In vitro antibacterial and antifungal activities of different solvent extracted samples from the stem of *Euphorbia heliscopia*

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Abstract: This paper presents the anti-microbial potentials of methanol, ethyl acetate, n-hexane, butanol and water extracted samples from the stem of *Euphorbia heliscopia* against *S. aureus* (Gram positive), *B. subtilis* (Gram positive), *P. aeruginosa* (Gram negative), *K. pneumonia* (Gram negative), *E. coli* (Gram negative), *C. albicans* (fungal specie) by discs diffusion susceptibility assay using 0.5 and 1mg disc^{-1} concentrations. Our results showed that all the extracted samples from the stem of *E. heliscopia* exhibited varying degree of antimicrobial activity. Ethyl acetate extracted samples measured maximum activity against the studies microbial species followed by the n-butanol and crude methanolic extract. n-hexane extracted samples inhibited the growth of all microbial species except *P. aeruginosa* and *E. coli* at lower concentration. Aqueous fractions showed inhibitory activity against *B. subtilis*, *K. pneumonia* and *C. albicans*. The most susceptible gram positive bacteria were *S. aureus* while *B. subtilis* was the most resistant one. Among Gram negative bacteria, *P. aeruginosa* showed more susceptibility while *K. pneumonia* was resistant.

Keywords: Antibacterial, antifungal, *Euphorbia heliscopia*, disc diffusion.

INTRODUCTION

For a long time, plant oils and extracts are in use for a wide range health related activities. These activities included from the use of rosewood and cedar wood in perfumery to flavoring drinks with lime, fennel or juniper berry oil, and the utilization of lemongrass oil for the preservation of stored food products. The antimicrobial activity of oils and extracts from plants in particular has many uses, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies (Hammer *et al.*, 1999). It is estimated that more than 80% of the world's population depends on herbal medicine for their primary health related issues (WHO, 2005). Among 6000 plant species in Pakistan, 700 are considered medicinally important (Shinwari and Qaiser, 2011; Bakht *et al*., 2011 a, b, c and d; 2012; 2013 a,b; 2014 a, b,c; 2015; Nasir *et al*., 2015; Ullah *et al*., 2015; Zakir *et al*., 2015; Chaun *et al*., 2015; Bilal *et al*., 2016; Wajid *et al*., 2016 a, b; Amjad *et al*., 2016; Anwar *et al*., 2016). Plant contains a wide range of bioactive compounds which can be used to cure chronic as well as infectious diseases. Alkaloids, flavonoids, tannins and phenolic compounds are the most important one present in plants. The use of herbal medicines is a common practice in the rural areas of many developing countries due to its low cost and less side effects (Duraipandiya *et al*., 2006). It is reported that water extracted samples from the leaves of *C. alata* revealed antimicrobial activity against *Candida albicans* and *Escherichia coli* in patients afflicted with *Acquired Immunodeficiency Syndrome* (AIDS). These results were at par to the commercially available antifungal drug amphotericin B and antibiotic chloramphenico (Somchit *et al.*, 2003).

The family *Euphorbiaceae* (Spurge family) consists of 300 genera and 5000 species. Within the family of *Euphorbaceae* with over 2000 species, the genus *Euphorbia* is the largest and the 6th largest genus among flowering plants (Haq *et al*., 2012). *Euphorbia helioscopia* (Sunspurge) is a common weed in Pakistan. *E. helioscopia* L. has been in use in china for the treatment of malaria, bacillary dysentery, and osteomyelitis (He *et al.*, 2010). *Euphorbia heliscopia*, which grows wild in different parts of Iran and used by local people for skin disorders was investigated for antiviral activity using plague reduction assay. This is the first report on antiviral activity of *Euphorbia heliscopia* (Ramezani *et al.*, 2008). Native Americans use *Euphorbia heliscopia* to cure skin infections (applied on the skin) and gonorrhea (internally). Traditionally, *Euphorbia* species have been used as laxatives as well as for rheumatism and skin conditions (Mahmood *et al*., 2013). The present research study was carried out to examine the effect of different solvent extracted samples of *Euphorbia heliscopia* on bacteria (both gram negative and gram positive species) and fungi.

MATERIALS AND METHODS

Plant material

The current research was carried out at the Institute of Biotechnology and Genetic Engineering, The University of Agriculture Peshawar Pakistan. Plant materials of *Euphorbia heliscopia* were obtained from the

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Malakandher Research Farms of the University of Agriculture Peshawar KPK during the month of March-April. The plant specimen was identified by Prof. Dr. Furrukh Hussain, Department of Botany University of Peshawar. After collection, the plant materials (stem) were washed thoroughly with distilled water to remove any dust particles, dried for seven days and grounded with electric grinder.

Crude extract preparation

Five hundred grams of dried plant materials were mixed with five liters of methanol, kept at 25ºC in dark for one week and the mixture was three times daily. The mixture was filtered through Whatman filter paper No.1. To the solid residue, twenty five hundred ml fresh methanol was added and the whole procedure was repeated three times. The filtered methanolic solution was dried at 45ºC under vacuum pressure in a rotary evaporator to obtain crude methanolic extract. The crude methanolic extract was divided into two parts. One part (10g) was used as crude extract and the second part (80g) was fractionated using different solvents.

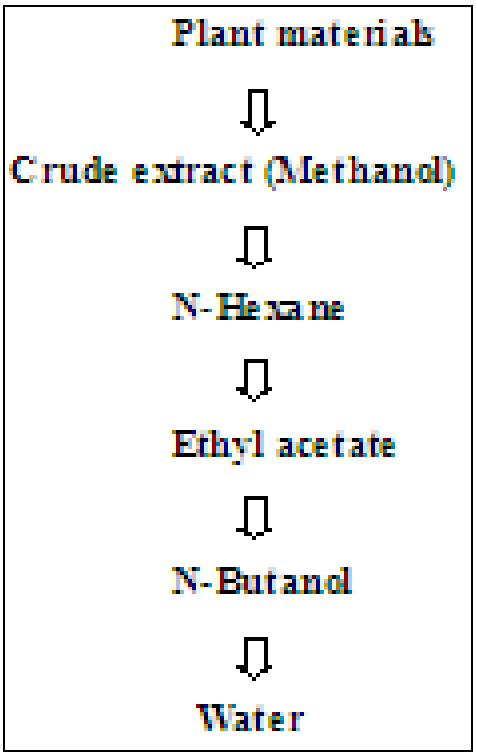

Fig. 1: Flow chart showing crude extracts preparation and different fractions by various solvents.

Fractionation of crude extract

Eight grams of crude extract was dissolved in 500ml sterile distilled water, mixed with n-hexane (300ml), shaken gently and allowed to stand for 15 minutes to separate the two phases. The upper n-hexane phase was obtained and the lower aqueous phase was re-extracted three times with fresh n-hexane. Different fractions of n-hexane were pooled together and dried at 45°C under vacuum pressure with a rotary evaporator. The same procedures were carried out to obtain ethyl acetate and butanol fractions. The lower aqueous phase at the end of the procedure was dried at 45ºC under vacuum pressure through rotary evaporator (fig. 1).

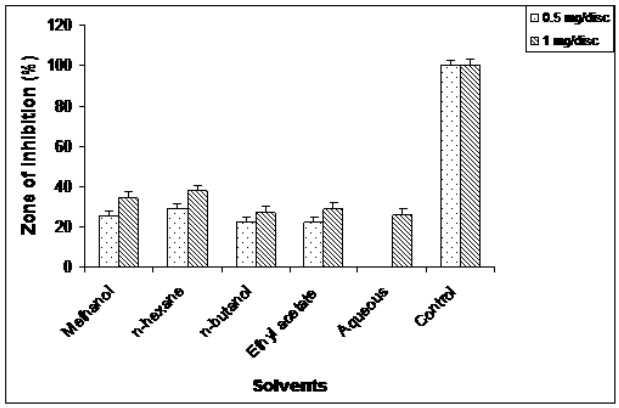

Fig. 2: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from the stem of *E. heliscopia* against *Bacillus subtilis* by disc diffusion assay (Bar shows LSD at $p<0.05$).

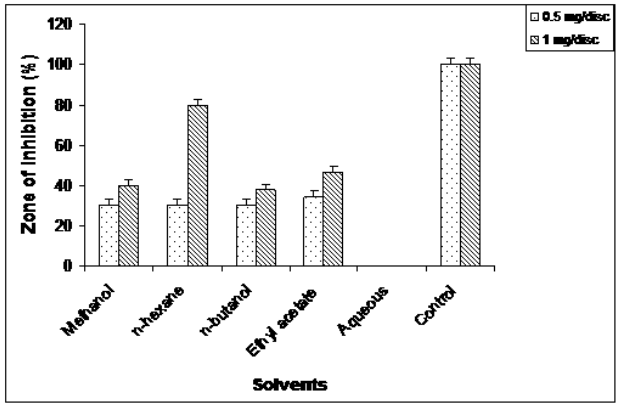

Fig. 3: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from the stem of *E. heliscopia* against *Staphylococcus aureus* by disc diffusion assay (Bar shows LSD at $p<0.05$).

Preparation of media

Nutrient broth was used for shaking incubation and standardization and nutrient agar medium for the culturing and growth of all microorganisms. The required amount of nutrient agar and nutrient broth were poured into conical flasks. Twenty ml of the nutrient broth was also poured into separate test tubes. All the media in the flasks and test tubes after sterilization was poured aseptically into sterilized petri plates and allowed to solidify for about an hour. After 24 hrs, uncontaminated plates were used for culturing of bacteria and fungi.

Disc diffusion susceptibility assay

The antibacterial activity of different solvent extracted samples from the stem of *Euphorbia heliscopia* was

Table 1: Microbial strains used during the present research

Microbial Species	Gram strain type	Details of the Microbial strains used
Klebsiella pneumoniae	Negative	Clinical isolate obtained from the Department of Microbiology, Quaid-I-Azam University Islamabad Pakistan
Pseudomonas aeruginosa	Negative	ATCC # 9721
Staphylococcus aureus	Positive	ATCC # 6538
Bacillus subtilis	Positive	Clinical isolate obtained from the Department of Microbiology, Quaid-I-Azam University Islamabad Pakistan
Escherichia coli	Negative	ATCC # 25922
Candida albicans		ATCC # 10231. Plant Pathology Department, The University of Agriculture Peshawar KPK Pakistan

carried by disc diffusion assay as described in Bauer *et al*. (1966) and antifungal activity by Ramdas *et al*. (1998) against different bacterial and fungal strains (table 1). Nutrient agar media plates were inoculated with 18-24 hrs cultures of microbial inoculums (a standardized inoculums 1-2 × 107 CFUml-1 0.5 McFarland Standard). Three discs of Whatman No. 1 filter paper (6mm in diameter) were placed on the media in petri plates. Different plant extracts in concentration of 0.5 and 1mg in 6 and 12µl volumes were applied on the discs. Antibiotics (6µl $disc^{-1}$) as positive control and DMSO (6µl $disc^{-1}$) as negative control were also applied on the discs in separate petri plates. Inoculated plates were then incubated at 37ºC for 18-24hrs. The next day zones of inhibition were recorded in mm around the discs in each plate.

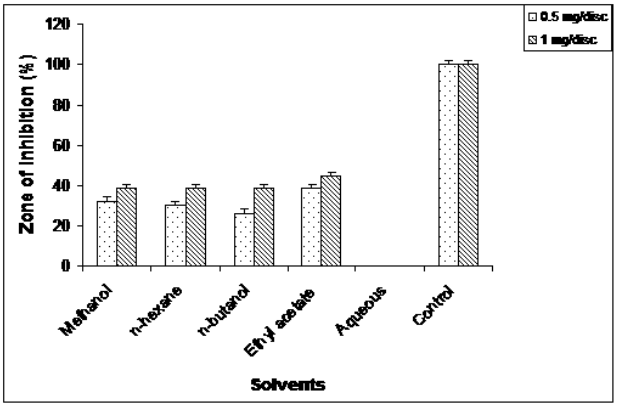

Fig. 4: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from the stem of *E. heliscopia* against *Escherichia coli* by disc diffusion assay (Bar shows LSD at $p<0.05$).

Positive controls

For Gram-positive bacteria; Ciprofloxacin 50µg per 6µl
For Gram negative-bacteria; Ciprofloxacin 50µg per 6µl
For Fungal strain; Fluconazole 50µg per 6µl

STATISTICAL ANALYSIS

Data are presented as mean values of three replications. MSTATC computer software was used for to carry out statistical analysis (Russel and Eisensmith, 1983). Least Significant Difference (LSD) test was employed to compare significant difference among means (Steel *et al*., 1997).

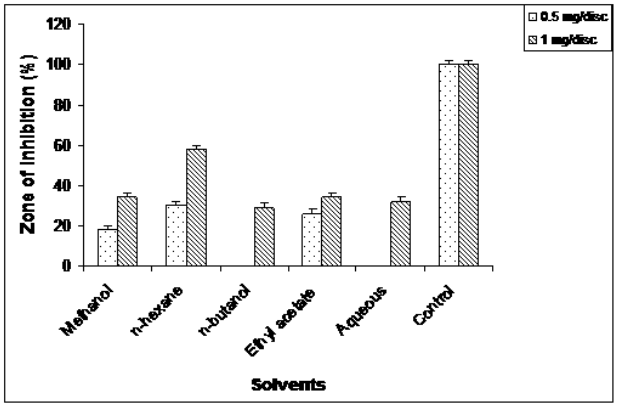

Fig. 5: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from the stem of *E. heliscopia* against *Klebsiella pneumoniae* by disc diffusion assay (Bar shows LSD at $p<0.05$).

RESULTS

Data regarding the antibacterial potential of different extracted samples from the stem against *Bacillus subtilis* is shown in fig. 2. The data suggested that different solvent extracted samples reduced the growth of *Bacillus subtilis* at both concentrations except aqueous extracted samples which showed activity at higher concentrations only. Maximum activity was measured by n-hexane extracted samples at both lower and higher concentrations (29 and 38% ZI at 0.5 and 1mg $disc^{-1}$), while methanol extracted samples showed 34% activity against the same bacteria. Ethyl acetate and n-butanol showed lower activity against the same bacteria at lower concentration (22% ZI at 0.5mg $disc^{-1}$). Aqueous extracted samples revealed no activity at 0.5mg $disc^{-1}$ concentration measuring 0% ZI, however, was effective at higher concentrations (26% ZI at 1mg $disc^{-1}$). The data indicated that *Staphylococcus aureus* revealed maximum susceptibility in case of n-hexane extracted samples at both concentrations used (30% and 80% ZI at 0.5mg and 1mg $disc^{-1}$ respectively; fig. 3). n-butanol and methanol extracted samples also revealed similar activity against the same bacterium measuring 30% ZI at concentration of 0.5mg $disc^{-1}$, while at 1mg $disc^{-1}$ the same extracts

showed 38% and 40% ZI respectively. Aqueous extracted samples did not show any activity at both concentrations measuring 0% ZI.

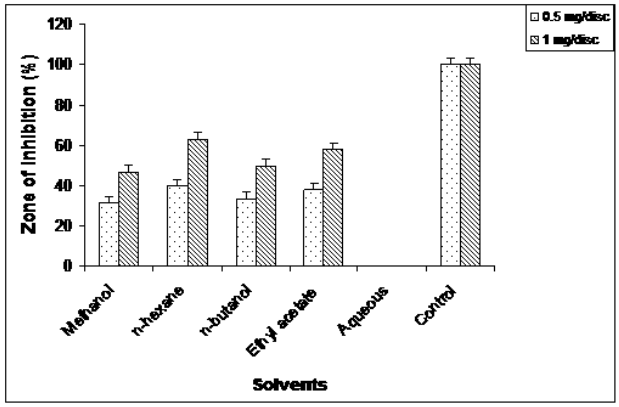

Fig. 6: Antibacterial activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from the stem of *E. heliscopia* against *Pseudomonas aeruginosa* by disc diffusion assay (Bar shows LSD at $p<0.05$).

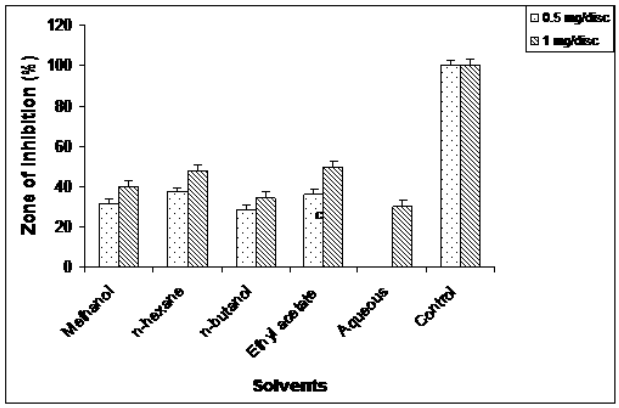

Fig. 7: Antifungal activity of crude methanol, n-hexane, ethyl acetate, butanol and water extracted samples from the stem of *E. heliscopia* against *Candida albicans* by disc diffusion assay (Bar shows LSD at $p<0.05$).

The anti-bacterial activity of different solvent extracted samples from the stem of *E. heliscopia* against *E. coli* is shown in fig. 4. The data revealed that all solvent extracted samples reduced the growth of *E. coli* at both higher and lower concentrations. The data also suggested that ethyl acetate extracted samples showed the highest inhibitory activity i.e. 45% at 1mg disc^{-1} when compared with controls. n-butanol, n-hexane and methanol extracted samples reduced the growth of the same microbe by 39%, while aqueous extracted samples did not show any activity at both concentrations revealing 0% ZI. Our results also revealed that all extracted samples inhibited the growth of *Klebsiella pneumoniae* at both concentrations except n-butanol and aqueous extracted samples at higher concentrations only. n-hexane extracted samples measured highest activity (58% ZI at 1mg disc^{-1}). Ethyl acetate and methanol extracted samples revealed similar activity against the same bacterium at higher concentrations. However, different inhibiting activity was shown by these two samples at concentration of 0.5 milligram disc^{-1} i.e. 26% and 18% ZI respectively. n-butanol and aqueous extracted samples on the other hand did not show activity at concentration of 0.5 mg disc^{-1} measuring 0% ZI (fig. 5).

The antibacterial potential different solvent extracted samples from the stem of *E. heliscopia* against *Pseudomonas aeruginosa* is shown in fig. 6. All the tested samples were more effective to control the growth of *Pseudomonas aeruginosa* at both concentrations except aqueous fractions. *Pseudomonas aeruginosa* was highly inhibited by n-hexane extracted samples measuring 63% inhibitory zone at 1 mg disc^{-1}. Ethyl acetate and n-butanol samples revealed 58% and 50% ZI respectively at higher concentrations. n-butanol and crude methanolic extract showed lowest activities i.e. 33% and 31% ZI at 0.5 milligram disc^{-1} concentrations when compared with other samples and controls. Analysis of the data also indicated that highest inhibitory zones were shown by ethyl acetate fractions (50% ZI) against *Candida albicans* followed by n-hexane fractions (48% ZI) when applied in concentration of 1mg discs^{-1}. Lowest inhibitory zones were measured by aqueous extracted samples (30% ZI) when applied in 1mg disc^{-1} concentrations. The data further showed that n-butanol and methanol extracted samples showed 34% and 40% ZI respectively at concentration of 1mg disc^{-1} when compared with other samples and controls (fig. 7).

DISCUSSION

Different solvent extracted samples from the stem of *E. heliscopia* were screened for their antimicrobial properties against *B. subtilis, S. aureus, E. coli, K. pneumoniae, P. aeruginosa* and *C. albicans*. The percent zone of inhibition values for the tested microbes were measured as an evaluation of the antimicrobial activity of the different samples under study. The data suggested that different solvent extracted samples inhibited the activity of *Bacillus subtilis* at both lower and higher concentrations except aqueous extracted fractions which was effective at higher concentrations only. Maximum activity was measured by n-hexane extracted samples at higher concentrations followed. Ethyl acetate and n-butanol showed minimum activity against the same bacterium at lower concentration. *Staphylococcus aureus* revealed maximum susceptibility in case of n-hexane extracted samples at higher concentrations used. n-butanol and methanol extracted samples also revealed similar activity against the same microbe at concentration of 0.5mg disc^{-1}, while at 1mg disc^{-1} the same extract showed varying degree of activity. Aqueous extracted samples did not show any activity at both concentrations

measuring 0% ZI. These results agree with Ogbulie *et al*. (2007), Khan *et al*. (2011) and Lone *et al*. (2013). These authors concluded that extracts obtained from *E. heliscopia* effectively control the growth of *Bacillus subtilis* and *Staphylococcus aureus.*

Our result also revealed that all solvent extracted samples from the stem of *E. heliscopia* inhibited the activity of *E. coli* at both concentrations. Ethyl acetate extracted samples measured maximum inhibitory activity at 1mg $disc^{-1}$ when compared with controls. n-butanol, n-hexane and methanol extracted samples also reduced the growth of the same microbe while aqueous extracted samples did not show any activity at both concentrations. Rajeh *et al*. (2010), Khan *et al*. (2011) and Lone *et al*. (2013) reported that different extracted samples from *E. heliscopia* effectively control the activity of *E. coli* in dose dependent manner. Our results also suggested that different solvent extracted samples reduced the activity of *Klebsiella pneumoniae* at both concentrations except n-butanol and aqueous extracted samples which revealed activity at higher concentrations only. n-hexane extracted samples showed highest activity at 1mg $disc^{-1}$. Ethyl acetate and methanol extracted samples were equally effective against the same bacterium at higher concentrations. However, varying degree of activity was revealed by these two samples at concentration lower concentrations. n-butanol and aqueous extracted samples did not reduced the growth of *Klebsiella pneumoniae* at concentration of 0.5mg $disc^{-1}$. These results agree with Rajeh *et al*. (2010), Khan *et al*. (2011) and Lone *et al*. (2013).

Our results also indicated that all the tested samples were more effective to reduce the growth of *Pseudomonas aeruginosa* at both concentrations except aqueous extracted samples which were effective at higher concentrations only. *Pseudomonas aeruginosa* was more susceptible to n-hexane extracted samples at 1 mg $disc^{-1}$ followed by ethyl acetate and n-butanol samples at higher concentrations. n-butanol and crude methanolic extract showed lowest activities against the same microbe at lower concentrations when compared with other samples and controls. Our results agree with Ogbulie *et al*. (2007), Khan *et al*. (2011) and Lone *et al*. (2013) who concluded that samples extracted with different solvents from *E. heliscopia* inhibited the growth of *Pseudomonas aeruginosa*. *Candida albicans* was more susceptible to ethyl acetate fractions at highest concentrations followed by n-hexane fractions. Aqueous extracted samples on the other hand revealed less susceptibility when exposed to the same concentrations. Our findings are in agreement with Khan *et al*. (2011) and Lone *et al*. (2013) who reported antifungal activity in the same plant species. It has been reported that the family of *Euphorbaceae* contain a wide range of different bioactive compounds including tannins, phenolic, alkaloids, anthraquinones, saponins, falvonids etc. These compounds have a vital application against human pathogens (Abubakar, 2009). Several researchers have the linked the presence of these bio-molecules to their antibacterial properties of different plant extracts (Adesokan *et al*., 2007; Ogbolie *et al*., 2007; Oyeleke *et al*., 2008).

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