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Bioactivity of *Xanthium indicum koen.* (Compositae) leaf

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Abstract

The objective of the present study was to explore the bioactivities (antidiarrheal, cytotoxic and antibacterial) of the ethanol extract of *Xanthium indicum (Compositae) leaf. Different phytochemical tests were carried out for the detection of different chemical groups present in the ethanol leaf extract of Xanthium indicum. By the preliminary phytochemical tests, reducing sugar, alkaloid, tannin, gum, glycoside and flavonoid were found. In vivo antidiarrhoeal activity was investigated on castor oil induced diarrhoea in mice. Brine shrimp lethality bioassay and disc diffusion method was carried out for the cytotoxic and antibacterial activity test respectively. The crude leaf extract showed mild antidiarrhoeal effect at the dose of 250 and 500 mg/kg body weight compared to the standard drug Loperamide (50 mg/kg). The extract revealed strong cytotoxic effect having LC50 of 20 µg/ml and LC90 of 80 µg/ml and appeared very potent in term of zone of inhibition against Staphylococcus aureus (20 mm), Streptococcus pyogenes (20 mm) and Staphylococcus epidermidis (20 mm) compared to standard antibiotic (Kanamycin 30 µg/disc) which was Staphylococcus aureus (22 mm), Streptococcus pyogenes (20 mm) and Staphylococcus epidermidis (29 mm). All these findings established the pharmacological value of the leaf of Xanthium indicum and justified the traditional use of this plant.*

Key Words: *Xanthium indicum*, Antibacterial activity, Antidiarrhoeal activity, Swiss Albino Mice, Brine shrimp, Cytotoxicity.

Introduction

Xanthium species have been reported to possess anti-inflammatory, analgesic, anti-ulcerogenic and antioxidant activity. Two species of Xanthium, *Xanthium indicum and Xanthium strumarium have been reported in South Asia.*[1] *Xanthium indicum* (Composite) (Xi) locally known as Ghagra, Banokra, Chota-Gokhru or Bichphal, is a coarse unarmed annual herb which grows as a gregarious weed in paddy field and by the canal or ditch bank of all the areas of Bangladesh. It is also found in India, Malaysia and Indonesia. The roots, leaves and fruits of the plant are used in Ayurvedic preparations. The leaves have diaphoretic, sedative and sudorific activity and useful in long standing causes of malaria. Root is bitter, tonic and useful in strumous diseases and different cancers like urinary cancer. Fruits are rich in vitamin C and have cooling and demulcent properties. It is given in smallpox and for eye aliments as ointment. Leaf, boiled in water is given in dysentery. Tender stems and petioles of the plant are used as vegetables.[2-4] The plant is reported to contain alpha and gamma tocopherols, polyphenols, glucoside, xanthostrumarin and xanthonolides as the principal constituents.[5] The leaf of *Xanthium strumarium* is also used as medicine. According to Ayurveda, *Xanthium strumarium* has cooling, laxative, fattening, anthelmintic, alexiteric, tonic, digestive and antipyretic activities. It also cures leucoderma, biliousness, epilepsy, salivation, fever and poisonous bites of insects.[2, 4] The Xanthium species yields Xanthinin which acts as a plant growth regulator. Isolation and Identification of the phytochemical properties of *Xanthium strumarium* has also been reported.[1]

In previous study, crude extract of Xi possessed significant in vitro antioxidant activity and *in vivo* analgesic and CNS depressant actions. The ethanol crude extract showed the antioxidant property by the presence of strong yellow spot on a purple background on the TLC plate and free radical scavenging activity in the DPPH assay (IC50 -141.25 µg/ml) which is comparable to standard, Ascorbic acid (IC50 -14.12 µg/ml). The extract also showed dose dependent reduction ability (Fe^{3+} to Fe^{2+} transformation) in reducing power assay. The extract inhibited the acetic acid induced writhing in mice (47.5% P<0.001 and 24.55% P<0.05) at the dose of 500 and 250 mg/kg respectively.[6] Hydromethanol extract of the leaves of Xi was investigated for possible CNS depressant actions using Hole cross and Open field test. The extract displayed dose dependent suppression of motor activity and exploratory behavior in mice.[7] Ullah et al reported the antibacterial activity and brine shrimp lethality bioassay of methanol extracts of the whole plant of Xi.[8]

However, the leaf of Xi has been used in traditional medicine men, locally known as "Kabiraj", in the treatment of diarrhoea and dysentery, cancer and tumor, gastrointestinal disorders for a long, yet there is no reported investigation of the leaves of Xi on antidiarrhoeal, cytotoxic and antibacterial activities. As a part of our ongoing investigation on medicinal plants of Bangladesh,[9-10] we investigated the boactivities (antidiarrheal, cytotoxic and antibacterial) of ethanol extract of Xi leaves for the first time to justify its traditional use in the remedy of diarrhoea, dysentery, bacteria borne diseases and cancer. Moreover, as some of these pharmacological activities of Xi were previously studied,[8] we also tried to explore the exact plant part responsible for these actions. Intending this, we evaluated only the leaves of Xi excluding stem, bark, fruits and other parts.

Materials and Methods

Plant material collection and extraction

For this study, fresh leaves of Xi was collected from the healthy plants from Khulna University campus, Bangladesh and was identified by Bangladesh National Herbarium, Mirpur, Dhaka (Accession number DACB-35522). A voucher specimen has been deposited in Pharmacy Discipline, Khulna University, Khulna-9208, Bangladesh. The collected leaves were separated from undesirable materials. They were shade dried for four week. The plants were ground into a coarse powder with the help of a suitable grinder (Capacitor start motor, Wuhu motor factory, China). The powder was stored in an airtight container and kept in a cool, dark and dry place until analysis commenced. About 150 gm of powered material was taken in a clean, flat-bottomed glass container and soaked in 900 ml of 95% ethanol.

The container with its contents was sealed and kept for a period of 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 (Ethanol extract) obtained was evaporated under ceiling fan and in a water bath until dried. After extraction a total amount of 2.50 g leaf extract of Xi was found.

Phytochemical tests

Small amount of dried ethanolic Xi leaf extract was appropriately treated to prepare sample solution and then subjected to the specific phytochemical tests using standard methods.[11-12] Fehling's and Benedict's test were followed to investigate the presence of reducing sugar. Hager's, Wagner's and Dragendroff's reagent test was performed to identify alkaloids. Salkowski's test was performed to identify steroid and terpenoid. Ferric chloride, Lead acetate and Potassium dichromate test was carried out to identify tannin. Molish's test was followed to investigate the presence of gum. Keller Killiani (cardiac glycoside) and Borntrager's test (anthraquinone glycosides) were performed to identify glycoside. Shinoda and alkaline reagent test were performed to identify flavonoid. Frothing test was followed to investigate the presence of saponin.

Chemicals and Drugs

Ethanol (≥99.5%, Merck KGaA, Darmstadt, Germany) was used as solvent for maceration of the plant material. Lead acetate, Potassium dichromate, Ferric chloride, Hydrochloric acid, Sulphuric acid, Mayer's reagent, Dragendroff's reagent, Wagner's reagent, Hager's reagent, Molish reagent, Benedict's reagent and Fehling's solutions were standard chromogenic reagents and purchased from Himedia (Mumbai, India) for preliminary phytochemical screening. Chloramphenicol was collected from Square Pharmaceuticals Ltd. Bangladesh. Loperamide was purchased from Beximco Pharmaceuticals Ltd. Bangladesh.

Kanamycin discs were collected from Oxoid Ltd. UK and all other chemicals were of analytical grade.

Animals

Young Swiss-albino mice of either sex, weighing 20-25 gm, purchased from the Animal Research Branch of the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B) were used for the test. 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 25.0 ± 2.0° C and 12 hours light-dark cycle) and fed with standard diets (ICDDR, B formulated) and had free access to tap water. This study was approved by ethics committee of Khulna University which gave it consent in absolute accordance with the international recommendation. The investigation for cytotoxic property of the ethanol extract was done on Artemia salina (Brine shrimp). The Brine shrimp eggs of Artemia cysts (Brine shrimp eggs of Artemia cysts, Ocean Star International Inc. P.O. Box 643, Snowville, UT, USA) were purchased from the M/S. Jalil Hatchery and Nursing, Debhata, Satkhira. One spoon of cyst was hatched for 48 hours in sea water. The cyst became nauplii.

Microorganisms

Sixteen species of both gram positive and gram negative bacteria were used for antibacterial test. The bacterial strains were collected from the International Centre for Diarrhoeal Disease and Research, Bangladesh (ICDDR, B).

Preparation of sea water

20 g pure NaCl and 18 g table salt were weighed accurately, dissolved in distilled water to make 1 (one) liter and then filtered off to get a clear solution.

Antidiarrhoeal activity test

Antidiarrhoeal activity of the ethanol leaf extract of Xi was tested using the model of castor oil induced diarrhoea in mice.[13-14] The mice were all screened initially by giving 0.5 ml of castor oil and only those showing diarrhoea were selected for the final experiment. The test animals were randomly chosen and divided into three groups having five mice in each.

Results

Phytochemical activity test

Different qualitative phytochemical tests were carried out for the detection of different chemical groups present in the ethanol leaf extract of Xi. The results are summarized in Table 1. It showed that the ethanol leaf extract of Xi contains reducing sugar, alkaloid, tannin, gum, glycoside and flavonoid. These chemical groups are supposed to be responsible for the biological activities of Xi.

Antidiarrhoeal activity test

Antidiarrhoeal activity of the ethanol leaf extract of Xi was tested by castor oil induced diarrhoea in mice. Diarrhoeal initiation time and the number of stools excreted by the animals in 4 hours were collected. The extract caused an increase in latent period 1.01 and 1.23 hour i.e. delayed the onset of diarrhoeal episode at dose of 250 & 500 mg/kg of body weight which was comparable to the standard drug Loperamide at the dose of 50 mg/kg body weight in which the resulted value was 1.97 hour ($P<0.001$) (Table 2). The selected concentration of the extract also showed a decrease mean of feces 5.75 and 5.25 at a dose of 250 & 500 mg/kg of body weight whereas Loperamide, standard antidiarrhoeal agent, showed 3.30 mean of feces. (Table 2)

Cytotoxic activity test

The ethanol leaf extract of Xi showed cytotoxic activity against the brine shrimp nauplii. The LC50 of the test sample and standard drug Chloramphenicol were found to 20 mg/ml (Table 4) and 18 mg/ml (Table 3) respectively and the LC90 of the test sample and standard drug Chloramphenicol were found to 80 mg/ml (Table 4) and 125 mg/ml (Table 3) respectively.

Antibacterial activity test

The antibacterial activity of the leaf extract of Xi was assessed against 16 pathogenic bacterial strains (Both gram positive and gram negative) at the dose of 500 µg/disc and the results were compared with the activity of the positive control, Kanamycin (30 µg/disc) (Table 5). Plant extract of 500 µg/disc showed antibacterial activity against *Proteus spp.* (15 mm), *Vibrio cologet* (16 mm), *Staphylococcus saprophyticus* (15 mm), *Shigella sonnei* (12 mm), *Shigella dysenteriae* (8 mm), *Staphylococcus aureus* (20 mm), *Streptococcus pyogenes* (20 mm) and *Staphylococcus epidermidis* (20 mm).

Discussion

By tradition the leaf of Xi is boiled in water is given in dysentery by the local folklore medicinal practitioners.[3] *In vivo* antidiarrhoeal activity of the ethanol leaf extract of Xi was tested by using the model of castor oil induced diarrhoea in mice.[13] Number of mechanisms have been previously proposed to explain the diarrhoeal effect of castor oil including inhibition of intestinal Na^+, K^+-ATPase activity to reduce normal fluid absorption,[23] activation of mucosal cAMP mediated active secretion,[24] stimulation of prostaglandin formation,[25] platelet activating factor and recently nitric oxide has been claimed to contribute to the diarrhoeal effect of castor oil.[26] However, castor oil induced diarrhoea when it mixes with bile and pancreatic enzymes liberate ricinolic acid from the triglycerides upon oral administration. Most of the ricinolic acid remains in the intestine and produces its absorptive or secretory effect. The ricinolic acid thus liberated readily forms of ricinoleate salts with Sodium and Potassium in the lumen of the intestine. The salt formed as such behaves like a soap or surfactant within the gut and at the mucosal surface. Generally ricinoleate salts stimulates the intestinal epithelial cells adenyl cyclase[27] or released prostaglandin [28, 13]. The extract caused and increased in latent period and decreased the frequency of defecation as well as the number of total stool count in a dose dependent manner.

Diarrhea also can be caused by a range of pathogens. Now a days, *rotavirus* is the major causative agent for infectious diarrhoea, particularly in young children, however, other viral (*adenovirus, enterovirus and norovirus*), bacterial (*Escherichia coli, Salmonella sp., Shigella sp., Campylobacter* and *Vibrio cholerae*) and parasitic (*Cryptosporidium* and *Giardia*) agents are important pathogens responsible for diarrhoea.[29, 30]

In our present study, Xi was found to increase mean latent period and decreased the frequency of defecation in a dose dependent manner. Moreover, it inhibited some responsible bacteria of *Shigella and Vibrio species.* The study validates the use of this plant in traditional medicine against diarrhoea and dysentery.

The brine shrimp lethality bioassay can be recommended as a guide for the detection of antitumor and pesticidal compounds because of its simplicity and low cost. It indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, pesticidal, antitumor etc of the compounds.[21,31-32] An approximate linear correlation was observed when logarithm of concentration versus percentage of mortality was plotted on the graph. The results tend to suggest its possible cytotoxic activity *in vivo*. Therefore, ethanol extract of Xi might possess a significant cytotoxic activity. Traditionally this plant is used against scrofulous tumors and different cancers like urinary cancer.[7, 2] Research result strongly supports this traditional use and opens the possibility to find out effective anticancer agents from this plant. [33-34]

In vitro antibacterial activity was tested by using the disc diffusion method which is widely acceptable for the preliminary screening of antibacterial activity. It is essentially a qualitative or semi qualitative test indicating the sensitivity or resistance of microorganisms to the test materials.[35, 32] The antibacterial activity was assessed against a panel of 16 pathogenic bacterial strains (both gram positive and gram negative) at the dose of 500 µg/disc and the results were compared with the activity of Kanamycin (30 µg/disc). The extract showed activity against both gram positive and gram negative bacteria. The zone of inhibition varies within the ranges of 8-20 mm. The highest zone of inhibition was found against 3 (three) gram positive bacteria; *Staphylococcus aureus*, *Streptococcus pyogenes* and *Staphylococcus epidermidis* (20 mm) at 500 µg/disc. The extract showed a mild activity against *Vibrio cologet*, *Proteus spp. and Staphylococcus saprophyticus.* The *Xanthium* species yields Xanthinin which has antibacterial activity.[1]

In India, paste made from the leaves of Xi is mixed with water and used for mouth wash to treat toothache.[36] Moreover, traditionally Xi is effective in treating small pox, herpes and bladder infections.[5] The research result supports this traditional use of this plant strongly.

Conclusion

To conclude, it can be said that the ethanol leaf extract of Xi possesses mild antidiarrhoeal activity in a dose dependent manner and has strong cytotoxic and antibacterial properties which correlates well with the traditional uses of this plant. Previously Ullah *et al. reported the antibacterial and cytotoxic activity of methanol extracts of the whole plant of Xi.*[8] *There is no research report available regarding the antidiarrhoeal, cytotoxic and antibacterial activities of the fruits, bark, seed or steam of Xi, however, they are also used. For these reasons, it can be claimed that among the other parts of Xi, leaf is mainly responsible for the above mentioned pharmacological activities. However, direct pharmacological comparison of different plant parts of Xi should be carried out to establish these results. Liquid chromatography–Mass Spectrometry (LC-MS) can be carried out to get a bigger picture of the chemical constituents present in the leaf. Screening methods applying on various cell lines or bacterial enzymes can be carried out to find the underlying mechanism for the observed biological activities.*[12] *On the basis of the present investigation, bioassay guided approach can be undertaken to isolate and identify the active components. So, further research is required on different plant parts of Xi to find out the causative compounds of these pharmacological activities.*

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Table 1: Results of Phytochemical group tests

Presence of phytochemical groups	Chemical group tests	Results
Reducing sugar	Fehling's test	+
	Benedict's test	+
Alkaloid	Hager's test	+
	Wagner's test	+
	Dragendorff's test	+
Steroid and Terpenoid	Salkowski's test	–
Tannin	Ferric chloride test	+
	Lead acetate test	+
	Potassium dichromate test	+
Gum	Molish's test	+
Glycoside	Keller Killiani test (cardiac glycoside)	+
	Borntrager's test (Anthraquinone glycosides)	+
Flavonoid	Shinoda test	+
	Alkaline reagent test	+
Saponin	Frothing test	–

+: Positive result, - : Negative result

Table 2: Effect of Xi on mean latent period and mean of feces of castor oil induced diarrhoeal episode in mice

Animal Group/ Treatment	Dose (/kg.p.o)	Latent period (Hour)	Mean number of feces
Group I - Negative control (1% tween-80)	10 ml/kg	0.98±0.15	6.15±0.15
Group II - Positive control (Loperamide)	50 mg/kg	1.97±0.13*	3.30±0.13*
Test group-I (EtOH. Extract)	250 mg/kg	1.01±0.10**	5.75±0.10**
Test group-II (EtOH. Extract)	500 mg/kg	1.23±0.18**	5.25±0.18**

Values are expressed as Mean S.E.M (n=5), *P<0.001, **P<0.005, % = Percentage, p.o. = per oral, EtOH = Ethanol

Table 3: Result of Brine Shrimp lethality bioassay of the standard drug Chloramphenicol

Test sample	Concentration (mg/ml)	Log con.	Number of alive shrimp		% Mortality	LC50 (mg/ml)	LC90 (mg/ml)
			Test-1	Test-2			
Standard (Chloramphenicol)	5	0.69	9	9	10	18	125
	10	1	8	8	20		
	20	1.3	5	4	55		
	40	1.7	4	3	65		
	80	1.9	2	1	75		
	160	2.2	0	1	95		

LC = Lethal concentration Con. = Concentration

Table 4: Result of Brine Shrimp lethality bioassay of the Xi crude extract

Test sample	Concentration (mg/ml)	Log con.	Number of alive shrimp		% Mortality	LC_{50} (mg/ml)	LC_{90} (mg/ml)
			Test-1	Test-2			
Ethanol extract of Xi	5	0.69	8	8	20	20	80
	10	1	6	6	40		
	20	1.3	5	5	50		
	40	1.7	3	3	70		
	80	1.9	1	1	90		
	160	2.2	0	0	100		

LC = Lethal concentration Con. = Concentration

Table 5: Zone of inhibition of Xi leaf extract against bacterial strains compared to Kanamycin (standard)

Bacterial Strains	Bacterial Types	Diameter of zone of inhibition in mm		
		Blank	Standard (Kanamycin) (30 µg/disc)	Plant extract (Xi) (500 µg/disc)
Salmonella typhi	Gm (-) ve	-	22	-
Salmonella paratyphi	Gm (-) ve	-	28	-
Escherichia coli	Gm (-) ve	-	14	-
Proteus spp.	Gm (-) ve	-	23	15
Vibrio cologet	Gm (-) ve	-	24	16
Shigella flexneri	Gm (-) ve	-	24	-
Staphylococcus saprophyticus	Gm (-) ve	-	28	15
Pseudomonas spp.	Gm (-) ve	-	12	-
Shigella sonnei	Gm (-) ve	-	23	12
Shigella boydii	Gm (-) ve	-	30	-
Shigella dysenteriae	Gm (+) ve	-	32	8
Staphylococcus aureus	Gm (+) ve	-	22	20
Streptococcus pyogenes	Gm (+) ve	-	20	20
Streptococcus agalectiae	Gm (+) ve	-	44	-
Enterococcus faecalis	Gm (+) ve	-	28	-
Staphylococcus epidermidis	Gm (+) ve	-	29	20

Gram (-):-Gram Negative Bacteria; Gram (+):-Gram Positive Bacteria; (-):- No inhibition