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COMPARATIVE ANTIMICROBIAL AND CYTOTOXIC ACTIVITY STUDY OF N-HEXANE, CHLOROFORM AND CARBON TETRA-CHLORIDE EXTRACTS OF *ADIANTUM INCISUM* FORSK

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ABSTRACT

In the present study the antimicrobial & cytotoxic activity of crude extracts (n-hexane, chloroform, carbon tetra-chloride) of *Adiantum incisum* Forsk were studied. Antimicrobial activity was tested against ten important pathogenic bacteria including both gram positive (+ve) and gram negative (-ve) bacteria and two fungi. The bacteria were *B. megaterium, B.cereus, B. subtilis, Staphylococcus aureus, Sarcina lutea, Escherichia coli, Shigella boydii, Pseudomonas aeruginosa, Vibrio mimicus* & *V. parahemolyticus*. Disc diffusion technique was used for in-vitro antibacterial and antifungal screening. Here kanamycin disc (30µg /disc) was used as standard for antibacterial study. The extracts showed antimicrobial activity against most of the bacterial strains with an average zone of inhibition of 8-14mm. The tested fungi were *Saccharromyces cerevaceae* and *Candida albicans*. The extracts showed mild to moderate antifungal activity with an average 8 -10 mm zone of inhibition. Among the three solvent extracts used, the most effective extract was found to be chloroform and maximum activity (14 mm, zone of inhibition) found against *Staphylococcus aureus*. Cytotoxicity test was also studied by Brine Shrimp Lethality Bioassay and compare with LC_{50} values of standard Vincristine sulphate as a positive control. The results illustrated significant cytotoxicity against *A. salina*, with LC_{50} 9.267µg/ml, 8.118µg/ml and 9.245µg/ml for n-hexane, chloroform and carbon tetra-chloride extracts respectively.

KEYWORDS: Antimicrobial activity, Pathogenic bacteria, Kanamycin, Cytotoxicity.

1. INTRODUCTION

Traditional medicine is a practice that has been developing for thousands of years and enriched by millions of traditional practitioners all over the world. Medicinal plants are frequently used in traditional medicine to treat different human diseases in different parts of the world. These medicines are used in the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations (Balick and Cox, 1997). They are the primary source of medicine still now in rural areas of the developing countries (Chitme *et al*., 2003) and about 80% peoples of these countries use this traditional therapy to treat their ailments (Kim, 2005; Hassan *et al*., 2009). According to world health organization (WHO, 1978), herbal medicine, composed mainly of medicinal plants, are still curing diseases of an estimated 80% (currently, it is said to be 88%) of the world population (Said, 1995). In developed countries, plant-based traditional medicines or phytotherapeuticals are often termed complementary or alternative medicine (CAM), and their use has increased steadily over the last 10 years.

Natural products and related drugs are used to treat 87% of all categorized human diseases including infectious disease, cancer & immunological disorders (Newman *et al*; 2000) and over 3000 species of plants have been reported to have anticancer properties. More than 250 medicinal plants are now in common use in the preparation of traditional medicine in Bangladesh (Mia and Ghani, 1990). Only 25% people in Bangladesh can avail themselves of allopathic treatment and the rest 75% depend on alternative sources of medicine for their treatment (Sharif and Banik, 2006). Current research on natural molecules and products primarily focuses on plants since they can be sourced more easily and selected on the basis of their ethno-medicinal use (Verpoorte *et al*., 2005). Continuing search of new therapeutic agent is geared toward the discovery and development of novel chemical structures such as therapeutic antimicrobial agents, antioxidants, hypoglycemic agent. The ongoing problem of development of resistance to existing antibacterial agents and the dearth of good antifungal agents motivates this effort toward innovation (Silver and Bostian, 1990). Therapeutics of plant origin is not associated with many side effects and has an enormous

therapeutic potential to heal many infectious disease (Iwu *et al*., 1999). The antimicrobial compounds produced by plants are active against plant and human pathogenic microorganisms (Mitscher *et al*., 1987). In the continuation of this strategy of new drug discovery we have studied the leaves of the plant *Adiantum incisum* for its antimicrobial and cytotoxic activity.

Adiantum incisum Forsk belongs to the genus adiantum of family Adiantaceae which is known as maiden-hair a fern and used in Ayervedic medicine. *Adiantum incisum*, locally known as 'Mayurshikha' in Bengali, is a widely used plant in folkloric medicine. It is well known for its antibacterial, antiviral, antifungal and other biological activities (Kshirsagar and Mehta, 1972; Wada *et al*., 1987; Hussain *et al*., 2008). *Adiantum incisum* Forsk grows abundantly in Bangladesh, India, Pakistan, South China and Yeman, Tropical Africa and many other countries (Husson *et al*., 1986). It has rhizome short and erect, 2-4 inches long stipes of brown color, 6-12 inches long fronds are simply pinnate, 0.5-0.75 inch long pinnae are 0.25 inch deep (Nasir and Ali, 1972; Beddone, 1892). It is a pteridophyte grown in slopes of hills in Chittagong, Syhlet, Rangamati, Bandorban etc. Fronds (leaves) of *Adiantum incisum* having a bud in its apical region, which serves the purpose of vegetable propagation that is why, this fern is called as "Walking fern' (Pradeep and Leena, 2006; Frank P *et al*., 2012). The major action of genus adiantum (maiden-hair fern) are expectorant, diuretic, emmenogogue and resonant. Their juice is used for remedy in all kinds of fever and chronic cough. *Adiantum incisum* is traditionally used for the treatment of malaria, hemicrania, diabetes, skin disease and bronchial diseases (Nadkarni, 1982; Nwosu, 2002; Hussain *et al*., 2008). The plant is also used as spasmolytic and anti-biotic (Brahmachari *et al*., 2003). Previous studies on this species reported that the plant contains several compounds such as adiantone, hentriacontanoic acid, 2-pentatriacontenoic acid, octoil, 2-(hexanoyloxy)-1-[(hexanoyloxy)methyl]ethyl(2Z,4Z)-2,4-ocat-dienoate, stigmasterol, β-sitosterol, stigmast-4-en-3-ol, ursolic acid, β-sitosterol 3-O-β-D-glucopyranoside (Hussain *et al*., 2008). There are very few reports regarding to its antimicrobial and cytotoxic effects using whole plant. The present study was aimed to evaluate the antimicrobial and cytotoxic potentiality of different solvent extracts of the whole plant of *Adiantum incisum*.

2. MATERIALS & METHODS

2.1 Plant material

The fresh green whole plant was collected from Chittagong hills tract in the month of February, 2014 and authenticated at Bangladesh National Herbarium, where a voucher specimen no DACB 42274 has been deposited.

2.2 Extraction and isolation

The air-dried leaves (500 gm) were finely pulverized and extracted by percolation with ethanol for seven days at room temperature. The extracts were filtered and concentrated under vacuum to obtain a crude extract of leaves. The extract was fractionated by the modified Kupchan partitioning method (Van Wagenen *et al*., 1993) into n-hexane, chloroform and carbon-tetra - chloride. The fractioned extracts were concentrated under vacuum to obtain solid extracts.

2.3 Antimicrobial assay

2.3.1 Microorganisms

Antimicrobial activity was tested against B. subtilis, *B. megaterium, B. cereus, Staphylococcus aureus, Sarcina lutea, Pseudomonas aeruginosa, Vibrio mimicus, Shigella boydii, Escherichia coli, V. parahemolyticus, Saccharromyces cerevaceae*, and *Candida albicans*. These microbial strains were isolated from clinical samples and obtained as pure cultures from the Institute of Nutrition and Food Science (INFS), University of Dhaka, Bangladesh.

2.3.2 Determination of the diameters of inhibition zone

The crude extracts were tested in vitro for antimicrobial activity by the standard disc diffusion method (Bauer *et al*., 1966, Rahman *et al*., 2008) against the bacteria. Solutions of known concentration (500µg/ 10µl) of the test samples were made by dissolving measured amount of the samples (50 mg) in 1 ml of solvents. Dried and sterilized filter paper discs (6 mm diameter) were then impregnated with known amounts of the test substances (500µg/ disc) using micropipette and the residual solvents was completely evaporated. Discs containing the test materials were placed on to nutrient agar medium uniformly seeded with the test microorganisms. Standard disc of kanamycin (30 µg/disc) and blank discs (impregnated with solvents followed by evaporation) were used as positive and negative control, respectively. These plates were then kept at low temperature (4^{o}C) for 16 hours to allow maximum diffusion of the test materials and kanamycin. The plates were then incubated at 37^{o}C for 24 hours to allow maximum growth of the organisms. The test material having antimicrobial activity inhibited the growth of the microorganisms and a clear, distinct zone of inhibition was visualized surrounding the discs. The antimicrobial activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm. The experiment was carried out in triplicate and the mean value was taken.

2.3.3 Antifungal screening

The n-hexane, chloroform and carbon tetra-chloride extracts of *Adiantum incisum* were tested for their antifungal activity against the selected fungi by the standard disc diffusion method (Bauer *et al*., 1966, Rahman *et al*., 2008). In this antifungal screening each of the crude extract was used at a concentration of 500µg/disc. Following the above mentioned procedure, the antifungal activity of the test agents was determined by measuring the diameter of zone of inhibition expressed in mm.

2.4 Cytotoxicity screening

Cytotoxic activity of the plant extracts was determined by brine shrimp lethality bioassay method (Meyer *et al*., 1982). This method detects the lethality of crude extracts on Artemia salina, used as a convenient monitor for the screening. The eggs of Brine Shrimp (*A. salina*) were hatched in a tank in artificial seawater (3.8% NaCl solution) at a temperature around 37^0C with constant oxygen supply. Two days were allowed to hatch and mature the nauplii.

2.4.1 Preparation of Test Groups:

The cytotoxicity assay was performed on brine shrimp nauplii using Meyer method. For the experiment, the test samples (extracts) were prepared by dissolving them in DMSO (not more than 50 µl in 5 ml solution) and sea water to attain concentrations of 20, 40, 60, 80 and 100 µg/ml. A vial containing 50 µl 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. Each test tube contained about 5 ml of seawater and 10 shrimp nauplii.

2.4.2 Counting of nauplii

After 24 hours, the vials were inspected using a magnifying glass and the number of survived nauplii in each vial was counted. The rate of mortality of nauplii was found to be increased in concentration of each of the samples. From this data, the percent (%) of lethality of the brine shrimp nauplii was calculated for each concentration.

2.4.3 Lethality concentration determination

The percentage lethality was determined by comparing the mean surviving larvae of the test and control tubes. LC_{50} values were obtained from the best-fit line plotted concentration verses percentage lethality. Vincristine sulphate was used as a positive control in the bioassay.

3. RESULT

3.1 Result of Antimicrobial screening

The extracts of the *Adiantum incisum* were screened against ten human pathogenic bacteria to check antibacterial activities by disc diffusion method .The extracts showed variable zone of inhibition against tested pathogenic bacteria which was shown in Table 1. All the extracts showed significant antimicrobial activity. The Chloroform extract of *Adiantum incisum* showed moderate to maximum antibacterial activity against all tested pathogenic bacteria. This extract showed very good antimicrobial activity against the Gram-(-) *Escherichia coli* (14 mm), *Shigella boydii* (13mm) and the Gram-(+) *Bacillus subtilis* (12mm), *Staphylococcus aureus* (14mm) bacteria with an average zone of inhibition of 8-14mm.

The carbon tetra-chloride extract showed moderate to good antibacterial activity against all tested pathogenic bacteria with an average zone of inhibition of 8-12 mm. The maximum zone of inhibition exhibited against *E. coli* (12mm) and *Pseudomonas aeruginosa* (12mm). The minimum zone of inhibition showed 9 mm against *Shigella boydii* and *V. mimicus*. Whereas an average zone of inhibition of 8-12 mm, against all the tested bacteria was showed by n-hexane. This extract exhibited moderate antimicrobial activity against *Staphylococcus aureus* (12mm).

Table 1: In vitro antibacterial activity of different extracts of the whole plant of *Adiantum incisum*

Test Organisms	Diameter of zone of inhibition (mm)			
	chloroform extract (500µg/disc)	carbon tetra-chloride extract (500µg/disc	n-hexane extract (500µg/disc)	Kanamycin (30µg/disc)
Gram (+) ve bacteria				
Bacillus subtilis	12	11	8	30
Bacillus megaterium	8	8	-	32
Bacillus cereus	9	9	8	33
Staphylococcus aureus	14	10	12	29
Sarcina lutea	8	-	-	31
Gram (-) ve bacteria				
Pseudomonas aeruinosa	13	12	11	25
Vibrio mimicus	11	10	8	30
Shigella boydii	13	10	9	30
Escherichia coli	14	12	9	22
V. parahaemolyticus	-	8	-	32

(-)= No activity.

3.2 Result of antifungal screening

The results of the extracts displaying antifungal effect against tested fungus are shown in Table 2. All the extracts showed mild to moderate activity against the fungus. The exhibited diameter of zone of inhibitions were 8mm, 10mm, 9mm against *Candida albicans*, 8mm, 9mm, 9mm against *Sacharomyces cerevaceae* for the n –hexane, Chloroform and carbon tetra-chloride extracts respectively.

Table 2: In vitro antifungal activity different extracts of the whole plant of *Adiantum incisum*.

Test organisms	Diameter of zone of inhibition (mm)		
	chloroform extract (500µg/disc)	carbon tetra-chloride extract (500µg/disc)	n-hexane extract (500µg/disc)
Sacharomyces cerevaceae	9	9	8
Candida albicans	10	9	8

3.3 RESULT OF CYTOTOXICITY SCREENING

The lethal concentration (LC_{50}) of the test samples after 24 hours was determined by a plot of percentage of the brine shrimps died against the logarithm of the sample concentration (toxicant concentration) and the best-fit line was obtained from the curve data by means of regression analysis. The lethality of the extracts to brine shrimps was determined and the results are ploted in Figure 1. The percent mortality increased with an increase in concentration. Vincristine sulphate (VS) was used as positive control and the LC_{50} value was found 8.907µg/ml. The crude n-hexane, chloroform and carbon tetra-chloride extract showed better cytotoxic activity with LC_{50} values of 9.267µg/ml, 8.118µg/ml, 9.245µg/ml in comparision with vincristine sulphate as standard.

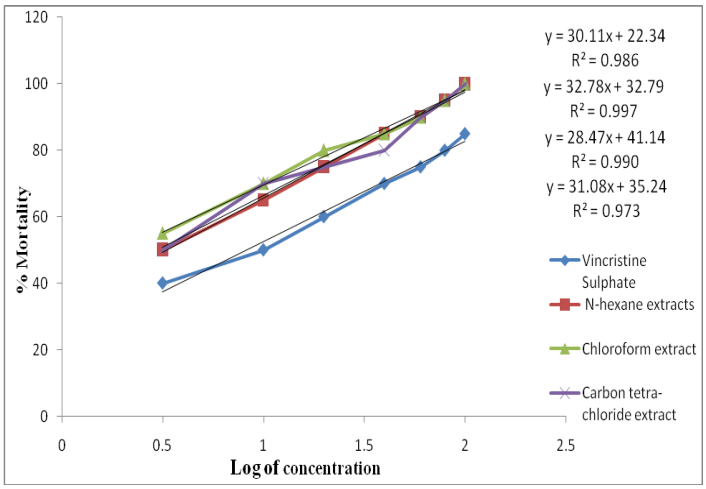

Figure 1: Determination of LC_{50} values for standard and crude n-hexane, chloroform and carbon tetra-chloride extracts of *A. incisum* from linear correlation between logarithms of concentration versus percentage of mortality.

4. DISCUSSION

4.1 Antimicrobial potential

In the present study the result of antimicrobial activity reveals that all the extracts showed significant antimicrobial activity towards both bacteria and fungi suggested the presence of antimicrobial compounds. Among the three extracts of *Adiantum incisum*, the chloroform extract exhibited more antibacterial potential than the n-hexane & carbon tetra-chloride extracts. Evidence showed that plant extracts contain sterols (including stigmasterol and β-sitosterol), fatty acids, hentriacontanoic acid, 2-pentatriacontenoic acid and ursolic acid. A pyridine alkaloid, trigonelline and sterols have been isolated from tissue cultures of leaves. In case of antifungal activity all the three extract showed almost same potential.

4.2 Cytotoxic activity

From the results of the brine shrimp lethality bioassay it can be well predicted that all the crude extracts have considerable cytotoxic potency. Among the three fractions, n-hexane extract showed significant cytotoxicity than carbon tetra-chloride and chloroform extracts of *Adiantum incisum*. Previous phytochemical screening indicated the presence of alkaloids and flavones, which have been shown to possess cytotoxic activity, may be responsible in part for the antitumour effect on Ehrlich ascites carcinoma (Brown, 1980). The variation in results may be due to the difference in the amount and kind of cytotoxic substances (e.g. tannins, flavonoids, triterpenoids, or coumarins) present in the different solvent crude extracts. The possible mechanism of cytotoxicity of *Adiantum incisum* against brine shrimp nauplii might be due to poisonous effect on cell mitosis.

5. CONCLUSION

Extensive practice of Traditional and alternative medicine has regained public attention over the past 20 years as this type of medicine is easily accessible in some regions. A postulation denoted that by 2010, at least two-thirds of Americans will opt for alternative therapies (Zhao *et al*., 1992**).** The non prescription use of medicinal plants is cited today as an important health problem, in particularly their toxicity to the kidney (Humber, 2002). So, if the plant extract found to show significant antimicrobial activities must take into account acceptable levels of toxicity.

The results obtained in our present study indicated that the crude extracts of *Adiantum incisum* leaves has got profound cytotoxic and antimicrobial effect and may have potential use in medicine. This finding will aid us to conduct pharmacological studies to understand the underlying possible mechanisms of the observed activities as well as bioactivity guided isolation and characterization of leading compounds in future.

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