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Antibacterial, Cytotoxic and Antioxidant Activity of Chloroform, n-hexane and Ethyl Acetate extract of plant *Amaranthus spinosus*

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Abstract: The main aim of this study was to find out the antibacterial, antioxidant and cytotoxic activity of chloroform , n-hexane and ethyl acetate extracts of *Amaranthus spinosus* (family: *Amaranthaceae*). Disc diffusion technique was used for in vitro antibacterial screening against gram positive and gram negative human pathogenic bacteria. In case of *Amaranthus spinosus* all extracts showed good antibacterial activity against both gram positive and gram negative & average zone of inhibition 8-15mm. The Brine shrimp lethality bioassay method was used to determine the cytotoxicity activities and Vincristine Sulphate was used as positive control. The LC_{50} values of standard vincristine sulphate, chloroform, n-hexane and ethyl acetate extract were 7.55 µg/ml, 18.15 µg/ml, 29.51 µg/ml & 18.15 µg/ml respectively for the *Amaranthus spinosus*. Antioxidant activity test of the crude extracts were assessed by means of DPPH free radical scavenging method where Ascorbic Acid was used as standard whose IC_{50} value was 43.22µg/ml. All the fractions of *Amaranthus spinosus* showed potent Antioxidant activity, of which the ethyl acetate extract fractions demonstrated the good Antioxidant activity with IC_{50} value of 53.68 µg/ml.
Key words: *Amaranthus spinosus,* antibacterial, antioxidant, cytotoxic.

Introduction

The plant kingdom comprises many species of plants containing substances of medicinal value, which are yet to be explored. A large number of plants are constantly being screened for their possible medicinal value[(1)]. The use of natural products with therapeutic properties is as ancient as human civilization and, for a long time, mineral, plant and animal products were the main sources of drugs[(2)]. Medicinal plants serve as an important therapeutic agents as well as important raw material for the manufacture of traditional & modern medicines. Plants are the important source of diverse range of bioactive principles. The in vitro antibacterial study was designed to investigate the antibacterial spectrum of the crude extracts by observing the growth response. The rationale for these experiments is based on the fact that bacteria and fungi are responsible for many infectious diseases, and if the test materials inhibit bacterial or fungal growth then they may be used in those particular diseases. The antioxidant study

was designed to investigate the free radical scavenging activities. Antioxidants which scavenge free radicals are known to possess an important role in preventing these free radical induced-diseases. There is an increasing interest in the antioxidants effects of compounds derived from plants, which could be relevant in relations to their nutritional incidence and their role in health and diseases. The brine shrimp cytotoxicity assay was considered as a convenient probe for preliminary assessment of toxicity, detection of fungal toxins, heavy metals, pesticides and cytotoxicity testing of dental materials[3]. It can also be extrapolated for cell-line toxicity and antitumor activity[4]. Brine shrimp lethality bioassay stands superior to other cytotoxicity testing procedures because it is a rapid method utilizing only 24 hours, inexpensive and requires no special equipment.

Amaranthus spinosus Linn.(Family: Amaranthaceae) grows annually as an erect, monoecious herb, up to 100–130 cm tall. The leaves and roots are applied as poultice to relief bruises, abscesses, burns, wound, inflammation, menorrhagia,gonorrhoea, eczema and inflammatory swelling[5]. *Amaranthus spinosus* is also used as antiinflammatory, antimalarial, antibacterial, antibacterial, antidiuretic, antiviral and in hepatic disorders[6]. *Amarnthus spinosus* have several active constituents like alkaloids, flavonoids, glycosides, phenolic acids, steroids, amino acids, terpenoids, lipids, saponins, betalains, b-sitosterol, stigmasterol, linoleic acid, rutin, catechuic tannins and carotenoids[6]. It also contains amaranthoside, a lignan glycoside, amaricin, a coumaroyl adenosine along with stigmasterol glycoside, betaine such as glycinebetaine and trigonelline[7] [8].

The plant is used in the treatment of abdominal pain, chicken pox, dysentry, dysurea, fever, hysteria, malaria, mania infantum, tonsillitis & vomiting. *A. spinosus* is also used as antiinflammatory, antimalarial, antibacterial, antibacterial, antidiuretic, antiviral and in hepatic disorders [6]. The plant possess hepatoprotective, antioxidant activity, aqueous extract has shown significant immunostimulating and stem extract has been credited with antimalarial activity [9]. The leaves part are used as a laxative and an applied as an emollient poultice to abscesses, boils and burns and reported as antimalarial, antioxidant and antihepatotoxic actions [10]. It is used internally in the treatment of internal bleeding, diarrhoea and excessive menstruation. The root is known as an effective diuretic. The leaves are also used for gastroenteritis, gall bladder inflammation, absesses, colic menorrhagia, arthritis and for the treatment of snakebites [11].

Materials and Methods

Plant material: The fresh leaf of *Amaranthus spinosus* was collected from Kushtia in the month of January 2009 and identified by Dr. M. A. Razzaque Shah, Tissue Culture Specialist, BRAC Plant Biotechnology Laboratory, Dhaka, Bangladesh.

Plant materials extraction and fractionation:

The fresh leaf was collected, sun dried for seven days and ground. The dried powder of *Amaranthus spinosus* leaf (200gm) was soaked in 600ml of methanol for 7 days and filtered through a cotton plug followed by Whatman filter paper number 1. The concentrated methanol extract of leaf (16 gm) was fractionated by the modified Kupchan partitioning method [12] into n-hexane, chloroform and ethyl acetate. The subsequent evaporation of solvents afforded n-hexane (450 mg), chloroform (700 mg) and ethyl acetate (350 mg) from leaf extract.

Antibacterial assay:

The disc diffusion method [13] was used to test antibacterial activity against eleven bacteria. Solutions of known concentration (µg/mL) of the test samples were made by dissolving measured amount of the samples in calculated volume of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances using micropipette. Discs containing the test materials were placed on nutrient agar medium uniformly seeded with the pathogenic test microorganisms. Standard antibiotic discs (Kanamycin 30µg/disc) and blank discs (impregnated with solvents) were used as a positive and negative control. These plates were then kept at low temperature (4^{o}C) for 24 hrs to allow maximum diffusion. There was a gradual change in concentration in the media surrounding discs. The plates were then incubated at 37^{o}C for 24 hrs to allow maximum growth of the organisms. The test materials having antibacterial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the medium. The antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment was carried out three times and the mean of the reading is required [13]. The antibacterial activity of n-hexane, chloroform and ethyl acetate of leaf was determined at a concentration of 500 µg/disc.

Cytotoxicity Screening:

Brine shrimp lethality bioassay is widely used in the bioassay for the bioactive compounds [3] [14]. Here simple zoological organism (*Artemia salina*) was used as a convenient monitor for the screening. The eggs of

the brine shrimp were collected from an aquarium shop (Dhaka, Bangladesh) and hatched in artificial seawater (3.8% NaCl solution) for 48 hrs to mature shrimp called nauplii. The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method [3]. The test samples (extract) were prepared by dissolving them in DMSO (not more than 50 µl in 5 ml solution) plus sea water (3.8% NaCl in water) to attain concentrations of 5, 10, 20, 40, and 80 µg/mL. A vial containing 50µl DMSO diluted to 5ml was used as a control. Standard Vincristine sulphate was used as positive control. Then matured shrimps were applied to each of all experimental vials and control vial. After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

Screening for antioxidant activity:
Antioxidant activity of n-hexane, chloroform and ethyl acetate of leaf extracts of *Amaranthus spinosus* was determined on the basis of their scavenging potential of the stable DPPH free radical in both qualitative and quantitative assay.

Qualitative assay:
A suitable diluted stock solutions were spotted on pre-coated silica gel TLC plates and the plates were developed in solvent systems of different polarities (polar, medium polar and non-polar) to resolve polar and non-polar components of the extracts. The plates were dried at room temperature and were sprayed with 0.02% DPPH in ethanol. Bleaching of DPPH by the resolved band was observed for 10 minutes and the color changes (yellow on purple background) were noted [15].

Quantitative assay:
The antioxidant activity of leaf extract of *Amaranthus spinosus* was determined using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay by the method of Blois [16]. DPPH offers a convenient and accurate method for titrating the oxidizable groups of natural or synthetic anti-oxidants [17]. DPPH solution was prepared in 95% methanol. The crude extracts of *Amaranthus spinosus* were mixed with 95% methanol to prepare the stock solution (5 mg/50mL). The concentration of the sample solutions was 100µg/mL. The test samples were prepared from stock solution by dilution with methanol to attain a concentration of 20µg/mL, 40µg/mL, 60µg/mL, 80µg/ mL & 100µg/mL respectively. Freshly prepared DPPH solution was added in each of these test tubes containing leaf extracts of *Amaranthus spinosus* and after 20 min, the absorbance was taken at 517 nm. Ascorbic acid was used as a positive control. The DPPH solution without sample solution was used as control. 95% methanol was used as blank. Percent scavenging of the DPPH free radical was measured using the following equation-

$$\% \text{ DPPH radical scavenging } (\%) = [1-(As/Ac)] \times 100.$$

Here, Ac =absorbance of control,
As =absorbance of sample solution.

Then % inhibitions were plotted against respective concentrations used and from the graph IC_{50} was calculated.

Result

This article describes the antibacterial, cytotoxicity and antioxidant activities of plant *Amaranthus spinosus* used in traditional medicine. Three different extracts (n-hexane, Chloroform, Ethyl acetate) of the plant were tested.

Antibacterial Activity:
The results of the extracts displaying antibacterial effect against different bacteria are shown in Table1.All extracts showed moderate to good activity against a range of gram positive and gram negative bacteria. The n-hexane extract of *Amaranthus spinosus* showed 15 mm zone of inhibition against gram positive *Sarcina lutea* and 14.5 mm zone of inhibition against gram positive *Staphylococcus aureus* which indicates good antibacterial activity. The n-hexane extract of *Amaranthus spinosus* also revealed 14 mm zone of inhibition against gram negative *Salmonella paratyphi* which indicates good antibacterial activity. The ethyl acetate extract was also active in varying degrees. It displayed 14 mm zone of inhibition against *Staphylococcus aureus* which indicates very good activity of the extract. The chloroform extract showed 11.5mm to 14 mm zone of inhibition which indicates presence of good antibacterial activity.

Table1: Antibacterial activity of extracts against both gram positive and gram negative bacteria.

Test organism	Diameter of Zone of inhibition (mm)			
	Chloroform extract (500 µg/disc)	Ethyl acetate Extract (500µg/disc)	n-hexane extract (500µg/disc)	Kanamycin (30µg/disc)
Gram positive bacteria				
Sarcina lutea	14	11	15	25
Staphlococcus aureus	11.5	14	14.5	33
Bacillus subtilis	13	9	8	30
Bacillus megaterium	13.5	10	8	32
Gram negative bacteria				
Vibrio mimicus	8	8	8	30
Salmonella paratyphi	8	9	14	
Vibrio parahemolyticus	8	8	9	32
Salmonella typhi	9	9	9.5	32
Shigella dysenteriae	9.5	9	8	28
Shigella boydii	9	8	8	30
Pseudomonas aeruginosa	9	9	9	28

Cytotoxicity Screening:

Brine Shrimp Lethality Bioassay:

Following the procedure of Meyer [3] the lethality of the crude chloroform, n-hexane and ethyl acetate extracts of *Amaranthus spinosus* to brine shrimp was determined on *A. salina* after 24 hours of exposure the samples and the positive control, Vincristine sulphate. This technique was applied for the determination of general toxic property of the plant extractive. The LC_{50} values for standard vincristine sulphate, chloroform, n-hexane & ethyl acetate extract were found to be 7.55µg/ml, 18.15 µg/ml, 29.15 µg/ml, 18.15 µg/ml respectively for *Amaranthus spinosus* (Figure-1) which indicates that the plant has potent cytotoxic effect.

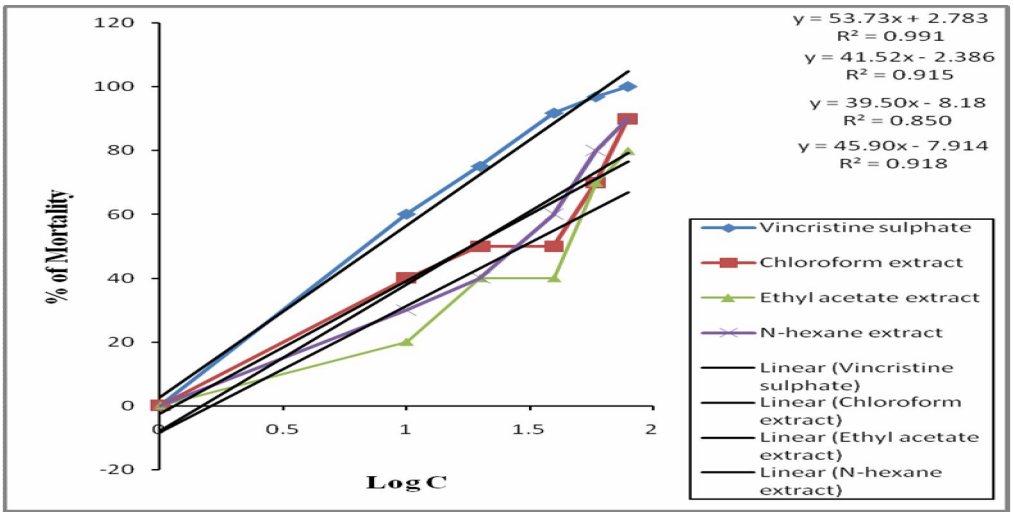

Figure 1: Determination of LC_{50} values for standard and chloroform, n-hexane, ethyl acetate extracts of leaves *Amaranthus spinosus* from linear correlation between logarithms of concentration versus percentage of mortality.

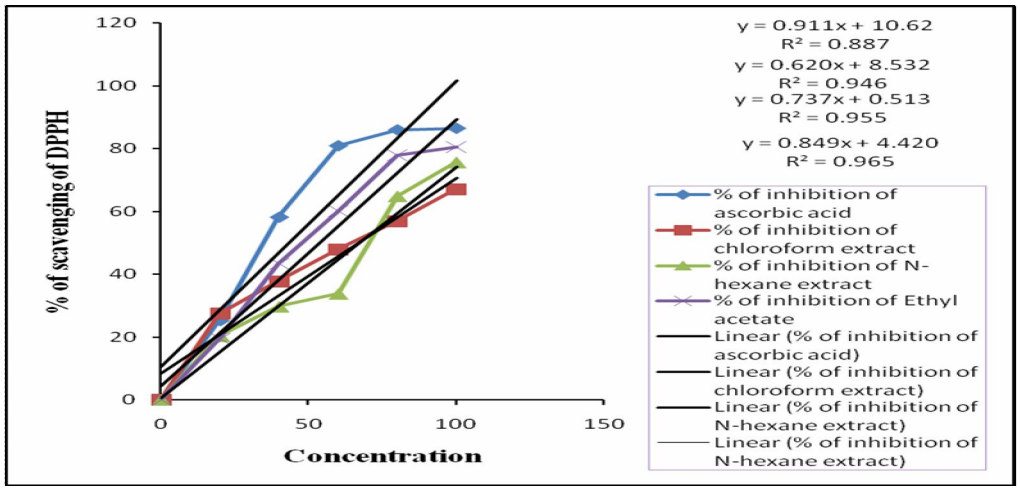

Figure 2: Determination of IC_{50} values for standard and chloroform, n-hexane, ethyl acetate extracts of leaves *Amaranthus spinosus* from linear correlation between logarithms of concentration versus percentage of scavenging of DPPH.

Antioxidant Activity:

To test their antioxidant activity, all three extracts were analyzed by DPPH free radical scavenging assay by the method of Blois [16]. Different fractions of ethanolic extract of *Amaranthus spinosus* was evaluate for the antioxidant activity. Here ascorbic acid was used as reference standard. The percentage reduction of DPPH radical exhibited by different extract was calculated and subsequently its IC_{50} was determined (Figure 2). The Ethyl acetate extract of *Amaranthus spinosus* scavenged 50% DPPH free radical at the lowest inhibitory concentration (IC_{50}:53.68 µg/ml). The chloroform & n-hexane extract of the plant also revealed moderate antioxidant activity IC_{50}: 66.88 µg/ml and 67.14 µg/ml respectively.

Discussion

The results of our investigation confirmed the rationale for the medicinal use of the studied plant. The remarkable aspect of the results was that all the extracts inhibited the growth of both gram positive and gram negative bacteria at their concentration tested. Among all extracts, the n-hexane extract of *Amaranthus spinosus* showed highest activity against gram positive *Sarcina lutea*, *staphylococcus aureus* and gram negative *Salmonella paratyphi*.

In the present study we also demonstrate the cytotoxic effect of the extracts of A*maranthus spinosus*. All the extracts exhibited a remarkable cytotoxic effect among them n-hexane extract showed highest cytotoxic activity (LC_{50} = 29.15 µg/ml). These cytotoxic samples may have clinical and therapeutic proposition in the most life threatening diseases like tumor, cancer and further studies are required to investigate these plant samples as antineoplastic agents.

Preliminary phytochemical investigation revealed the presence of phenolic compounds and flavanoids [6] which have been reported to be associated with antioxidative action in biological systems acting as scavengers of singlet oxygen and free radicals.

In summary, we conclude that most of the results of this study are in good agreement with the traditional uses of the investigated plant. All the extracts showed significant antibacterial, cytotoxic and antioxidant activity. It was postulated that an increase in the antibacterial activity of pure compounds occurred when they are combined with antioxidants. Therefore, we consider that if both antibacterial and antioxidant compounds exist in the extracts, they could interact and enhance the antibacterial activity. The bioassay-guided fractionation of these extracts in order to isolate and to identify the compounds responsible for each of these activities, followed by a study of their interaction, is highly desirable.

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