. The percent inhibition of writhing was measured using the formula,

$$\text{Percent inhibition of writhing} = (1 - W_t/W_c) \times 100$$

where, W_c and W_t represent the average number of writhing produced by the control and test group, respectively.

Formalin test

The test was performed according to method described previously (16 – 17) with a minor modification. Fifty microlitres of formaldehyde 2.5% (v/v in distilled water) was injected subcutaneously into a hind paw of mice, 30 min after administration of the extract (250 and 500 mg/kg, p.o.) and diclofenac sodium (25 mg/kg, p.o.) or vehicle. The time (in seconds) spent in licking, biting and scratching responses of injected paw was considered as an indicator of pain response. Responses of the first 5 min was considered as early phase (neurogenic phase) and the period of 15–30 min as the late phase (inflammatory phase). The following formula was used for the calculation of percent inhibition,

$$\text{Percent inhibition} = (1 - T_t/T_c) \times 100$$

where, T_c and T_t represent the average time (in seconds) spent for licking, biting and scratching by the control and test group, respectively.

Hot plate test

The hot plate test was performed in accordance with the method described previously (18, 19) with minor modification. In this test, the experimental animals were placed into a perspex cylinder on the heated surface. The temperature of the heated surface was maintained at 55 ± 2 °C. The antinociceptive response latency was recorded from the time between placement and licking of hind paws or jumping movements of the animals. 30 minutes before the beginning of the experiment, the test sample (250 and 500 mg/kg, p.o.) and the standard drug, morphine (5 mg/kg, i.p.) were administered. Mice were observed before and at 30, 60, 120, 180, 240 and 300 min after administration. The cut-off time was 20 s.

Statistical analysis

Student’s *t* - test was used to determine a significance difference between the control group and experimental groups. *P* values of 0.05 or less were considered statistically significant.

RESULTS

Preliminary phytochemical screening

Preliminary phytochemical screening showed the presence of various classes of constituents, such as flavonoids, saponins, tannins, glycosides and steroids. The result of preliminary phytochemical test is presented in Table 1.

Table 1. Phytochemical investigation of MeOH extract of AI.

Test	Observations
Flavonoids	+
Tannins	+
Steroids	+
Saponins	+
Alkaloids	-
Glycosides	+
Gums	-

+: Positive result, -: Negative result, AI: *A. ilicifolius*

Acetic acid-induced writhing test

In the acetic acid-induced writhing assay the extract produced 33.0% ($P < 0.01$) and 51.1% ($P < 0.001$) writhing inhibition at the doses of 250 and 500 mg/kg of body weight respectively, which was comparable to the standard drug diclofenac sodium where the inhibition was 65.0% ($P < 0.001$) at the dose of 25 mg/kg of body weight (Table 2).

Table 2. Effect of MeOH extract of *A. ilicifolius* on acetic acid-induced writhing in mice.

Animal group/Treatment	Dose (mg/kg; p.o.)	No. of writhings	Inhibition (%)
Control (vehicle; 10 ml/kg)		51.5 ± 4.1	
Diclofenac Sodium	25	18.0 ± 2.2 ^{**}	65.0
MeOH extract	250	34.5 ± 2.5 [*]	33.0
	500	25.2 ± 1.9 ^{**}	51.1

Values are expressed as mean ± S.E.M; (Number of animals, n = 6); vehicle = 1% tween-80 solution in distilled water p.o. = per oral; * $P < 0.01$; ** $P < 0.001$ vs. control (student's *t*-test)

Formalin test

In the formalin test model, administration of the extract produced significant and dose dependent inhibition in both early (37.54%, and 50.18%) and late phases (45.5% and 67.24%) licking responses, at the tested doses of 250 and 500 mg/kg respectively (Table 3). Similarly, morphine exhibited noticeable inhibition of both the early phase (88.64%) and late phase (78.26%). In contrast, the treatment of animals with diclofenac sodium showed significant inhibition (76.81%) of the late phase, but not the early phase.

Table 3. Effect of MeOH extract of *A. ilicifolius* on formalin-induced pain in mice.

Animal group/Treatment	Dose (mg/kg; i.p.)	Total time spent in licking (s)			
		0 - 5 min.	Inhibition (%)	15 – 30 min.	Inhibition (%)
Control(vehicle; 10 ml/kg)		54.6 ± 5.8		138.0 ± 5.7	
Diclofenac Sodium	25	50.1 ± 7.2	8.24	32.0 ± 7.3**	76.81
Morphine	5	6.2 ± 0.5**	88.64	30.0 ± 2.3**	78.26
MeOH extract	250	34.1 ± 2.2*	37.54	75.2 ± 4.2**	45.5
	500	17.2 ± 2.4*	50.18	45.2 ± 3.9**	67.24

Values are expressed as mean ± S.E.M; (Number of animals, n = 6); vehicle = 1% tween-80 solution in distilled water p.o. = per oral; **P*<0.01; ***P*<.001 vs. control (student's *t* -test)

Hot plate test

The result of hot plate test is summarized in Table 4. It showed significant prolongation of latency time in both doses of oral administration of the extract at 250 and 500 mg/kg body weight. This effect begun early at 30 min after administration of MeOH extract and persists until the following fifth hour that was comparable to the standard drug morphine. The value *P* < 0.05 was considered to be significant.

Table 4. Effect of MeOH extract of *A. ilicifolius* on hot plate test in mice.

Animal group/Treatment	Dose (mg/kg; i.p.)	Latency time (s)						
		0 min.	30 min.	60 min.	120 min.	180 min.	240 min.	300 min.
Control (vehicle; 10 ml/kg)		6.2 ± 1.2	7.5 ± 1.1	7.7 ± 0.9	7.4 ± 0.7	6.8 ± 1.0	6.9 ± 0.8	6.4 ± 0.5
Morphine	5	7.1 ± 1.1	14.2 ± 0.9*	15.3 ± 1.1**	16.1 ± 0.8**	15.8 ± 0.7**	15.4 ± 0.6**	15.1 ± 0.4**
MeOH extract	250	6.3 ± 1.2	9.1 ± 0.6	9.7 ± 1.0	10.1 ± 0.9 [#]	10.0 ± 0.6 [#]	9.8 ± 0.7 [#]	9.6 ± 0.6*
	500	6.1 ± 1.3	11.9 ± 0.8*	12.1 ± 0.9**	12.4 ± 0.8**	12.3 ± 0.8*	11.5 ± 0.9*	11.6 ± 1.0**

Values are expressed as mean ± S.E.M; (Number of animals, n = 6); vehicle = 1% tween-80 solution in distilled water p.o. = per oral; [#]*P* < 0.05; **P*<0.01; ***P*<.001 vs. control (student's *t* -test)

DISCUSSION

The antinociceptive activity of MeOH extract of *A. ilicifolius* was tested by using three models (acetic acid-induced, formalin and hot plate) so that both the centrally and peripherally mediated effects could be investigated. The acetic acid-induced pain involves the peripheral mechanism whereas the hot plate test involves the central mechanism (20, 21). The formalin test is believed to show the involvement of both peripheral and central mechanisms (22).

The acetic acid induced writhing response is a sensitive procedure to evaluate peripherally acting analgesics. The possible mechanism is thought to be mediated by inhibition of lipooxygenase and/or cyclooxygenase in peripheral tissues induced by acetic acid, thereby reducing PGE2 synthesis and interfering with the mechanism of transduction in primary afferent nociceptor (23, 24). Since the MeOH extract of *A. ilicifolius* significantly inhibited the acetic acid-induced writhing in mice it suggests that the analgesic effect of the extract may be peripherally mediated.

It is postulated that the formalin test may involve sensorial C-fibers in early phase and a combined process generated by peripheral inflammatory tissue and functional changes in the dorsal horn in late phase (25, 26). The drugs that show their activity through central mechanism,

such as narcotics, inhibited both phases almost equally, while peripherally acting drugs only inhibited the second phase (17). Inhibition of both phases of pain as observed in this study demonstrated that the MeOH extract of *A. ilicifolius* contain active analgesic principles acting both centrally and peripherally.

Nociceptive reaction towards thermal stimuli in hot plate test using mice is a well-validated model for the detection of opiate analgesic as well as several types of analgesic drugs from spinal origin (27, 28). Nociceptive pain inhibition was noticed highest at 120 minutes after administration of the extracts and the response time was increased from 6.1 seconds to 12.4 seconds that was comparable to the standard drug morphine where the response time was 16.1 seconds at 120 minutes of study. As the hot plate method is selective to screen centrally acting opiate analgesic drugs (29), the effect of the extract on this pain model indicates that it might be centrally acting.

Preliminary phytochemical screening showed the presence of various classes of constituents, such as flavonoids, saponins, tannins and steroids in the plant extract. So, the observed analgesic activity may be attributed to these compounds. Moreover recent studies suggested that the inflammatory tissue damage is due to the liberation of reactive oxygen species from phagocytes invading the inflammation site (30). There are also reports on the role of flavonoid, a powerful antioxidant, in analgesic activity primarily by targeting prostaglandins (31,32). Since several flavonoids and tannins isolated from medicinal plants have been discovered for their significant antinociceptive and/or anti-inflammatory activity (33,34), therefore, possible that the antinociceptive effects observed with this extract in the present study may be attributing to its flavonoids and tannins component.

CONCLUSION

The results obtained in this study indicate that the MeOH extract of *A. ilicifolius* possess considerable antinociceptive activity at the investigated doses on the experimental laboratory animal. This could provide a rationale for traditional uses of this plant in the management of pain and suggests for further investigation and isolation of biologically active constituents responsible for the activity.

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