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In vitro antibacterial and antifungal potential of methanolic crude extracts of some *Heliotropium* spp.

Yasir Ihtesham[1,2]*, Uzma Khan[3], Ikram Ullah[4], Wajiha Khan[5], Rizwana Aleem Qureshi[1]

[1]Department of Plant Sciences, Quaid-i-Azam University Islamabad, Pakistan.
[2]Department of Pharmacognosy, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey.
[3]Department of Botany, Hazara University, Mansehra, Pakistan.
[4]Department of Biotechnology, Quaid-i-Azam University Islamabad, Pakistan.
[5]Department of Environmental Sciences, COMSATS Institute of Information Technology, Abbottabad, Pakistan,

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ABSTRACT

Natural products obtained from plants are best source of remedies for many infectious diseases. The active ingredients are found in each part of the plant. In the present study the methanolic crude extracts of three plants, that is, *Heliotropium europeaum* L., *Heliotropium curassavicum* L. and *Heliotropium crispum* Desf. of family Boraginaceae were tested against five pathogenic bacterial and two fungal strains using agar well diffusion and tube dilution methods respectively. Different concentrations (5.0, 7.5, 10.0, 12.5 and 15.0 mg/ml) against bacterial strains and only one concentration of 15 mg/10 ml for fungal strains were tested. The activity of *Heliotropium* spp. was compared with the broad spectrum commercially available antibiotic doxycyclin (DOX). The results indicate that the different concentrations of extract of the three plants have a wide range of antibacterial and antifungal activity. Among the different concentration used, 15 mg/ml was found best against bacterial strains. *Staphylococcus aureus* was the most sensitive strain and *Bacillus subtilis* was the most resistant strain towards all the extracts of three plants. In the present study, methanolic extract of these plants showed good growth inhibition at 15 mg/10 ml concentration against the two fungal species used. By analyzing the overall data it could be concluded that the sensitivity of the microbes increase with increase in the crude extract concentration in the well. All the concentration has high efficiency and broad spectrum activity but lower than that of standard antibiotic.

Keywords: *Heliotropium europeaum* L. *Heliotropium curassavicum* L., *Heliotropium crispum,* antibacterial activity, antifungal activity.

*Corresponding author. E-mail: yasir.ihtesham@yahoo.com. Tel: 0090-534-7016373.

INTRODUCTION

The use of plants as source of remedies for the treatment of many diseases dated back to prehistory and people of all continents have this old tradition (Ogbonna et al*.,* 2001). Despite the remarkable progress in synthetic organic chemistry of the twentieth century, over 25% of prescribed medicines in industrialized countries derived directly from plants (Newman et al., 2000). In developing countries, where medicines are quite expensive, investigation on antimicrobial activities from ethno medicinal plants may still be needed. Plants produce a diverse range of bioactive molecules, making them a rich source of different types of medicines (Fazal et al*.,* 2011), and these molecules will find their way in the arsenal of antimicrobial drugs prescribed by physicians (Cowan, 1999). In recent years, drug resistance to human pathogenic bacteria has been commonly and widely reported in literature. Because of the side effects and the resistance that pathogenic microorganisms build against antibiotics (Bandow et al., 2003), many scientists have paid attention to biologically active extracts from plant species used in herbal medicines (Essawi and Srour, 2000). The screening of plant extracts and plant products

for antimicrobial activity has shown that plants represent a potential source of new anti-infective agents (Amani et al., 1998; Salvat et al., 2001; Kloucek et al., 2007). In literature various other plants, *Cordia dichotoma* L. (Khond et al*.,* 2009), *Arnebia euchroma* Rolye (Pirbalouti et al*.,* 2009), *Echium amoenum* Fisch (Mehrabania et al*.,* 2005) of Boraginaceae family have significant amount of antimicriobial, antifungal and anti inflammatory activities. *Heliotropium* spp. are known to contain pyrrolizidine alkaloids and have a wide variety of biological activities such as antitumor, antibacterial, antifungal, insecticide, antispasmodic, mydriatic, mutagenic, teratogenic and hepatotoxic activity (Hernandez et al*.,* 2007; Okusa et al., 2007). Medicinal plant still needs to be testifying according to the modern parameters to ensure their activity and efficacy. The selectivity of the three species of *Heliotropium* is based on the abundance and only availability of all the three species in the area. Pakistani medicinal plants, for the purpose of drug development, are one of the least investigated sources of natural compounds. In the present study, three plant species (*Heliotropium europeaum* L*; Heliotropium curassavicum* L., and *Heliotropium crispum* Desf.) of Boraginaceae family were tested against five pathogenic bacterial (*Bacillus subtilis, staphylococcus aureus, Escherichia coli*, *vibrio cholerae* and *Enterobacter aerogenes*) and two fungal strains (*Aspergillus nigar and Aspergillus fumigatus*).

To the best of our knowledge, here we report for the first time, the *in vitro* antibacterial and antifungal activity of the methanolic crude extracts of the *Heliotropium* spp. against certain pathogenic bacterial and fungal strains.

MATERIALS AND METHODS

Plant collection and preparation of extracts

Three medicinal plant species (*H. europaeum*, *H. curassavicum*. and *H. crispum*) of Boraginaceae family were collected from Bahawalpur District of Pakistan where plants were identified by Professor Dr. Rizwana Aleem Qureshi and each plant was deposited in the herbarium of Quaid-i-Azam University Islamabad [Vouchers No. RAW. 2251, 369 and 76, respectively]. The entire plants were rinsed with distilled water and kept under shade till drying. Shed drying method was applied to not to lose heat sensitive compounds. The dried plant materials were grounded to fine powder using kitchen blender. Methanol was added to it and kept for three days at room temperature in extraction bottle. After three days, mixture was filtered twice, using Whatman-41 filter paper. Methanol was then completely evaporated by rotary evaporator to obtain the extract. 15 mg of the extract was dissolved in 10 ml of DMSO and diluted to 15, 12.50, 10, 7.5 and 5 mg/ml. Dilution (2 mg/ml) of standard antibiotic Doxycycline (DOX) and DMSO was used as reference for positive and negative control.

Antibacterial assay

Tested bacterial strains

Five strains of bacteria were used. Two were gram-positive i.e. *Bacillus subtilis* and *staphylococcus aureus* while three were gram-negative; *Escherichia coli*, *Vibrio cholerae* and *Enterobacter aerogenes*. The organisms were maintained on nutrient agar medium at 40°C.

Preparation of inoculum

Nutrient broth medium (Merck) was used to grow bacteria for inoculums preparation. Centrifuged palates of bacteria from 24 hour old culture in nutrient broth (SIGMA) of selected bacterial strains were mixed with physiological normal saline solution until a McFarland turbidity standard [10^{-6} colony forming unit (CFU) ml^{-1}] was obtained.

Preparation and inoculation of seeded agar plates

Nutrient agar medium was prepared by suspending nutrient agar (Merck) 2.8 g in 100 ml of distilled water; pH was adjusted to 7.0 and autoclaved at 121°C for 20min. It was allowed to cool up to 45°C. Petri plates were prepared by pouring 75 ml of nutrient agar and allowed to solidify. Then it was seeded with 10ml of prepared inocula. Ten wells per plate were made with sterile cork borer (5 mm). Using micropipette, 100 µl of test solutions was poured in respective wells. These plates were incubated at 37°C. After 24 hours of incubation; the diameter of the clear zones of inhibitions were measured by a ruler. Antibacterial activity of each dilutions of plants extract was determined against five bacterial strains.

Antifungal assay

For antifungal assay initial stock of 15 mg of each plant extract per 10 ml of DMSO was prepared. Two fungal strains**,** *Aspergillus nigar and Aspergillus fumigatus* was tested against plants extract using agar tube dilution method. Sabouraud dextrose agar (MERCK) was used to grow fungus for inoculums preparation.

The sabouraud dextrose agar (MERCK) dispensed as 4ml volume into screw capped tubes or cotton plugged test tubes and were autoclaved at 121°C for 20 min. Tubes were allowed to cool to 50°C and non-solidified SDA were loaded with 100 µl of 15 mg/10ml plant extracts using compound pipette from the stock solution. Tubes were then allowed to solidify in slanting position at room temperature.

The tubes containing solidified media and test compound were inoculated with 4mm diameter piece of inoculums, taken from a seven days old culture of fungus. Negative control test tubes without extract were also inoculated. The test tubes were incubated at 28°C for 7 days. Cultures were examined twice weekly during the incubation. Reading was taken by measuring the linear length of fungus in slant by measuring growth (mm) and growth inhibition was calculated with reference to negative control.

Data analysis

Each treatment consists of three replicates, repeated twice. Data are presented in tables. Zone of inhibition was based on readings. Mean and standard deviation was calculated using statistical software SPSS. Percent inhibition of fungal growth for each plant extract was determined by the following formula:

$$\% \text{ inhibition} = \frac{100 - \text{Linear growth in test tube (mm)}}{\text{Linear growth in control (mm)}} \times 100$$

RESULTS

Methanolic extracts of three plants *H. curassavicum* L., *H. crispum* Desf. and *H. europaeum* L. were tested against five strains of bacteria. Two strains were gram-positive, that is, *Bacillus subtilis* and *Staphylococcus aureus* and three were gram-negative, that is, *Vibrio cholerae, Enterobacter aerogenes* and *Escherichia coli.* The inhibitory zone of methanolic crude extract of *Heliotropium* species is presented in Table 1 and Figure 1 only showed the inhibition at highest concentrations i.e. 15 mg/ml. DMSO were used as solvent and it was found that to have no effects on the growth of bacterial species tested. Standard antibiotic (2 mg/ml) DOX (Doxycycline) was used as reference.

The different methanolic crude extract concentrations of *H. curassavicum* L. shows that as we increase the concentration of crude extract, the activity against bacteria increase. The highest activity of (15 mg/ml) crude extract of *H. curassavicum* L., was found (25 ± 0.21 mm) and (15 ± 0.17 mm) against *S. aureus and E. aerogenes*, respectively.

Similarly the different crude extracts concentrations of *H. crispum* Desf. and *H. europaeum* L. shows that antibacterial activity increase with increase in the crude extract concentration. *Enterobacter* aerogenes was found resistant to all the crude extract concentrations of *H. crispum* Desf. The highest activity was found against *S. aureus* (30 ± 0.09 mm) and *E. coli* (15±0.39 mm) at 15 mg/ml concentration and the lowest was (05 ± 0.04 mm) for *Bacillus subtilis.*

The methanolic crude extract (15 mg/ml) of *Heliotropium europaeum* L. was found to be effective against *S. aureus* (25 ± 0.16 mm) and *E. coli* (20 ± 0.11 mm). Over all the different crude extract concentration of three plants shows activity against the tested micro organism, but all the concentration was most effective against *S. aureus, Enterobacter aerogenes* and *Escherichia coli.* However, the efficacy of the extracts was lower as compared to the standard antibiotic used. This may be due to the fact that, we used crude extract and it requires further purification for higher efficiency.

Table 1. Zones of inhibition (mm) of methamolic crude extracts (mg/ml) of *Heliotropium curassavicum* L., *Heliotropium crispum* Desf., and *Heliotropium europaeum* L. against pathogenic bacteria.

Plant species	Methanolic extract (mg/ml)	Mean of zones of inhibition (mm) against bacterial strains used ± SD				
		Vibrio cholerae	***Enterobacter aerogenes***	***Staphylococcus aureus***	***Bacillus subtilis***	***Escherichia coli***
H. curassavicum L.	15.0	14 ± 0.12	15 ± 0.17	25 ± 0.21	12 ± 0.32	12 ± 0.14
	12.5	12 ± 0.09	14 ± 0.32	25 ± 0.16	12 ± 0.22	12 ± 0.12
	10.0	11 ± 0.16	14 ± 0.16	25 ± 0.17	12 ± 0.07	12 ± 0.02
	7.5	10 ± 0.05	14 ± 0.31	25 ± 0.32	11 ± 0.09	11 ± 0.31
	5.0	10 ± 0.20	13 ± 0.15	20 ± 0.19	10 ± 0.14	11 ± 0.45
H. crispum Desf.	15.0	10 ± 0.21	00	30 ± 0.09	05 ± 0.04	15 ± 0.39
	12.5	10 ± 0.07	00	20 ± 0.05	03 ± 0.06	15 ± 0.25
	10.0	10 ± 0.13	00	20 ± 0.04	03 ± 0.13	14 ± 0.22
	7.5	08 ± 0.11	00	20 ± 0.31	02 ± 0.11	13 ± 0.31
	5.0	07 ± 0.23	00	20 ± 0.23	02 ± 0.21	12 ± 0.41
H. europaeum L.	15.0	10 ± 0.11	14 ± 0.14	25 ± 0.16	15 ± 0.22	20 ± 0.11
	12.5	10 ± 0.03	14 ± 0.15	23 ± 0.07	10 ± 0.01	18 ± 0.21
	10.0	10 ± 0.06	13 ± 0.11	20 ± 0.16	10 ± 0.02	17 ± 0.35
	7.5	10 ± 0.23	12 ± 0.21	20 ± 0.25	10 ± 0.21	16 ± 0.33
	5.0	10 ± 0.14	12 ± 0.02	20 ± 0.07	7 ± 0.25	10 ± 0.07

H = Heliotropium, SD = standard deviation.

Antifungal assay

The methanolic crude extract (15 mg/10 ml) of *H. crispum*, showed 15.38% (110 mm) and 16% (105 mm) growth inhibition against *Aspergillus nigar* and *Aspergillus fumigatus* respectively. *Heliotropium curassavicum* exhibited 15.38% (110 mm) growth inhibition against the fungal strain, that is, *Aspergillus niger*, and 20% (100 mm) growth inhibition against *A. fumigatus.* Similarly *H. europaeum* L. indicated 11.53% (115 mm) growth inhibition against the fungal strain *Aspergillus nigar* while 16% (105 mm) growth inhibition against *A. fumigatus* as shown in Table 2 and Figure 2. From the results it is clear that the crude extract has antifungal activity against the

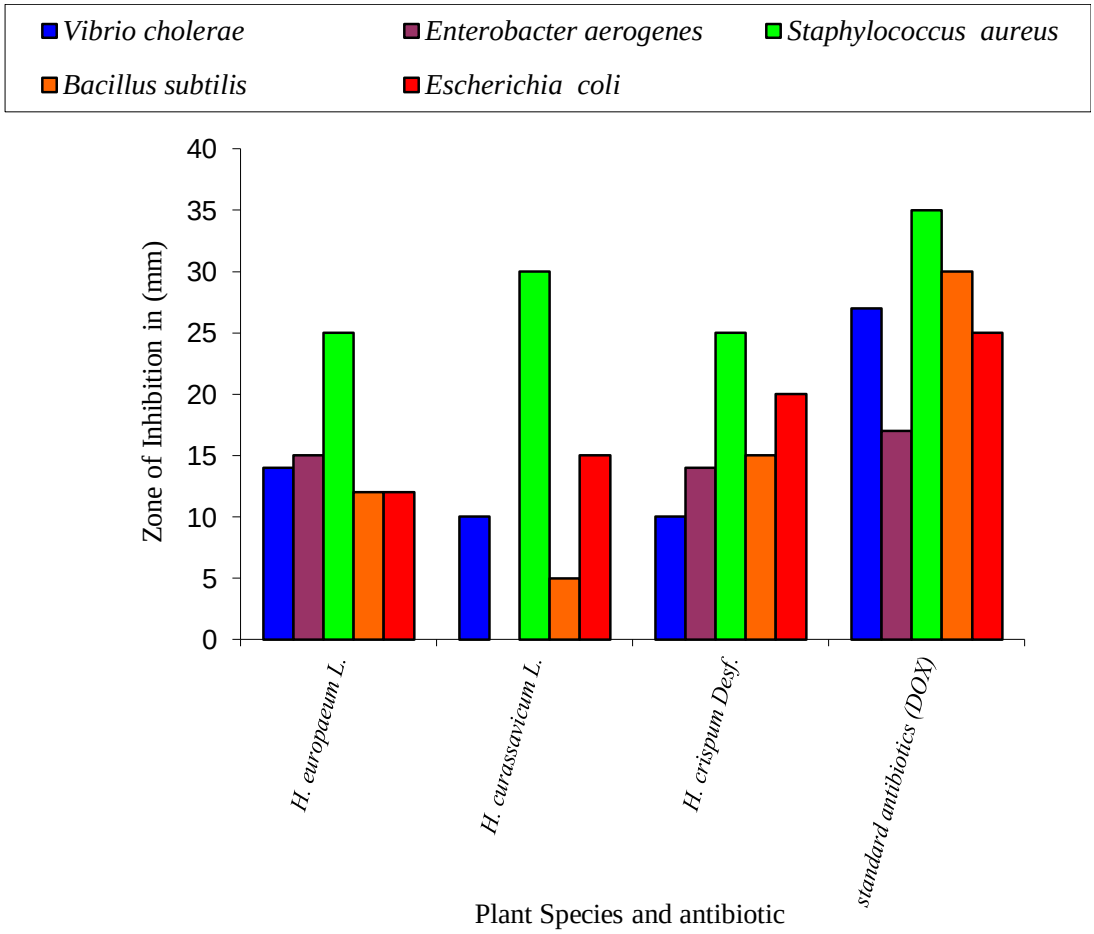

Figure 1. Zone of inhibition of three plant extracts (15 mg/ml) against tested bacterial strains.

Table 2. Data showing antifungal activity of methanolic crude extract (15 mg/10ml) of three plants of family Boraginaceae.

Plant species	Fungal strain	Control growth (mm)	Growth inhibited (mm)	% Inhibition
H. curassavicum L.	*Aspergillus niger*	130	110	15.38
	Aspergillus fumigatus	125	105	16.00
H. crispum Desf.	*Aspergillus niger*	130	110	15.38
	Aspergillus fumigatus	125	100	20.00
H. europaeum L.	*Aspergillus niger*	130	115	11.53
	Aspergillus fumigatus	125	105	16.00

tested fungal strains but there are no significant differences in the growth inhibition of the three plants against respective fungal strain. The plants extracts with media in the test tubes were considered as tests and only media in the test tubes without any plants extracts were taken as control. The species *Aspergillus niger* gave 130 mm growth and it was consider as control. Whereas *A. fumigatus* showed 125 mm growth and it was also taken as control. All antifungal results were compared with the two controls of each fungus strain.

DISCUSSION

Plant crude extracts are a mixture of various active and non-active compounds (Brantner and Grein, 1994).

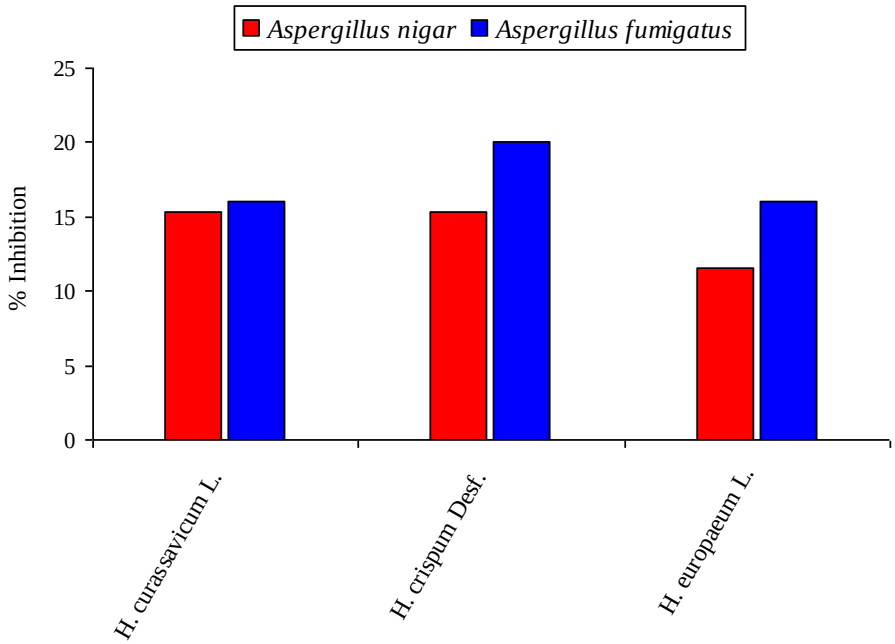

Figure 2. Percent growth inhibition of fungal strains against respective plant extracts.

Family Boraginaceae chemical constituents include pyrrolizidine alkaloids, naphthaquinones, flavonoids, terpenoids, triterpenoids and phenols (Sharma et al., 2009). Ethno medicinal use of plants can be helpful for biological screening of natural products, because many infectious diseases are known to have been treated with herbal remedies throughout the history of mankind. In the present study three plants of Boraginaceae family, that is, *H. europaeum* L., *H. crispum* Desf., and *H. curassavicum* L. were selected and appeared to have potential for testing as a plant of high medicinal values for various antimicrobial activities. The bacteria selected for this study were three gram-negative (*Escherichia coli, Enterobacter aerogenes* and *Vibrio cholerae*) and two gram-positive (*B. subtilis* and *S. aureus*). The fungal strains used in this study were *A. niger* and *A. fumigatus*. The antibacterial activity was tested on the basis of the magnitude of zones of inhibition (in mm). The activity of *Heliotropium* spp. was compared with the broad spectrum commercially available antibiotic DOX. The overall result showed that *S. aureus* was the most sensitive strain and *B. subtilis* was the most resistant strain towards all the extracts of three plants. A study conducted by Mughal et al. (2010) using methanolic extract of *H. sterigosum* Willd showed good antimicrobial activity against certain pathogenic Gram positive and negative bacterial strain. By analyzing the overall data it could be concluded that the sensitivity of the microbes increase with increase in the crude extract concentration in the well. All the extracts concentration has high efficiency but lower than that of standard antibiotic. This may be due to the crude extract need to be further purified. Two new benzoquinones, heliotropinones A and B, have been isolated from the aerial parts of *H. ovalifolium* Forssk and posses antifungal activities against *Cladosporium cucumerinum* and *Candida albicans* as well as antibacterial activity against *B. subtilis* (Guntern et al*.,* 2001). In comparison to this, also in the present study, methanolic extract of these plants showed good growth inhibition at 15 mg/ml concentration against the used bacterial and fungal species..

Conclusion

From the study it is concluded that extracts of these plants have broad spectrum activity and these plants will be helpful in the development of alternative remedial system against various pathogenic organism without side effects.

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