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Research Article

INVITRO ANTIBACTERIAL AND ANTIFUNGAL ACTIVITIES ALONG WITH X-RAY IRRADIATION STUDIES OF MEDICINAL PLANT *HYGROPHILA AURICULATA*

CHRISTIBAI JULIET ESTHER V*[1], SARASWATHI R[2], DHANASEKAR S[3]

*[1]Department of Chemistry, PRIST University, Vallam, Thanjavur 613403, Tamilnadu, India, [2]Department of Chemistry, Kunthavi Nachiyar Govt. Arts College for Women, Thanjavur 613007, Tamilnadu, India, [3]Department of Chemistry, V.R.S College of Engineering and Technology, Arasur 607107, Tamilnadu, India. *Email: christychemisty@yahoo.com

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ABSTRACT

In the present study, the medicinal plant *Hygrophila auriculata* was selected and the major phytochemicals and amino acids present in this plant were analysed using standard qualitative tests. The presence of amino acids such as glycine, proline, valine and phenylalanine was confirmed by paper chromatography. The antimicrobial activity of the ethanolic extract of the plant was studied against five selected bacterial and five selected fungal pathogens. The well method employed using various microorganisms. The effects of x- ray on the extract were also screened, where higher dosages gave maximum activity compared to lower and normal. This study gives a new route to discovery of new drug with and without x- ray interaction.

Keywords: *Hygrophila auriculata*, Antimicrobial, Antifungal, Paper chromatography, X-ray irradiation

INTRODUCTION

Medicinal plants are gifts of nature to cure limitless number of diseases in human beings[1]. The abundance of plants on the earth's surfaces has led to an increasing interest in the investigation of different extracts obtained from traditional medicinal plants as potential sources of new therapeutic agents[2]. The use of plant extracts and phytochemicals with known antimicrobial properties can be of great significance in therapeutic treatments[3]. In the last few years, a number of studies have been conducted to prove such efficiency[4-6].Many plants have been used because of their antimicrobial traits, which is due to compounds synthesized as secondary metabolites by the plant. These products are known by their active substances, for example, the phenolic compounds which are part of the essential oils[7], as well as tannins[8]. The plant *Hygrophila auriculata* (K. Schum) Heine (Acanthaceae) has been traditionally used for the treatment of inflammation, pain, urinary infection, edema, gout and as a diuretic. It is described in ayurvedic literature as Ikshura, Ikshugandha, and Kokilasha having eyes like the Kokila or Indian Cuckoo. It is classified in the ayurvedic system of medicine as Seethaveryam, Mathuravipaka and is used for the treatment of a number of conditions including diabetes and dysentery[9, 10].The plants are widely distributed throughout India, Sri Lanka, Burma, Malaysia and Nepal. Following various folk claims as a cure for numerous diseases, efforts have been made by researchers to verify the efficacy of the plant by scientific biological screening. The plant contains saponins, alkaloids, steroids, tannins, flavonoids and triterpenoids are the main phytoconstituents. A scrutiny of literature revealed some notable pharmacological effects like anti-nociceptive[11], antitumor[12,13], antioxidant[14], hepatoprotective[15-18], hypoglycemic[19], haematinic[20], diuretic[21], free radical scavenging[22], anthelmintic[23], anti-inflammatory[21], antipyretic[24] and antimotility activities [25]. The present review is an attempt to highlight the various ethanobotanical and traditional uses as well as phytochemical and pharmacological reports about *Hygrophila auriculata* (K. Schum) Heine. The aim of the present work is to carryout phytochemical studies on the leaves of the medicinal plant, *Hygrophila auriculata* (*Heine.) and* to study the in-vitro antibacterial and antifungal activities of the leaf-extract of the plant both before and after irradiation with x-ray.

MATERIALS AND METHODS

Collection of Plant Material

The plant material *Hygrophila auriculata* was collected from paddy fields in and around Kurunguloor village and Gurudayalsharma Kalyanamandapam in Manambuchavady area in Thanjavur District on different days. The whole plants were collected and refrigerated for 2 to 4 days to keep them fresh.

Fig. 1: Photograph of *Hygrophila auriculata*

Preparation of Plant Extract

The plant leaves were dried in shadow. The dried plant leaves were subjected to pulverization using a grinder, to get coarse powder. To 20g of the coarse powder, 100ml of absolute alcohol (Analytical Reagent) is added. The mixture was kept soaking for 24 hours. Then the mixture was filtered using Whatmann- 41 filter paper. The filtrate of the ethanol extract was stored in air tight container in a refrigerator. The extract was used for phytochemical screening of compounds, and for antimicrobial studies before and after exposure to x-rays.

Qualitative tests were carried out with the extract to examine the presence of the following classes of compounds.

Alkaloids

The alcoholic extract (corresponding to 2.5g of plant on a material) was evaporated to dryness and the residue was heated on a boiling water bath with 2N HCL (5ml). After cooling, the mixture was filtered and the filtrate was divided into two equal portions. One portion was treated with a few drops of Mayer's reagent (LR – National chemicals) and the other with equal amounts of Wagner's reagent (LR-National Chemicals). The samples were then observed for the presence of turbidity or precipitation. A(+) score was recorded if the reagent produced only a slight opaqueness; a(++) score was recorded if a definite turbidity, but no flocculation was observed and a (+++) score was recorded if a definite heavy precipitate or flocculation was produced.

Flavonoids

The alcoholic extract (5ml, corresponding to 1g of plant material) was treated with a few drops of concentrated HCL and magnesium turnings (LR, National chemicals) (0.5g). The presence of flavonoids was indicative if pink or magenta-red color developed within 3min.

Saponins

About 2.5g of the plant material was extracted with boiling water. After cooling, The extract was shaken vigorously to froth and was then allowed to stand for 15-20min and classified for saponin content as follows: no forth = negative; froth less than 1cm = Weakly positive; forth 1.2cm high = positive; and froth greater than 2cm high = strongly positive.

Tannins

The alcoholic extract (corresponding to 1g of plant material) was evaporated and the residue was extracted by 10ml of hot 0.9% NaCl solution, filtered and divided into 3 equal portions. A sodium chloride solution was added to one portion of the text extract, 1% gelatin solution to a second portion and the gelatin salt reagent (LR, National Chemicals) to a third portion precipitation with both the second and third reagent is indicative of the presence of tannins. Positive tests are confirmed by the addition of $FeCl_3$ solution to the extract and should result in a characteristic blue, blue-black, green or blue-green color and precipitate.

Determination of Amino acids by Chromatographic Technique

Preparation of the Sample

5g of fresh plant leaf material of the same size and age from 4th node from the apex in all the cultivars and types were crushed with 10ml of alcohol and hydrochloric acid mixture (99:1) in a glass mortar. 30ml of chloroform (LR, SD fine-chem. limited) was added and shaken thoroughly. Afterwards the supernatant was separated, using a separating funnel and centrifuged to remove debris. The supernatant was used for spotting.

Paper Chromatography

Chromatograms were developed in each cultivar and type to know the nature of protein and amino acids. The solvent was a mixture of n-butanol, acetic acid (LR, Hi-pure fine chem. Industries) and distilled water (BAW solution) in the ratio 4:1:5. Grade: 1 CHRO (Biomed Instrument Industry) chromatography paper of 10x18cm dimension and 0.18mm thickness were used. A pencil line is drawn about 1.5cm from one end. A drop of the concentrated solution of the sample was spotted using a capillary pipette. Spot should be about 5mm in diameter. The solvent was allowed to evaporate. The paper is hung from a horizontal metallic rod vertically so that about 0.5cm of the paper is dipped in the solvent mixture taken in a metallic tray. Then the solvent flows through the paper to produce separation. When the solvent front has traveled almost to the top of the paper, the paper was taken out and dried. The chromatogram was spotted using Ninhydrin spray.

The spray regent used was 0.1 percent Ninhydrin (Loba-Chemie Indoaustranal co.) in acetone. After spraying, the paper was heated at 105^{0}C for two minutes. The colors of the spots were recorded from the chromatogram. The Rf values of the spots were determined.

$$Rf = \frac{\text{Distance traveled by the sample}}{\text{Distance traveled by the solvent}}$$

Antimicrobial Studies

Media Preparation

Bacterial Media (Muller Hinton Media)

36g of Muller Hinton Media (Hi-Media) was mixed with distilled water and then sterilized in autoclave at 151b pressure for 15minutes. The sterilized media were poured in to Petri dishes. The solidified plates were bored with 5mm dia. cork borer. The plate wells were used for the antibacterial studies.

Fungal Media (PDA)

200gm of potato slices were boiled with distilled water. The potato infusion was used as water source of media preparation. 20g of dextrose was mixed with potato infusion. 20g of agar was added as a solidifying agent. These constituents were mixed and autoclaved. The solidifying plates were bored with 6mm dia. cork borer.

Antibacterial activity of the plant extract

The ethsssanolic extract of *Hygrophila auriculata* was used throughout the study. 50, 100, & 200µl of the extract were tested against different bacterial pathogens such as *Escherichia Coli, Citrobacter divergens, Enterobacter faecalis, Klebsiella pnemoniae and serratia marcescens.*

Antifungal activity of the plant extract

The ethanolic extract of *Hygrophila auriculata* was used throughout the study. 50, 100,& 200µl of the extract were tested against different fungal pathogens, such as *Aspergillus niger, aspergillus flavus, Aspergillus fumigatous, Rhizobus inducum and Fusarium sps.*

Antibacterial and Antifugal Activity of plant Extract after Exposure to x- ray radiation

The ethanolic extract of the *Hygrophila auriculata* was poured into two separate Petri dishes. Each plate contained approximately 15ml of extract. They were placed one by on the x-ray table. The two plates were exposed two different strength of x-rays especially. The dosages were 55KVP 16MAS and 82 KVP 120MAS. The duration of x-ray exposure to plant extract was around 15minutes. And after that they were used to screen for antimicrobial activities. Their effects were compared with the normal extract values.

Well Diffusion Method

Antibacterial and antifungal activity of the plant extract were tested using well diffusion method[26]. The prepared culture plates were inoculated with different selected strains of bacteria and fungi using streak plate method. Wells were made on the agar surface with 6mm cork borer. The extracts were poured into the well using sterile syringe. The plates were incubated at 37$\pm$2^{0}C for 24 hours for bacterial and 25$\pm$2^{0}C for 48 hours for fungal activity. The plates were observed for the zone formation around the wells. The extract of the dried leaves was used throughout the study. The ethanolic extract was dissolved in sterile distilled water to form dilution such as 50, 100, & 200. Each concentration of the extracts was tested

against different bacterial pathogens. It was demonstrated by well diffusion assay[26]. The zone of inhibition was calculated by measuring the diameter of the inhibition zone around the well (in mm) including the well diameter. The readings were taken in three different fixed directions in all three replicates and the average values were tabulated in table 7.

RESULTS AND DISCUSSION

For all human ailments, herbal medicines are available in our environment itself. The present investigation had been undertaken to find out the screening of the availability of phytochemicals, amino acids and antimicrobial activity of ethanol extract of *Hygrophila auriculata* L. with and without exposure to different strengths of x-ray radiation.

Phytochemical Studies

Alkaloids, flavonoids, tannins and saponins were qualitatively evaluated in the alcoholic extracts of *Hygrophila auriculata* L. All the above substances were found to be present in this extract. Fresh leaf extract was screened for the presence of amino acids by paper chromatography. The amino acids detected were glycine, proline, valine and phenylalanine.

Table 1: Experimental & Theoretical values of Rf

S. No.	Amino acids present	Rf values Experimental	Rf values Literature
1	Glycine	0.170	0.17
2	Proline	0.297	0.30
3	Valine	0.468	0.47
4	Phenylalanine	0.594	0.58

The leaf extract of the herb, *Hygrophila auriculata* was screened for antimicrobial activity against different pathogenic prokaryotic and eukaryotic micro organism in terms of zone inhibition. The leaf extract was subjected to irradiation with low and high doses of x-rays separately and the irradiated leaf extracts were also tested for antimicrobial activity against the same selected set of bacterial and fungal strains. The leaf extract showed inhibition against *Aspergillus niger* but did not inhibit the other pathogenic fungi tested- *Aspergillus flavus, Aspergillus fumigatous, Rhizobus indicum* and *Fusarium* sps. Similar results are found in the literature. Amer Jamil *et al.*,[27] have screened 7 medicinal plants including *Hygrophila auriculata* against 4 fungal strains. Their results show that the leaf extract of *Hygrophila auriculata* shows antifungal activity against the fungal strains, *Mucor mucedo* and *Aspergillus niger* and is inactive against *Aspergillus tamarii* of *Rhizopus solani*. They have also shown that the seed extract of the plant inhibits all the above four fungi. In the present work, the leaf-extract alone was tested and the results are consistent with those found in earlier literature. The leaf-extract was also screened against gram +ve and gram -ve bacterial strains and inhibition of bacterial growth was observed in the case of *E-coli* and *Klebsiella pneumoniae* whereas there was no apparent antibacterial activity by the leaf extract of *Hygrophila auriculata* against *Citobactor divergens*, *Enterobactor faecalis* and *Serratia marcescens*.Samples of the leaf extract of *Hygrophila auriculata* were separately exposed to a single dose of x-ray radiation each at two different strengths (55 Kvp & 82 Kvp) and the irradiated the leaf extract of *Hygrophila auriculata* showed some interesting inhibition trend against the fungal and bacterial strains. Inhibition of *Aspergillus niger* showed a linear correlation with the plain the leaf extract of *Hygrophila auriculata* and with the irradiated the leaf extract of *Hygrophila auriculata* and greater the dose strength greater was the inhibition. In the case of *Aspergillus fumigatous*, while the plain the leaf extract of *Hygrophila auriculata* showed no detectable antibacterial activity, the irradiated the leaf extract of *Hygrophila auriculata* exhibited the same trend of enhanced antifungal activity with the strength of x-ray dose.The trend shown by the leaf extract of *Hygrophila auriculata* with regard to antibacterial activity was unique in its own way. While the inhibition of *E-coli* was exactly the same by the plain as well as by the irradiated the leaf extract of *Hygrophila auriculata*, there was a non-linear trend shown against *Klebsiella pneumoniae*. The leaf extract of *Hygrophila auriculata* irradiated with low dose of x-ray showed a lower zone of inhibition as compared to plain the leaf extract of *Hygrophila auriculata* whereas the high dose x-ray irradiated the leaf extract of *Hygrophila auriculata* exhibited higher zone of inhibition. Further, the fungal strain *Enterobactor faecalis*, whose growth was not apparently inhibited by plain and low dose x-ray irradiated the leaf extract of *Hygrophila auriculata* was inhibited by high dose irradiated the leaf extract of *Hygrophila auriculata*.The antifungal activity of plant extract have been attributed mainly to bioactive protein/peptide. The alteration in the activity of protein/peptides or enzymes on being subjected to ionizing radiation could have resulted in increased anti microbial activity of the x-ray irradiated leaf extract. More work is needed to identify the key factor responsible for the effect. X-ray treated extracts gave greater responses than the normal extract. Higher dosage of extract gave maximum and minimum from the lower level of extract (Fig.1 & Fig.2).

Table 2: Antifungal activity of *Hygrophila auriculata*

S. No.	Name of the Organism	Zone inhibition (mm)		
		50µl	100µl	200µl
1	Aspergillus niger	8	9	11
2	Aspergillus flavus	-	-	-
3	Aspergillus fumigatous	-	-	-
4	Rhizobus inducum	-	-	-
5	Fusarium sps	-	-	-

Table 3: Antibacterial activity of *Hygrophila auriculata*

S. No.	Name of the Organism	Zone inhibition (mm)		
		50µl	100µl	200µl
1	Escherichia coli	8	11	14
2	Citrobacter divergens	-	-	-
3	Enterobacter faecalis	-	-	-
4	Klebsiella pneumoniae	11	13	19
5	Serratia marcescens	-	-	-

Table 4: Antifungal activity of Low x-ray exposed extract of *Hygrophila* auriculata

S. No.	Name of the Organism	Zone inhibition (mm)		
		50µl	100µl	200µl
1	Aspergillus niger	9	11	13
2	Aspergillus flavus	-	-	-
3	Aspergillus fumigatous	8	12	16
4	Rhizobus inducum	-	-	-
5	Fusarium sps	-	-	-

Table 5: Antibacterial activity of Low x-ray exposed extract of *Hygrophila* auriculata

S. No.	Name of the Organism	Zone inhibition (mm)		
		50µl	100µl	200µl
1	Escherichia coli	8	11	14
2	Citrobacter divergens	-	-	-
3	Enterobacter faecalis	-	-	-
4	Klebsiella pneumoniae	9	11	15
5	Serratia marcescens	-	-	-

Table 6: Antifungal activity of High x-ray exposed extract of *Hygrophila auriculata*

S. No.	Name of the Organism	Zone inhibition (mm)		
		50µl	100µl	200µl
1	Aspergillus niger	11	14	17
2	Aspergillus flavus	-	-	-
3	Aspergillus fumigatous	10	14	19
4	Rhizobus inducum	-	-	-
5	Fusarium sps	-	-	-

Table 7: Antibacterial activity of High x-ray exposed extract of *Hygrophila* auriculatas

S. No.	Name of the Organism	Zone inhibition (mm)		
		50µl	100µl	200µl
1	Escherichia coli	8	11	14
2	Citrobacter divergens	-	-	-
3	Enterobacter faecalis	9	15	23
4	Klebsiella pneumoniae	14	17	21
5	Serratia marcescens	-	-	-

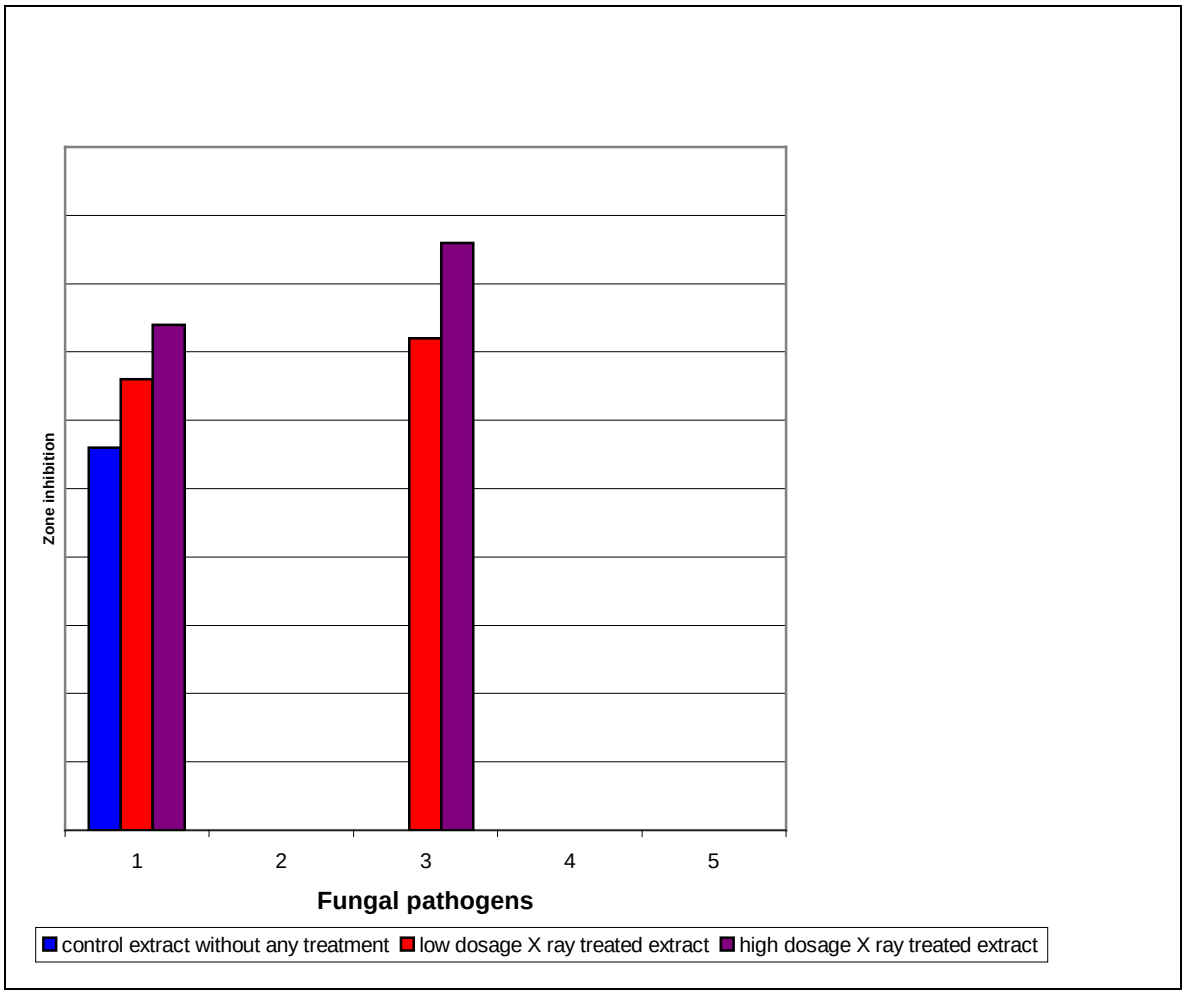

Fig. 1: Antifungal activity of *Hygrophila* auriculata

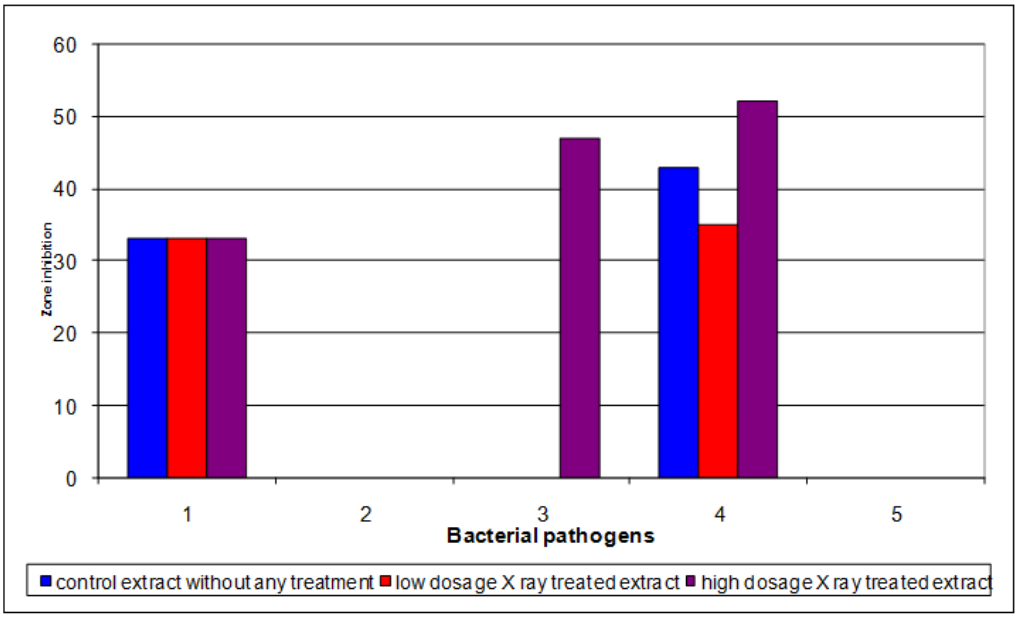

Fig. 2: Antibacterial activity of *Hygrophila* auriculata

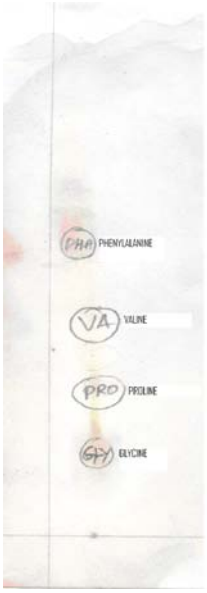

Fig. 3: Paper chromatography for amino acid screening

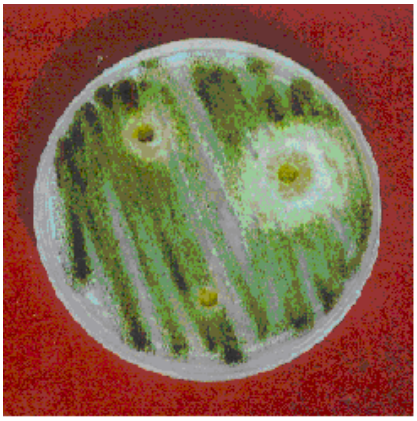

Fig. 4: In vitro antifungal activity of alcoholic extract of Hygrophila auriculata L. on Aspergillus niger

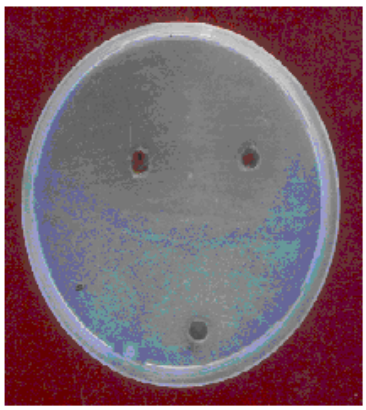

Fig. 5: In vitro antibacterial activity of alcoholic extract of Hygrophila auriculata L. on Escherichia coli.

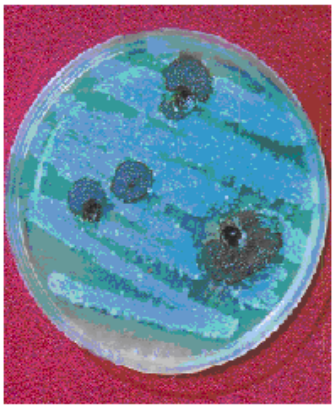

Fig. 6: In vitro antibacterial activity of alcoholic extract of Hygrophila auriculata L. on Klebsiella pneumoniae.

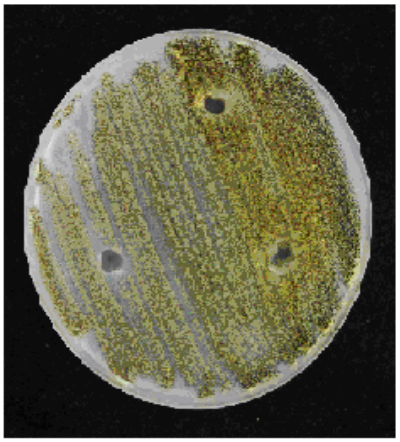

Fig. 7: In vitro antifungal activity of Low X ray treated alcoholic extract of Hygrophila auriculata L. on Aspergillus fumigatus.

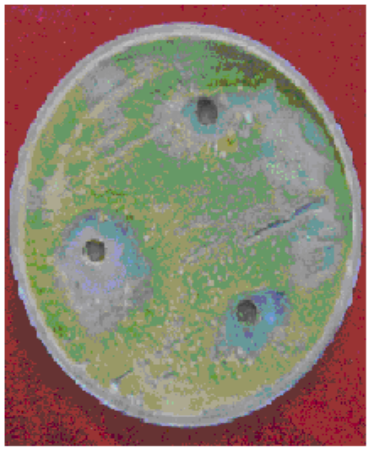

Fig. 8: In vitro antifungal activity of Low X ray treated alcoholic extract of Hygrophila auriculata L. on Aspergillus fumigatus.

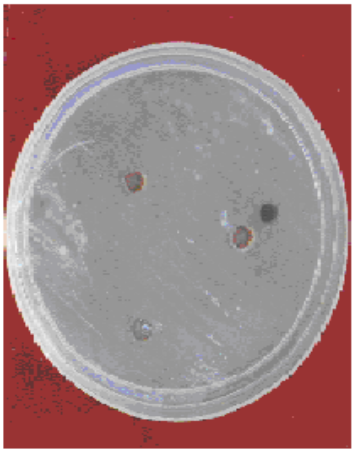

Fig. 9: In vitro antifungal activity of Low X ray treated alcoholic extract of Hygrophila auriculata L. on Escherichia coli.

Fig. 10: In vitro antibacterial activity of Low X ray treated alcoholic extract of Hygrophila auriculata L. on Klebsiella pneumoniae.

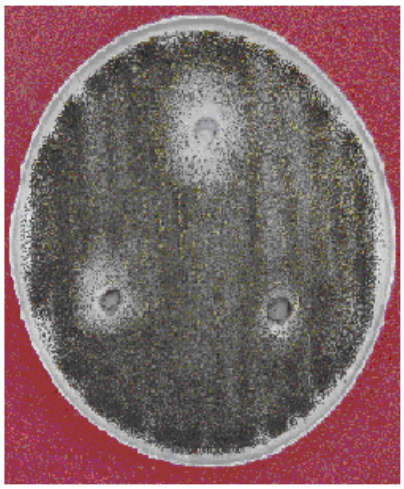

Fig. 11: In vitro antifungal activity of High X ray treated alcoholic extract of Hygrophila auriculata L. on Aspergillus niger.

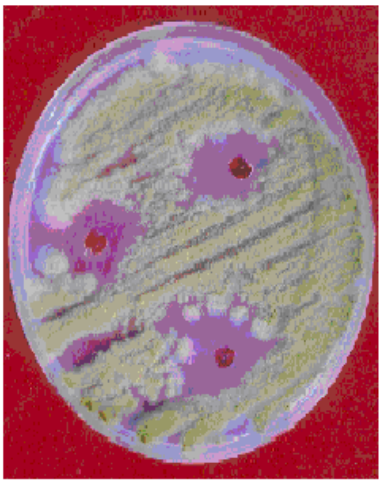

Fig. 12: In vitro antifungal activity of High X ray treated alcoholic extract of Hygrophila auriculata L. on Aspergillus fumigatus.

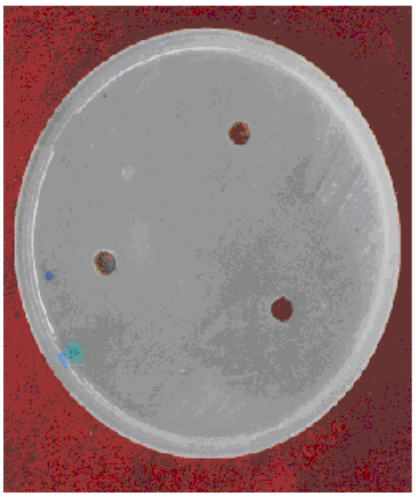

Fig. 13: In vitro antibacterial activity of High X ray treated alcoholic extract of Hygrophila auriculata L. on Enterococcus feacalis.

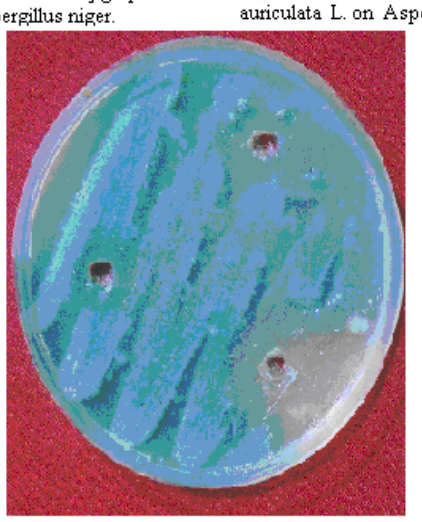

Fig. 14: In vitro antibacterial activity of High X ray treated alcoholic extract of Hygrophila auriculata L. on Klebsiella pneumoniae.

As a preliminary step, in the present study, the medicinal plant *Hygrophila auriculata* was selected and the major phytochemicals and amino acids present in this plant were analysed using standard qualitative tests. The antimicrobial activity of the ethanolic extract of the plant was studied against five selected bacterial and five selected fungal pathogens. The effects of x-ray on the extract were also

screened, where higher dosages gave maximum activity compared to lower and normal. This study gives a new route to discovery of new drug with and without x-ray interaction.

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