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EVALUATION OF ANTINOCICEPTIVE, ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF ETHANOLIC EXTRACT OF *Acacia nilotica* LEAVES FROM BANGLADESH

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Abstract

The crude ethanolic extract of the leaves of *Acacia nilotica* (family: Mimosaceae) was evaluated for its possible phytochemical nature (group determination of plant constituent) and selected pharmacological activities (Antinociceptive, Antimicrobial and Cytotoxic activity) growing in Bangladesh. Phytochemical analysis of the ethanolic extract of the leaves of *Acacia nilotica* Linn. (*A. nilotica*) indicated the presence of alkaloid, steroid, reducing sugars, tanin & saponin types of compounds. The ethanolic extract of leaves of *A. nilotica* on acetic acid induced writhing model in mice. The extract produced about 10.99% ($P < 0.01$) and 14.29% ($P < 0.01$) writhing inhibition at the dose of 250 and 500 mg/kg of body weight respectively, which were comparable to the standard drug diclofenac sodium where the inhibition was about 71.43% ($P < 0.001$) at the dose of 25 mg/kg of body weight. The ethanolic extract of the leaves of *A. nilotica* (500 µg/disc) showed moderate anti-microbial activity against *Staphylococcus saprophyticus, Proteus spp., Staphylococcus aureus, Shigella flexneri, Shigella sonnei, Shigella boydii, Enterococcus faecalis, Streptococcus pyogenes, Staphylococcus epidermidis, Streptococcus agalactiae*. The extract of leaves of *A. nilotica* showed significant ($p<0.001$) toxicity to the brine shrimp nauplii. The concentrations of crude extract for 50% mortality (LC_{50}) and 90% mortality (LC_{90}) were 32µg/mL and 74.29 µg/mL respectively.

Key words: *Acacia nilotica,* Antinociceptive, Antimicrobial and Cytotoxic activity

Introduction

A. nilotica is a common, medium sized tree, locally known as 'Babul' or 'Kikar' belongings to the family Mimosaceae. *Acacia* is the most significant genus of family Leguminosae firstly described by Linnaeus in 1773. It is estimated that there are roughly 1380 species of *Acacia* worldwide[1,2]. It is known as "Bagaruwa" among the "Hausa" speaking people of northern Nigeria. The plant is a tree with yellow mimosa-like flowers and long grey pods constricted between seeds. The bark and branches are dark with fissures. The branches bear spikes about 2 cm long. The leaves are five and densely hairy with 3 - 6 pairs of pinnae consisting of 10 - 20 pairs of leaflets that are narrow with parallel margins that are rounded at the apex and with a central midrib closely crowded. The inflorescence consists of bright yellow flowers in auxillary head on stalks that are half way up. The flowering period of the plant is between November and March[3]. The powdered bark of the plant with little salt is used for treating acute diarrhea[4].

Acacia species contains secondary metabolites including amines and alkaloids, cyanogenic glycosides, cyclitols, fatty acids and seed oils, fluoroacetate, gums, nonprotein amino acids, terpenes (including essential oils, diterpenes, phytosterol and triterpene genins and saponins), hydrolyzable tannins, flavonoids and condensed tannins[5].

Babul plant is therapeutic used as anti-cancer, antitumor, antiscorbutic, astringent, anti-oxidant, natriuretic, anti-spasmodial, diuretic, intestinal pains, nerve stimulant, cold, congestion, coughs, dysentery, fever, hemorrhages, leucorrhea, ophthalmia and sclerosis[6].

The plant is considered to be antispasmodic and antidysenteric[7]. Pods and tender leaves are reported to treat diarrhoea[8]. The plant has been shown to exhibit antibacterial[9], antiinflammatory[10], antiplatelet aggregatory activity[11], cestocidal activity[12], antibacterial effects[13], spasmogenic, vasoconstrictor actions[14], antihypertensive, antispasmodic activities[15], inhibitory effect against hepatitis C virus[16] and cytotoxic activity[17].

Since there is no sufficient data currently available to substantiate antinociceptive, antimicrobial and cytotoxic activities from *Acacia nilotica*, therefore the present study was designed to provide scientific evidence for its use as a traditional folk remedy by investigating the antinociceptive, antimicrobial and cytotoxic activities that also confirm its use as pain killer and antimicrobial activity.

Materials and Methods

Collection and identification of plant materials: *A. nilotica* was collected from market of Khulna. The time of collection was December 2010. The leaves were fresh. The plant leaves were collected for identification. The Bangladesh National Herbarium Dhaka identified the plant (Herbarium Accession No-DACB-38290).

Preparation of ethanolic extract: The leaves of *A. nilotica* were freed from any of the foreign materials. Then the roots were chopped and air-dried under shed temperature followed by drying in an electric oven at 40° C. The dried plant materials were then ground into powder. About 500g of powdered material was taken in a clean, flat-bottomed glass container and soaked in 1.4 liters of 80% ethanol. The container with its contents was sealed and kept for a period of 4 days accompanying occasional shaking and stirring. The ethanolic extract was filtered by Buchner funnel and the filtrate was concentrated with rotary evaporator at bath temperature not exceeding 40°C to have gummy concentrate of extract (yield approx. 13.26%).

Animal: For the experiment, twenty swiss albino mice of either sex, weighing between 20-25 g, were collected from the animal research branch of the International Center for Diarrheal Disease and Research, Bangladesh (ICDDRB). Animals were maintained under standard environmental conditions (temperature: (24.0 ± 1.0°C), relative humidity: 55-65% and 12 h light/ dark cycle) and had free access to feed and water *ad libitum*. The animals were acclimatized to laboratory condition for one

week prior to experiments. All protocols for animal experiment were approved by the institutional animal ethical committee.

Phytochemical screening: The freshly prepared crude extract was qualitatively tested for the presence of chemical constituents, by using the following reagents and chemicals, for example, alkaloids were identified by the Dragendorff's reagent, flavonoids with the use of Mg and HCl, tannins with ferric chloride and potassium dichromate solutions, steroids with Libermann-Burchard reagent and reducing sugars with Benedict's reagent[18].

Chemical Group Tests of the Extract: Testing different chemical groups present in the extract are performed the preliminary phytochemical studies. The chemical group test that was performed along with the results obtained is as follows[19]. In each test 10% (w/v) solution of extract was taken unless otherwise mentioned in individual test.

Reagents used for the different chemical group test: The following reagents were used for the different chemical group test[18,20].

i) Mayer's reagent

1.36 gm mercuric iodide in 60 ml of water was mixed with a solution containing 5 gm of potassium iodide in 20 ml of water.

ii) Dragendroff's Reagent

1.7 gm basic bismuth nitrate and 20 gm tartaric acid were dissolved in 80 ml water. This solution was mixed with a solution containing 16 gm potassium iodide and 40 ml water.

iii) Fehling's solution A

34.64 gm copper sulphate was dissolved in a mixture of 0.50 ml of sulphuric acid and sufficient water to produce 500 ml.

iv) Fehling's solution B

176 gm of sodium potassium tartarate and 77 gm of sodium hydroxide were dissolved in sufficient water to produce 500 ml. Equal volume of above solution were mixed at the time of use.

v) Benedicts Reagent

1.73 gm cupric sulphate, 1.73 gm sodium citrate and 10 gm anhydrous sodium carbonate were dissolved in water and the volume was made up to 100 ml with water.

vi) Molisch Reagent

2.5 gm of pure á-naphthol was dissolved in 25 ml of ethanol.

vii) Libermann-Burchard Reagent

5 ml acetic anhydride was carefully mixed under cooling with 5ml concentrated sulphuric acid. This mixture was added cautiously to 50 ml absolute ethanol with cooling.

Tests performed for identifying different chemical groups

The following tests were performed for identifying different chemical groups.

Tests for tannins

i) Ferric Chloride Test

5 ml solution of the extract was taken in a test tube. Then 1 ml of 5% Ferric chloride solution was added.

ii) Potassium dichromate test

5 ml solution of the extract was taken in a test tube. Then 1 ml of 10% Potassium dichromate solution was added.

iii) Lead Acetate Test

1 ml of 10% Lead acetate solution was added to 5 ml of extract solution.

Test for Flavonoids

A few drops of concentrated hydrochloric acid were added to a small amount of extract of the

plant material.

Test for Saponins

1 ml solution of the extract was diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes.

Test for gums

5 ml solution of the extract was taken and then Molisch reagent and sulphuric acid were added.

Tests for Steroids

i) Libermann-Burchard test

1 ml solution of chloroform extract was taken and then added 2 ml Libermann-Burchard reagent.

ii) Sulphuric acid test

1 ml solution of chloroform extract was taken and then 1ml Sulphuric acid was added.

Tests for alkaloids

i) Mayer's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Mayer's reagent was added.

ii) Dragendroff's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of Dragendroff's reagent was added.

iii) Wagner's test

2 ml solution of the extract and 0.2 ml of dilute hydrochloric acid were taken in a test tube. Then 1 ml of iodine solution (Wagner's reagent) was added.

iv) Hager's test

2 ml. solution of the extract and 0.2 ml. of dilute hydrochloric acid were taken in a test tube. Then 1 ml of picric acid solution (Hager's reagent) was added.

Tests for Carbohydrates

i) Benedict's Test (Test for Reducing Sugar)

0.5 ml of aqueous extract of the plant material was taken in a test tube. 5 ml of benedict's solution was added to the test tube, boiled for 5 minutes and allowed to cool spontaneously.

ii) Fehling's Test (Standard Test for Reducing Sugar)

2 ml of aqueous extract of the plant material was added to 1ml of a mixture of equal volumes of Fehling's solutions A and B, boiled for few minutes.

iii) Combined Reducing Sugar Test

1 ml of aqueous extract of plant material was boiled with 2 ml of dilute HCl acid for 5 minutes. The mixture was cooled and neutralized with NaOH solution and performed the Fehling's test as described above.

Antinociceptive activity

Antinociceptive activity of the crude extract was tested using the model of acetic acid induced writhing in mice[21-22]. The experimental animals were randomly divided into three groups, each consisting of ten animals. Group I was treated as 'control group' which received 1% (v/v) Tween-80 in water at the dose of 10 mL/kg of body weight; group II was treated as 'positive control' and was given the standard drug diclofenac sodium at dose of 25 mg/kg of body weight; group III was test groups and was treated with the extracts at dose 500 mg/kg of body weight respectively. Each mouse was weighed properly and the dose of the test samples and control materials were adjusted accordingly. Control vehicle, standard drug and extracts were administered orally, 30 min prior to acetic acid (0.7%) injection in peritoneum. Then after an interval of 10 min, the number of writhes (squirms) was counted for 5 min.

Antimicrobial assay

The antimicrobial activity was investigated using disc diffusion assay[23-24]. Reference microorganisms from the stock were streaked onto nutrient agar plates and the inoculated plates were incubated overnight at 37°C. Using a sterile loop, small portion of the subculture was transferred into test tube containing nutrient broth and incubated (2-4 h) at 37°C until the growth reached log phase. Nutrient agar media seeded with standard inoculum suspension was poured in Petri-dishes and allowed to solidify. Measured amount of each test samples (Table-1) were dissolved in specific volume of solvent (chloroform or methanol) to obtain the desired concentrations in an aseptic condition. Sterilized metrical (BBL, Cocksville, USA) filter paper discs were taken in a blank Petri-dish under the laminar hood. Then discs were soaked with solutions of test samples and dried. Discs impregnated with extract, standard antibiotic disc (Ciprofloxacin 30 µg/disc, Oxoid Ltd, UK) and blank (solvent chloroform or methanol) discs were placed on the Petri-dishes with sterile forceps and gently pressed to ensure contact with the inoculated agar surface. Finally the inoculated plates were incubated at 37° C for 24 h and the zone of inhibition was measured in millimeters[23,25].

Cytotoxicity Test

The brine shrimps used for cytotoxicity test were obtained by hatching 5 mg of eggs of *Artemia salina* in natural seawater after incubation at about 29°C for 48h. The larvae (nauplii) were allowed another 48 h in seawater to ensure survival and maturity before use. Six doses of plant extract (20, 40, 60, 80, 120 and 140 µg/ml) in 5% DMSO and/or seawater were tested. Each extract preparation was dispensed into clean test tubes in 10 ml volumes and tested in duplicates. The concentration of DMSO in the vials was kept below 10 µl/ml. For control, same procedure was followed except test samples. After marking the test tubes properly, 10 living shrimps were added to each of the 20 vials with the help of a Pasteur pipette[26]. The test tube containing the sample and control were then incubated at 29°C for 24 h in a water bath, after which each tube was examined and the surviving nauplii counted. From this, the percentage of mortality was calculated at each concentration.

Results

Preliminary phytochemical analysis: Results of different chemical tests on the methanol crude leaves extract of *A. nilotica* showed the presence of Alkaloid, Steroid, Reducing sugars,Tanin & Saponin (Table-1).

see Table 1.

Antinociceptive activity

Table 2 showed the effect of dried leaves of *Acacia nilotica* on acetic acid-induced writhing model in mice. The extract produced about 10.99% and 14.29% writhing inhibition at the dose of 250 and 500 mg/kg of body weight respectively, which were comparable to the standard drug diclofenac sodium where the inhibition was about 71.43% at the dose of 25 mg/kg of body weight (Table 2).

see Table 2.

Antimicrobial activity

The ethanolic extract of barks of *Acacia nilotica* was showed significant activity against both Gram (+) & (-). So from this experimental study we can summarize the activity of this extract as a potent antibacterial agent. It is a preliminary investigation. Further study should be done for more scientific evidence.

see Table 3.

Cytotoxic Activity

In brine shrimp lethality bioassay, the extract showed lethality against the brine shrimp nauplii. It showed different mortality rate at different concentrations. From the plot of percent mortality versus

log concentration on the graph paper LC50 and LC90 were deduced (LC50 = 32 µg/ml; LC90 = 74.29 µg/ml) (Table 3)

see Table 4.

Discussion

Antinociceptive activity of the ethanol extract of *A. nilotica* leaves was tested by acetic acid induced writhing model in mice. Acetic acid-induced writhing model represents pain sensation by triggering localized inflammatory response. Acetic acid, which is used to induce writhing, causes algesia by liberation of endogenous substances, which in turn excite the pain nerve endings[27]. Increased levels of PGE_2 and $PGF_{2\alpha}$ in the peritoneal fluid have been reported to be responsible for pain sensation caused by intraperitoneal administration of acetic acid[28]. The extract produced significant writhing inhibition comparable to the standard drug diclofenac sodium (Table 2). The polar compounds present in the plant extract may be responsible for the obtained antinociceptive activity. Based on this result it can be concluded that the ethanol extract of *A. nilotica* might possess antinociceptive activity. Tannins are important compounds known to be potent cyclooxygenase-1 inhibitors and with anti-phlogistic activity[29].

The plant is also reported to contain saponins. There is growing interest in natural saponins caused as much by the scientific aspects extraction and structural analysis of these compounds, as by the fact of their wide spectrum of pharmacological activities; for instance, bactericidal, antiviral, cytotoxic, analgesic, anti-inflammatory, anti-cancer and antiallergic[30]. Phytochemical constituents such as tannins, flavonoids, alkaloids and several other aromatic compounds of plant that serve as defense mechanisms against predation by many microorganisms, insects and herbivores. The antibacterial activity of flavonoids is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls[31-33]. Several reports are available in support of antimicrobial activity of saponins against bacterial and fungal pathogens[34]. The alkaloids are known to have antimicrobial and antiparasitic properties. Verpoorte have reported about 300 alkaloids showing such activity[35]. Similar results on antibacterial activity were reported on related species of the genus Mahonia by Duraiswamy *et al.* (2006)[36].

The cytotoxic activity of the ethanol extract of dried leaves of *A. nilotica* was tested by using brine shrimp lethality bioassay. It is a recent development in the bioassay for the bioactive compounds. Brine shrimp lethality bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, pesticidal, antitumor[37]. The extract was found to show potent activity against the brine shrimp nauplii. Therefore the positive response obtained in this assay suggests that the extract may contain antitumor, antibacterial or pesticidal compounds.

Conclusion

In conclusion, the present study demonstrates that the ethanol extract of dried leaves of *A. nilotica* contains pharmacologically active substance(s) possessing significant antinociceptive, antimicrobial and cytotoxic activities. The present data provided a scientific support for the traditional use of this plant as painkiller. However, more detailed phytochemical analysis will be necessary to isolate and characterize the active compounds which are responsible for the antinociceptive, antimicrobial and cytotoxic activities and to understand exact mechanisms of action of these activities.

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Plant Extract	Alkaloid	Reducing Sugars	Tannins	Gums	Flavonoids	Saponin	Steroid
EE	+	+	++	+	-	++	+

Table 1: Results of different group tests of ethanolic extract of *A. nilotica* leaves.

EE: Ethanol extract of A. nilotica; +: Positive result; - : Negative result; ++: significantly positive

Group	Treatment and Dose	Number of writhes (% Writhing)	% Writhing Inhibition
Control	1% tween 80 solution 10 ml/kg, p.o.	18.20± 0.58 (100)	---
Positive Control	Diclofenac Na 50 mg/kg, p.o.	5.20 ± 0.80 ** (28.57)	**71.43**
Test Group- 1	Et. Extract of *A. nilotica* 250 mg/kg, p.o.	16.20 ± 0.37 * (89.01)	**10.99**
Test group- 2	Et. Extract of *A. nilotica* 500 mg/kg, p.o.	15.6 ± 0.51 * (85.71)	**14.29**

Table 2: Effects of the ethanolic extract *Acacia nilotica* on acetic acid induced writhing of mice (n=5).

*Values are expressed as mean±SEM (Standard Error Mean); Et.: Ethanolic; * indicates P < 0.01 and ** indicates P < 0.001; n = Number of mice; p.o.: per oral*

Serial No	Bacterial Strains	Diameter of Zone of Inhibition in mm		
		Blank	Kanamycin (30 µg/disc)	Extract of *A. nilotica* leaves (500µg/disc)
Gram Negetive (-) bacteria				
1	*Staphylococcus saprophyticus*	-	24	10
2	*Proteus spp.*	-	21	8
3	*Escherichia coli*	-	10	0
4	*Staphylococcus aureus*	-	21	9
5	*Shigella flexneri*	-	17	9
6	*Shigella sonnei*	-	14	10
7	*Shigella boydii*	-	21	12
Gram Positive (+) bacteria				
1	*Enterococcus faecalis*	-	24	9
2	*Streptococcus pyogenes*	-	-	12
3	*Staphylococcus epidermidis*	-	25	9
4	*Streptococcus agalactiae*	-	11	9

Table: 3 In vitro antibacterial activity of ethanol extract of *Acacia nilotica*

Test Sample	Conc. (µgm/ml)	Log conc.	Avg. no of alive shrimp (sample)	% mortality	LC_{50}	LC_{90}
Ethanolic extract of *A. nilotica*	5	0.698	08	25	32	74.29
	10	1.000	07	35		
	20	1.301	06	35		
	40	1.602	04	60		
	80	1.903	01	95		
	160	2.204	0	100		

Table: 4 Result of Brine Shrimp lethality bioassay of 95% ethanolic extract of *Acacia nilotica* bark.

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