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Original Article

Studies on the Antimicrobial Activity and Brine Shrimp Toxicity of the Leaves Extract of *Ageratum Conyzoides*

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The purpose of this study is to evaluate the antimicrobial and cytotoxic properties of the leaves extracts of *Ageratum conyzoides* L. *Ageratum conyzoides* has been utilized for the treatment of various ailments. The crude methanolic extract of *Ageratum conyzoides*, n-hexane, chloroform and ethyl acetate soluble fractions of methanolic extracts were screened for their antimicrobial activity against a number of gram positive and gram negative bacteria by disk diffusion method. The crude extract and n-hexane soluble fraction of crude extract showed significant to moderate antimicrobial activity and chloroform, ethyl acetate soluble fraction of crude extract showed moderate to mild antibacterial activity against gram positive and gram negative bacteria. The zones of inhibition produced by the crude extract (methanolic extract), n-hexane, chloroform and ethyl acetate soluble fractions were found to be 14.89 mm - 19.40 mm, 14 mm - 19.40 mm, 10.66 mm - 13.50 mm and 9.50 mm - 13.26 mm respectively at a concentration of 30 mg/disc. Chloroform, n-hexane and ethyl acetate soluble fractions of methanolic extract of *Ageratum conyzoides* were screened for antitumor properties using brine shrimp lethality bioassay. A reputed cytotoxic agent vincristine sulphate was used as a positive control. From the results of the brine shrimp lethality bioassay it can be well predicted that n-hexane, chloroform and ethyl acetate soluble fractions of methanolic extract of *Ageratum conyzoides* possess cytotoxic activity on shrimp nauplii. The median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) of n-hexane, chloroform and ethyl acetate were 245.42 mg/ml, 92.61 mg/ml and 6.35 mg/ml, respectively, comparison with positive control vincristine sulphate with 0.839 mg/ml.

***Key Words*: *Ageratum conyzoides* L, antimicrobial activity, cytotoxic activity, LC_{50} .**

Introduction

Even before the discovery of modern antibiotics and other chemotherapeutic agents, traditional medicine has served as man's resource when attacked by infective agents such as bacteria and fungi[1]. *Ageratum conyzoides* is derived from the Greek word '*agera*' meaning non-aging referring to the longevity of flowers or the whole plant, commonly called goat or billy goat weed. It belongs to the family *Astreracae*, which is an annual herbaceous plant with a long history of traditional use in several countries of the world[2,3]. It is native to Central America, Caribbean, Florida (USA), Southeast Asia, South China, India, Nigeria, Australia, and South America[4,5]. Traditional communities in India, Asia, South America and Africa use this species as an antidysenteric, antilithic[6], and an antibacterial agents[7,8]. *Ageratum conyzoides* has long been known in herbal or folk medicine[7] and its gastroprotective[9] antibacterial[10] anti-inflammatory, antianalgesic, antipyretic[5] anticoccidial,[11] and anticonvulsant[12] properties have been reported.

It is an erect, annual, branched, slender, hairy and aromatic herb, which grows to approximately 1 m in height (Figure 1.1 and 1.2). The stems and leaves are covered with fine white hairs, the leaves are stalked, ovate, 4-10 cm long and 1-5 cm wide, with tip and base somewhat pointed and with round-toothed margins long. The flowers are purple to white, less than 6 mm across and arranged in close terminal inflorescences. The fruit is black and are easily dispersed while the seeds are photoblastic and often lost within 12 months[4].

Cancer is one of the most widespread diseases in humans and there is considerable scientific and commercial interest in the continuing discovery of new anticancer agents from natural product of *Ageratum conyzoides*[13,14]. Currently, over 50% of drugs used in clinical trials for anticancer activity were isolated from natural sources or are related to them[15]. A number of active compounds of *Ageratum conyzoides* have been shown to possess anticancer activity; these include flavonoids, diterpenoids, triterpenoids, and alkaloids[16].

Ageratum conyzoides is very rich in polyoxygenated flavonoids. A total of 21 polyoxygenated flavonoids have been reported. This includes 14 polymethoxylated flavones that are tricin derivatives, 32, 42, 52 -oxygenated flavones, which include ageconyflavone A, B, C, other flavonoids include 52 -methoxynobiletin, linderoflavone B, 5, 6, 7, 32, 42, 52 -hexamethoxyflavone, 5, 6, 8, 32, 42, 52 -hexamethoxyflavone, eupalestin, nobiletin, 5, 6, 7, 52 -tetramethoxy-32, 42 -

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methylenedioxyflavone, sinensetin, 5, 6, 7, 8, 32 ,52 - hexamethoxyflavone, 5, 6, 7, 8, 32 -pentamethoxy-42 - hydroxyflavone and 5, 6, 7, 8, 32 ,52 -hexamethoxy-42 - hydroxyflavone. Conyzorigun originally believed to be a phenoxy chromone was found to be identical with eupalestin[17]. The polyhydroxyflavones include scutellarein-5, 6, 7, 42 - tetrahydroxyflavone, quercetin, quercetin-3-rhamanopiranoside, kaempferol, kaempferol-3-rhamnopiranoside and kaempferol-3,7-diglucopiranoside. A novel isoflavone glycoside, 5, 7, 22 ,42 -tetrahydroxy-6,32 -di-(3,3-dimethylallyl)-isoflavone 5-O-á-L-rhamnopyranosyl-(1'!4)-á-L-rhamnopyranoside isolated from the stems[18-28]. Lycopsamine and echinatine, two isomeric pyrrolizidine alkaloids, are the only alkaloids isolated from this plant[29].

This knowledge could enable more rational exploitation of the plant both in traditional medicine and in the empirical development of new antibacterials. This work was based on scientific investigation of the widely acclaimed medicinal value of *Ageratum conyzoides* to establish its antimicrobial and cytotoxic properties.

Figure 1. *The plant Ageratum conyzoides*

Figure 2. *Leaves of Ageratum Conyzoides*

Methods and Materials

Bacterial strain

Six gram positive and gram negative bacterial strains were obtained from the stock culture of the Department of Pharmacy, Noakhali Science and Technology University, Bangladesh (Table 1). These strain were grown in nutrient broth (Oxoid, USA) for 18 hour at 37º C and maintained on agar slant at 4º C.

Plant material

The plant *Ageratum conyzoides*. was collected from the Madaripur district, Bangladesh. The collected plant parts (leaves) were washed with water, separated from undesirable materials and then air-dried under shade to protect from sunlight for one week. The dried leaves were ground into a fine powder with the help of a hammer mill and fine powder was stored in cool and dry place in an airtight container until analysis commenced.

Preparation of extract

Plant leaves extracts were made by cold extraction method. 400 gm of powered material was soaked in 1300 ml of 90% methanol. The container with its contents was sealed and kept 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 whatman filter paper (Cat. No. 1440125) and the filtrate thus obtained was concentrated by using traditional spontaneous natural vaporization method at room temperature. Then, 5 gm of methanolic extract was dissolved in 100 ml of 90% methanol. This is the mother solution, which was partitioned off successively by three solvents of different polarity such as n-hexane, chloroform and ethyl acetate.

Antibacterial screening

Disc diffusion method is used for antimicrobial screening. Nutrient agar media is used as culture medium. The antimicrobial activity of the methanolic, n-hexane, chloroform and ethyl acetate extracts of *Ageratum conyzoides* leaves samples were evaluated using agar disc-diffusion according to the Kirby Bauer[30] method against the six Gram positive and Gram negative test organisms. The final concentrations of test samples were 3 µg/µl by dissolving the test samples with specific volume of solvents (Table 2).

Sterile Whatman filter paper discs (5 mm in diameter) were taken in petridish. One drop of sample solution of 3µg/ml concentration was applied on filter paper discs with the help of micropipette in an aseptic condition. These discs were left for few minutes in an aseptic condition for complete removal of sample. Kanamycin (30 µg/disc) discs were used as positive control. Blank discs and disc containing one drop of each solvent were used as negative control.

Tested bacterial sample from the stock culture were transferred to nutrient agar slants medium. The inoculated slants were then incubated at 37º C for 18-24 hours and then the fresh culture was transferred to the test tube containing nutrient broth to make a uniform suspension of organisms. The bacterial suspension was immediately poured onto nutrient agar plate to give a uniform layer of bacteria. Excess bacterial suspension was taken out with a sterile Pasteur pipette.

Impregnated test sample, positive and negative control discs were placed aseptically on the freshly seeded solidified agar

plate using sterile forceps. The spatial arrangement of discs were such that the discs were not closer than 20 mm to the edge of the plate and far enough apart to prevent the overlapping the zone of inhibition. The plates were kept at refrigeration temperature for 3-4 hour for better absorption, during this time microorganisms will not grow but absorption of extract would take place. Finally the plates were incubated upside down at 37^0C for 12-18 hours.

Table 1. *Lists of bacteria were used for antimicrobial screening*

Gram negative	Gram positive
Escherichia coli	*Staphylococcus aureus*
Vibrio cholera	*Bacillus subtilis*
Salmonella typhi	*Bacillus cereus*

Table 2: *Preparation of test solution*

Sample	Amount (mg)	Solvent type and their volume (ml)	Final concentrations (µg/µl)
Crude(Methanol)extract	30	Methanol,10	3
n-Hexane fraction	30	n-Hexane, 10	3
Chloroform fraction	30	Chloroform, 10	3
Ethylacetate fraction	30	Ethyl acetate, 10	3

Brine shrimp lethality bioassay

Brine shrimp lethality bioassay is used for screening of general toxic properties, which also indicates a range of bioactivities such as anticancer, antiviral and pesticidal properties. The Brine Shrimp Lethality Bioassay of the methanolic, n-hexane, ethylacetate and chloroform extract of *Ageratum conyzoide*s leaf sample were evaluated according to Meyer protocol[31] against the *Artemia salina* as a test organism to monitor the cytotoxicity of a compound. In this lethality bioassay it is estimated that LC_{50} values with 95% confidence intervals for statistically significant comparisons of potencies.

Hatching of brine shrimps

Thirty eight gram sea salt (pure NaCl) was weighed, dissolved in one litre of distilled water and filtered off to get clear solution. Seawater was taken in the small tank and *Artemia salina* leach (brine shrimp eggs) was added to one side of the tank and then this side was covered. Two days were allowed to hatch the shrimp and to be matured as nauplii. Constant oxygen supply was provided throughout the hatching time. The hatched shrimps were attracted to the lamp through the perforated dam and with the help of a pasteur pipette 10 living shrimps were added to each of the vials containing 5 ml of seawater.

Preparation of different concentration of n-hexane, ethylacetate and chloroform soluble fraction of methanolic extract of *Ageratum conyzoides*.

Different concentration of extract were prepared by dissolving them in DMSO to attain a concentration of 0.78125, 1.5625, 3.125, 6.25, 12.50, 25, 50, 100, 200 and 400 µg/ml. Then 100 µl of each concentration of different extracts were added to 5ml simulated seawater-shrimp naupalii.

Preparation of positive control and negative control group for leathality bioassay

Vincristine sulphate was used as the positive control. Measured amount of the vincristine sulphate is dissolved in DMSO to get an initial concentration of 40 mg/ml from which serial dilutions are made using DMSO to get 20 mg/ml, 10 mg/ml, 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml, 0.3125 mg/ml and 0.15625 mg/ml. Then the positive control solutions are added to the premarked vials containing ten living brine shrimp nauplii in 5 ml simulated sea water to get the positive control groups. 100 ml of DMSO was added to each of three pre marked glass vials containing 5 ml of simulated sea water and 10 shrimp nauplii to use as negative control groups. If the brine shrimps in these vials show a rapid mortality rate, then the test is considered as invalid as the nauplii died due to some reason other than the cytotoxicity of the compounds.

After 24 h, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. The mortality endpoint of this bioassay was defined as the absence of controlled forward motion during 30 s of observation. From this data, the percent (%) of mortality of the brine shrimp nauplii was calculated for each concentration using the following formula:

$$\text{Mortality} = \frac{N_t}{N_0} \times 100$$

Where, Nt = Number of killed nauplii after 24 hrs of incubation,

No = Number of total nauplii transferred i.e 10.

The LC_{50} (Median lethal concentration) was then determined using Regression analysis.

Results and Discussion

Antimicrobial test

The result of the antimicrobial activity was measured in terms of the diameter of zone of inhibition in mm (Millimeter) with standard deviation (Table 3). The crude sample (methanolic extract), n-Hexane, chloroform and ethyl acetate soluble fractions for antimicrobial activity was used in single concentration 30 µg/disc.

The crude extract showed significant activity against *Escherichia coli* (15.28 mm), *Salmonella typhi* (14.89 mm) and *Staphylococcus aureus* (19.40 mm), moderate activity against *Bacillus cereus* (15.25 mm), *Bacillus subtilis* (15.50 mm) and mild activity against *Vibrio cholera* (15.80 mm).

The n-hexane soluble fraction exhibited significant activity against *Escherichia coli* (14.00 mm), *Salmonella typhi* (14.70

mm), *Bacillus subtilis* (17.68 mm), and *Staphylococcus aureus* (19.40 mm).The n-hexane soluble fraction showed moderate activity against *Bacillus cereus* (16.21 mm), *and Vibrio cholera* (16.50 mm).

The chloroform soluble fraction exhibited significant activity against *Escherichia coli* (13.40 mm) and moderate activity against *Salmonella typhi* (10.66 mm), *Bacillus subtilis* (12.50 mm), and *Bacillus cereus* (13.50 mm). The chloroform soluble fraction showed mild activity against *Staphylococcus aureus* (11.15 mm) and *Vibrio cholera* (11.60 mm).

The ethyl acetate soluble fraction exhibited moderate activity against *Escherichia coli* (11.60 mm), *Bacillus subtilis* (13.26 mm) and *Salmonella typhi* (11.40 mm), and showed mild activity against rest of all test organisms.

Brine shrimp lethality bioassay

In the present bioactivity study, the n-hexane, chloroform and ethyl acetate fractions of methanolic extract showed positive results indicating that the test samples are biologically active (Table 4). Each of these test samples showed different mortality rates at different concentrations (Figure 1.3). Plotting of log of concentration versus percent mortality for these test samples showed an approximate linear correlation. From the graphs, the median lethal concentration (LC_{50}, the concentration at which 50% mortality of brine shrimp nauplii occurred) was determined for the samples. The positive control groups showed non linear mortality rates at lower concentrations and linear rates at higher concentrations. There was no mortality in the negative control groups indicating the test as a valid one and the results obtained are only due to the activity of the test agents.

Table 3. *Antimicrobial activity of the crude sample, n-Hexane, chloroform and ethyl acetate soluble fractions of Ageratum conyzoides*

Bacterial strains	Diameter of zone of inhibition in mm				
	Crude sample	n-Hexane	Chloroform	Ethyl acetate	Kanamycin
		30 µg/disc			30 µg/disc
Gram negative					
Escherichia coli	15.28±0.22	14.00±0.32	13.40±0.35	11.70±0.13	17.90±0.15
Vibrio cholerae	15.80±0.55	16.50±0.13	11.60±0.25	10.70±0.15	25.13±0.12
Salmonella typhi	14.89±0.45	14.70±0.15	10.66±0.40	11.40±0.25	17.50±0.15
Gram positive					
Bacillus cereus	15.25±0.45	16.21±0.22	13.50±0.48	10.10±0.12	21.60±0.25
Bacillus subtilis	15.50±0.48	17.68±0.25	12.50±0.32	13.26±0.22	22.10±0.34
Staphylococcus aureus	19.40±0.50	19.40±0.18	11.15±0.28	9.50±0.21	22.38±0.26

Table 4. *Effect of n-hexane, chloroform and ethyl acetate soluble fraction of Ageratum conyzoides on shrimp nauplii*

Test Sample	Log C	% Mortality			Vincristine Sulphate		
Conc (C) (µg/ml)		n-Hexen	Chloroform	Ethyl acetate	Conc(C) (µg/ml)	Log C	% Mortality
400	2.602	90	100	40	40	1.602	100
200	2.301	50	50	30	20	1.301	90
100	2	20	40	20	10	1.000	90
50	1.699	20	30	20	5	0.698	80
25	1.398	10	30	10	2.5	0.397	70
12.5	1.097	10	20	10	1.25	0.096	70
6.25	0.796	0	10	10	0.625	-0.204	50
3.125	0.495	0	0	0	0.3125	-0.505	30
1.5625	0.194	0	0	0	0.15625	-0.806	20
0.78125	-0.107	0	0	0	0.078125	-1.107	10

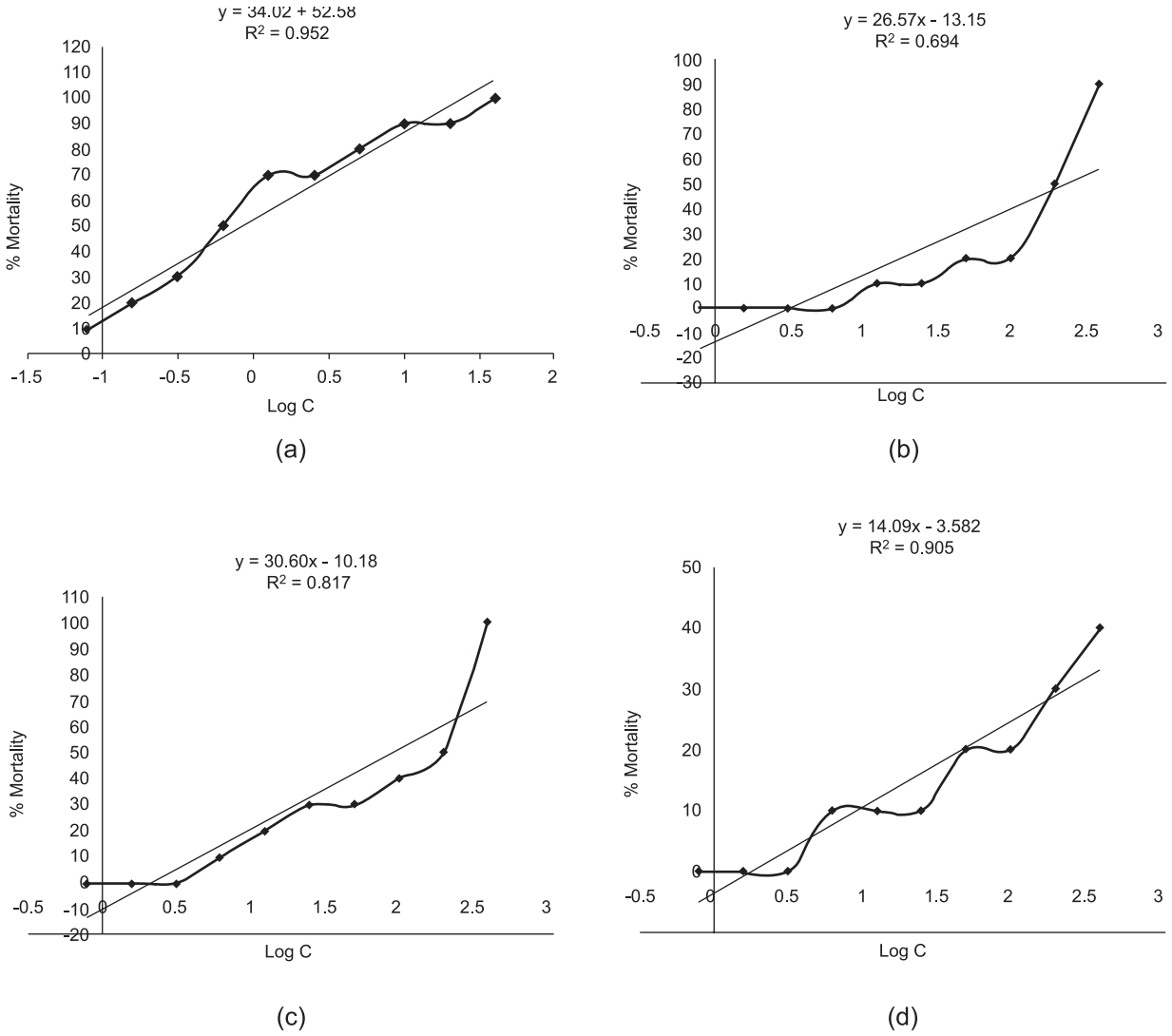

Figure 3. *Effect of test samples at different concentration on shrimp naupalii. a. Vincristine sulphate; b. n-hexane soluble fraction; c. Chloroform soluble fraction; d. Ethylacetate soluble fraction*

Table 5: *Results of the test samples of Ageratum conyzoides*

Sample	LC_{50} (mg/ml)	Regression equation	R^2
Vincristine sulphate (positive control)	0.839	y = 34.027x + 52.588	0.9168
n-hexane soluble fractions of methanolic extract	245.42	y = 26.57x - 13.15	0.694
Chloroform soluble fractions of methanolic extract	92.61	y = 30.60x – 10.18	0.817
Ethyl acetate soluble fraction of methanolic extract	6.35	y =14.09x – 3.582	0.905

The LC_{50} values of the n-hexane, chloroform and ethylacetate soluble extracts are 245.42 mg/ml, 92.61 mg/ml and 6.35 mg/ml respectively on shrimp nauplii (Table 5). The LC_{50} values positive control vincristine sulfate is 0.839 mg/ml. Comparism with the positive control indicates that n-hexane, chloroform and ethylacetate soluble fraction of methanolic extracts have mild antitumour activitiy. This may be due to the presence of flavonoids, diterpenoids, triterpenoids, and alkaloids in *Ageratum conyzoides* which was previously described by Han et al[16]. Ethyl acetate soluble fractions has a highest antitumour activity than n-haxane and chloroform soluble fractions of methanolic fractions.

The pharmacological examination for antimicrobial activity of crude extract (methanolic extract) of *Ageratum conyzoides and* n-hexane, chloroform and ethyl acetate soluble fractions of methanolic extract showed a significant to moderate microbial growth inhibitory effect against gram negative bacteria *Escherichia coli, Vibrio cholera, Salmonella typhi* and gram positive bacteria *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis*. As the pharmacological activity of any drug

substance depends on its applied dose and in this experiment the applied dose is 30 microgram/disc. The results of this experiments correlates with Almagboul[7] and Ekundayo[8] and indicated that the leaf extracts of *Ageratum conyzoides* exhibited antimicrobial activities both on Gram posiiive and Gram negative microorganisms.

It has been also investigated for antitumor properties of n-hexane, chloroform and ethyl acetate soluble fractions of methanolic extract of *Ageratum conyzoides* using brine shrimp lethality bioassay on shrimp nauplii[31]. The brine shrimp test correlates reasonably well with cytotoxic and anti-tumour properties Kinghorn[13] and Fouche[14]. From the results of the brine shrimp lethality bioassay it can be well predicted that the n-hexane, chloroform and ethyl acetate soluble fraction possess cytotoxic activity comparison with positive control vincristine sulfate and it signifies that the cytotoxicity exhibited by these soluble fractions might have mild antitumor and pesticidal activity. Further study is needed to identify the specific fraction of these extracts using column chromatoghaphy and desired chemical compounds from these fractions that inhibit microbes and tumour in life. Finally this experiments signifies that the leaves extracts of *Ageratum conyzoides* helps to discover low cost drugs against life threatening disease.

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