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Antifungal Potential of *Achyranthes aspera* Linn. Collected from Himachal Pradesh, Punjab and Haryana Region

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Antifungal activity of the whole plant extract of *Achyranthes aspera Linn.*, procured from three different locations namely, Himachal Pradesh, Punjab and Haryana, was evaluated using agar well diffusion method. The plants were collected in the month of January-2010. The methanolic extract of *Achyranthes aspera Linn.* (Family: Amaranthaceae) was tested for antifungal activity against various fungal spp. viz. *Aspergillus flavus, Aspergillus nidulans*, *Fusarium* and various isolates of *Aspergillus niger.* Qualitative analysis of phytochemicals showed presence of sugars, proteins, alkaloids, tannins, phenols, saponins, triterpenoids, glycosides and steroids in *Achyranthes aspera*. The plant showed maximum antifungal activity at 10000µg ml-1 concentration against *Aspergillus flavus, Aspergillus nidulans and Fusarium species* with the zone of inhibition upto 17.5mm, 16mm and 15.5 mm respectively from Punjab region. In case of Aspergillus niger, maximum zone of inhibition was observed in the isolate grown on pomegranate peels (15 mm). Among the three locations the best antifungal activity was obtained from the plants procured from Punjab region. The result obtained in the present study suggests the significant scope of developing novel broad spectrum antifungal formulations.

Key words: *Achyranthes aspera, Aspergillus isolates, Fusarium,* Antifungal herb, Agriwaste.

The practice of traditional medicine is wide spread in China, India, Japan, Pakistan, Srilanka and other African countries. In China about 40% of the total medicinal consumption is attributed to traditional tribal medicines. Infections particularly those involving microorganisms i.e. bacteria, fungi, viruses etc, they cause serious infections in tropical and subtropical countries of the world. Hence there is an urgent requirement to study the screening of antimicrobial properties of those herbs, which will be helpful in the treatment of several diseases caused by microorganism. Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources. The plants are potential source of medicines since ancient times. The use of medicinal plants to treat human diseases has its roots in pre historical times. Medicinal plants are used by 80% of the world population as the only available medicines especially in developing countries (Periyasamy *et al.,* 2010). There is a continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms of action for new and re-emerging infectious diseases (Rojas *et al.*,,

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2003). Therefore, researchers are increasingly turning their attention to folk medicine, looking for new leads to develop better drugs against microbial infections (Benkeblia, 2004). Reports are available on the use of several plant by-products, which posses antimicrobial properties, on several pathogenic bacteria and fungi (Bylka *et al.*,, 2004; Shimpi and Bendre, 2005; Kilani, 2006). *Ahyranthes aspera* Linn. belongs to the family *Amaranthaceae*. It is an annual, stiff erect herb, and found commonly as a weed throughout India and used by traditional healers for the treatment of fever, dysentery and diabetes. Leaf decoction for cardiovascular toxicity has been reported and the methanol crude extract showed high bacterial activity against *E.coli, Baccilus.* Root extract of the plant inhibited the growth of *Bacillus subtilis* and *Staphylococcus aureus* bacterial strains. Seeds are rich in protein, cooked and eaten. It acts as antispasmodic, astringent, diuretic, odontalgic (Dwivedi *et al.,* 2007*)*. The whole plant is used medicinally, but the roots are generally considered to be more effective. They contain triterpenoid saponins (Hariaran *et al.*, 1970). The juice of the plant is used in the treatment of boils, diarrhea, dysentery, hemorrhoids, rheumatic pains, itches and skin eruptions. Numerous surveys on antimicrobial medicinal plants had been made in United States and in many countries throughout the world. Such study had demonstrated the wide occurrences of active compounds in higher plants. The root extract is well reputed for its pronounced insect molting hormonal activity and the ethanolic extract of the leaves and stem of the plant inhibited the growth of *Bacillus subtilis* and *Staphylococcus aureus* bacterial strain Dhar *et al.,* 1968). Leaf extracts were reported to possess thyroid stimulating and antiperoxidative properties. Roots are also used as astringents to wounds, in abdominal tumor and stomach pain (Ghani *et al.,* 2003). The benzene extract of the stem bark shows abortifacient activity in the rat (Bhattrai *et al.,* 1984). Leaf extracts were reported to possess thyroidstimulating and antiperoxidative properties (Tahiliani *et al.,* 2000). The aqueous and methyl alcohol extracts of the plant also decreased blood glucose levels in normal and alloxan diabetic rabbits (Akhtar *et al.,* 1991). It is reported to contain alkaloids, flavonoids, saponins, steroids and terpenoids (Gokhale *et al.,* 2002). The water soluble alkaloid achyranthine isolated from *Achyranthes aspera* possess anti-inflammatory activity (Dwivedi *et al.,* 2008). The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants (Erdogrul 2002).

In the present study, we have collected *Achyranthus aspera* Linn. from Punjab, Himachal Pradesh and Haryana. Plants were screened for phytochemical constituents and antifungal activity against fungal species viz. *fusarium, Aspergillus flavus, Aspergillus* nidulans and various isolates of *Aspergillus niger* grown on agriwaste such as pomegranate peel, orange peel, tamarind seeds, apple and groundnut.

MATERIAL AND METHODS

Procurement of Plant material

Fresh plants of *Achyranthes aspera Linn.* were collected from three locations namely Punjab, Himachal Pradesh and Haryana in the month of January 2010 and were cleaned with distilled water followed by drying in shade at room temperature. The plants were identified taxonomically and authenticated in the Department of Biotechnology, Lovely Professional University, Phagwara, Punjab.

Extraction Process

After collecting fresh whole plants, thorough washing was done 3-4 times with running tap water finally with sterile water followed by shade drying at room temperature for 15-20 days. The dried plant material was made into coarse powder and passed through sieve and then used for crude extraction in methanol. The dried powder (100gm) was extracted to exhaustion in a soxhlet apparatus using methanol. Extract was filtered through a cotton plug followed by Whatman filter paper No.1 and then concentrated by using a rotary evaporator at low temperature (40-50°C) and reduced pressure to get 3.0gm yield. The extract was preserved in airtight container and kept at 4-5°C until further use. Further the extract was tested for antifungal activity against the fungal spp.

Phytochemical Tests

Molisch's Test for Carbohydrates

Few drops of alpha napthol were added to the portion dissolved in distilled water, this was

then followed by addition of 1 ml of conc. H_2S0_4 by the side of the test tube. Formation of a red – violet color at the junction of the two layers was a positive test. (Sofowora 1993)

Test for Alkaloids

Small quantity of the extract was stirred with 5 ml of 1% aqueous HCl on water bath and then filtered. Of the filtrate, 1 ml was taken separately into two test tubes. To 1 ml, Mayer's reagent was added and appearance of buff- colored precipitate will be an indicator for the presence of alkaloids (Sofowora, 1993)

Test for Tannins

500 mg of plant powder was stirred with about 10 ml of distilled water and then filtered. Few drops of 1% ferric chloride solution were added to 2 ml of the filtrate, occurrence of a blue black, green or blue-green precipitate indicates the presence of tannins. (Trease *et al.,* 2002)

Biuret Test

To 3 ml of extract added an equal volume of (3ml) 5% sodium hydroxide and then 3-4 drops of copper sulphate. Purple color indicated the positive result.

Liebermann-burchard Test for Steroids and Terpenoids

To 0.2 g of sample, 2 ml of acetic acid was added, the solution was cooled well in ice followed by the addition of conc. H_2SO_4 carefully. Color development from violet to blue indicated the presence of a steroidal ring (Sofowora, 1993).

Lead Ethanoate Test for Flavonoids

Some quantity of extract was dissolved in water and filtered. To 5 ml of filtrate, 3 ml of lead ethanoate solution was then added. Appearance of a buff –colored precipitate indicated the presence of flavonoids (Trease *et al.,* 2002)

Haemolysis Test for Saponins

About 2ml of blood was taken in two test tubes separately. To one of the test tubes, equal quantity of water was added. To the other test tube, an equal quantity of ethanolic extract dissolved in water was added. A clear red liquid was formed in the first tube, which indicated that red blood corpuscles were haemolysed. The extract in the second test tube also haemolysed. It indicates the presence of saponin (Kokate, 1991).

Test Organisms

The cultures of fungal spp were used for the test were *Aspergillus flavus, Aspergillus nidulans*, *Fusarium spp* and various isolates of *Aspergillus niger*, grown on agriwaste such as pomegranate peel, orange peel, tamarind seeds, apple and groundnut. The cultures were taken from the repository of Department of Biotechnology, Lovely school of Sciences, Lovely Professional University, Phagwara, India.

Culture media and inoculums preparation

Potato dextrose broth (PDB) was used as the media for the culturing of fungal strains. A loop full of all the fungal cultures was inoculated in the potato dextrose broth (PDB) at 37°C for 72 hrs.

Plating of media

The potato dextrose agar media was poured in petriplates and then innoculum was spread with the help of L- spreader. The wells were made in the petriplates and then the extract was poured in the well. Finally, the petriplates containing the extracts were incubated in B.O.D incubator for 72 hours.

Antifungal Activity

Agar well diffusion method

The extract obtained from the whole plant was used for studying their antifungal activity. A loop full of fungal strain was inoculated in 30 ml of potato dextrose broth in a conical flask and incubated for 72 hrs to get active strain by using agar well diffusion method. The media was poured into petri dishes. After solidification 0.25 ml of test strains were inoculated in the media separately. Care was taken to ensure proper homogenization. The experiment was performed under strict aseptic conditions. After the medium was solidified, a well was made in the plates with sterile borer (4mm).The extract was introduced into the well and plates were incubated at 37°C for 72 hrs. Microbial growth was determined by measuring the diameter of zone of inhibition.

RESULTS AND DISCUSSION

Fresh whole plants of *Achyranthes aspera* were collected from Punjab, Himachal Pradesh and Haryana region and investigated for antifungal activity against *Aspergillus flavus, Aspergillus nidulans*, f*usarium species* and various isolates of *Aspergillus niger* grown on agriwaste such as pomegranate peel, tamarind seeds, orange peel, groundnut, apple. The plants were collected from different locations to study the effect of

environmental conditions on the occurrence of various phytochemicals and hence on the antifungal potential as well. The plants were extracted in aqueous methanol (50%) keeping in view to pool water soluble constituents as well as organic solvent soluble constituents in single

Table 1. Inhibition zone in (mm) by minimum inhibitory concentration evaluation of extract procured from Punjab region

Fungal Species	Punjab			
Methanolic Extract (µg ml$^{-1)}$	10000	6000	4000	2000
A. flavus	17.5	10.0	7.0	0
A. nidulans	16.0	12.0	8.0	7.0
Fusarium species	15.5	8.0	0	0
A. niger isolates				
Pomegranate peel	15.0	12.0	7.0	0
Tamarind	13.5	7.0	0	0
Orange peel	12.0	9.0	7.0	0
Apple	14.5	12.0	10.0	8.0
Groundnut	10.0	7.0	0	0

Table 2. Inhibition zone in (mm) by minimum inhibitory concentration evaluation of extract procured from Himachal Pradesh region

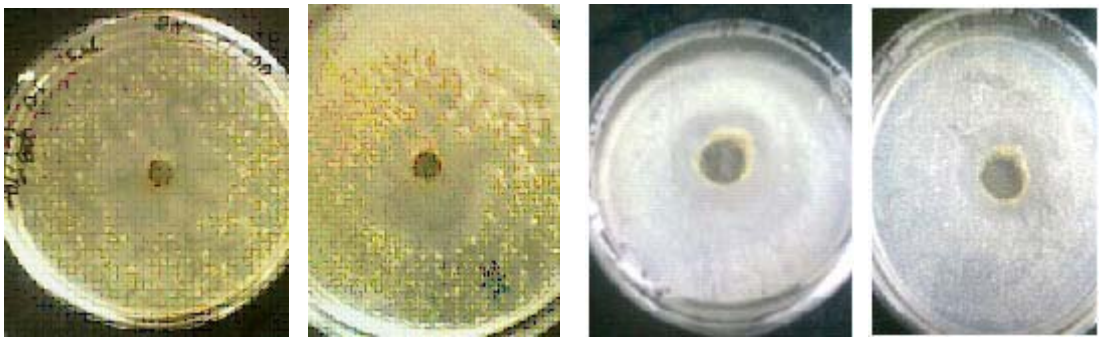
Fungal species	Himachal Pradesh			
Methanolic Extract (µg ml$^{-1)}$	10000	6000	4000	2000
A. flavus	15.0	13.0	8.0	7.0
A. nidulans	10.0	8.0	7	0
Fusarium species	10.0	7.0	0	0
A. niger isolates				
Pomegranate peel	11.5	10.0	7	0
Tamarind	12.5	10.5	8	0
Orange peel	10	7.0	0	0
Apple	7.0	0	0	0
Groundnut	10.0	8.0	0	0

Table 3. Inhibition zone in (mm) by minimum inhibitory concentration evaluation of extract procured from Haryana region

Fungal species	Haryana			
Methanolic Extract (µg ml$^{-1)}$	10000	6000	4000	2000
A. flavus	14.0	12.5	7.0	0
A. nidulans	9.0	7.0	0	0
Fusarium species	0	0	0	0
A. niger isolates				
Pomegranate peel	12.0	11.0	9.0	7.0
Tamarind	13.0	10.0	8.0	0
Orange peel	11.0	8.0	0	0
Apple	10.0	8.0	0	0
Groundnut	10.0	7.0	0	0

solvent extract. The preliminary qualitative screening of phytochemicals indicated the presence of flavonoids, alkaloids, glycosides saponins, triterpenoids, tannins, carbohydrates and proteins in all the extracts of different locations. However, the plants collected from Haryana, reflected low profile of phytochemicls, qualitatively which might be attributed to the different geographical and environmental conditions and the locations from where the plants were collected since *A. aspera* is a weed so owing to allelopathic affect the concentration of phytochemicals my vary. Phytochemicals such as alkaloids, saponins, tannins and flavonoids are known to have curative activity against several pathogens and therefore could suggest the use traditionally for the treatment of various illnesses. (Hassan *et al.*, 2004 and Usman *et al.*, 2007). The broad antibacterial activities of this extract could be as a result of the plant secondary metabolites present in the extract. Small protein or peptide present in the methanolic extract plays an important role in plants's antimicrobial defense system (Ceolho *et al.*, 2004). Several investigators have reported that the methanolic extract of leaves of *Achyranthes aspera* has significant antimicrobial activity against the fungal species (Londonkar *et al.*, 2011).

The results of antifungal determinations for methanolic extract of whole plant of *Achyranthes aspera* procured from different locations, against four fungal species and five various isolates were investigated by agar well diffusion method. The result showed maximum inhibition in fungal growth in terms of zone of inhibition. The increase in zone of inhibition was concomitant with the increase in the concentration of extract (Table 1, 2 &3) in line with the findings of Usman and Osuji 2007. The maximum zone of inhibition was noted for *Aspergillus flavus* (17.5mm) (Fig.1a.) at the concentration of 10000µg ml^{-1} followed by *Aspergillus nidulans* (16mm) (Fig. 1.c.) and *Fusarium* (15.5mm) (Fig.1d.) species collected from Punjab (Table 1).



(a) (b) (c) (d)

Fig. 1. (a) Zone of inhibition of *Aspergillus flavus* at 10000µg ml^{-1} methanol extract from Punjab (b) Zone of inhibition of *Aspergillus flavus* at 10000µg ml^{-1} methanol extract from Himachal Pradesh

Fig. 2. (c) Zone of inhibition of *Aspergillus nidulans* at 10000µg ml^{-1} methanol extract from Punjab region Punjab (d) Zone of inhibition of *fusarium species* at 10000µg ml^{-1} methanol extract from Punjab region

Different isolates of *Aspergillus niger* were also tested against extract of the varying concentration, the maximum zone of inhibition was observed in the isolate grown on pomegranate (Table 1). At 2000µg ml^{-1} *Aspergillus nidulans* and *Aspergillus niger* showed zone of inhibition (7 & 8mm respectively) (Table 1) from Punjab region. The extent of inhibition was decreased with the decrease in concentration of extract. Plants procured from Himachal Pradesh showed less antifungal activity as compared to the Punjab region. The zone of inhibition shown by *Aspergillus flavus* at 10000µg ml^{-1} was 15mm and 14mm from Himachal Pradesh and Haryana region respectively. The variation might be attributed to the difference in varieties of plants. The *Fusarium* species could not show any inhibition beyond 6000µg ml^{-1} from all the locations studied (Table 1, 2 & 3). Reports are available on the study of leaves and stem (Cragg *et al.*, 2001) so the present study

was done on whole plant keeping in view to study the combined effect of all plant parts. The result obtained from Punjab region is encouraging hence the extracts can be used to carry out further pharmacological evaluation.

CONCLUSION

The present investigation indicates that antifungal potential of *Achyranthus aspera* is affected by environmental conditions / allelopathy and increases with the concentration of extract. The plant collected from Punjab region were found with highest potential followed by Himachal Pradesh region and Haryana. The plants from Punjab region are recommended to explore further isolation of therapeutical antifungal compounds and carry out further pharmacological evaluation and the development of herbal antifungal drug.

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