Pak. J. Bot., 43: 123-127, Special Issue, December, 2011 (Medicinal Plants: Conservation & Sustainable use)

IN VITRO INHIBITION POTENTIAL OF FOUR CHENOPOD HALOPHYTES AGAINST MICROBIAL GROWTH

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Abstract

The present investigation deals with antibacterial and antifungal activities of four selected halophytes belonging to family Chenopodiaceae. Crude methanolic extracts from leaves of *Suaeda fruticosa* Forssk. ex J.F.Gmel., *Atriplex leucoclada* Boiss., *Haloxylon salicornicum* (Moq.) Bunge ex Boiss., and *Salicornica virginica* L. were used in two concentrations (100 mg/ml and 75 mg/ml) against four bacterial (*Escherichia coli* ATCC 25922, *Bacillus subtilis* ATCC 6059, *Pseudomonas aeruginosa* ATCC 7221 and *Staphylococcus aureus* ATCC 6538) and two fungal strains (*Candida tropicalis* and *Candida albicans*). These halophytes were collected from District Mardan. Plant extracts were less effective against selected bacterial and fungal strains in comparison to chloramphenicol and terbinafine. Extracts from *Haloxylon salicornicum* and *Salicornica virginica* showed less activity against bacterial and fungal species than extracts from *Suaeda fruticosa* and *Atriplex leucoclada*.

Introduction

The diverse range of climate and phytogeograpic conditions in Pakistan results in high floral diversity containing many medicinal plant species. The total estimated flora of Pakistan is approximately 6000 species (Shinwari *et al*., 2000). Interest in phytochemical studies of medicinal plants for pharmacological as well as nutritional purposes has gained momentum in the last few years (Oktay *et al*., 2003; Wangensteen *et al*., 2004). Phytochemicals derived from various parts of plants include phenolic compounds, essentials oils, proteins and antioxidants, which serve as biocontrol agents (Cragg *et al*., 1996). Extensive research has been conducted to check the potential of medicinal plants against different diseases (Qin & Xu, 1998). People dwelling in the hilly areas of Pakistan have used medicinal plants species for curing various diseases for a long time (Gilani *et al*., 2009; Mohy-ud-Din *et al*., 2010; Shinwari, 2010). Halophytes are salt-tolerant plants that may be potentially useful for economic applications as new sources of natural antioxidants in dietary food (Meot-Duros *et al.,* 2008). Halophytes are known for their ability to resist and quench toxic reactive oxygen species (ROS) because they are equipped with powerful antioxidant enzyme systems (Ksouri *et al.,* 2008). Crude methanolic extracts of halophytes have been investigated for their anticarcinogenic and chemopreventive activities by evaluating the levels of hepatic antioxidant defence (Sehrawat & Sultana, 2006). Several studies attributed the inhibition potential of plant extracts against bacterial pathogens to their phenolic composition (Baydar *et al.,* 2004; Rodriguez Vaquero *et al.,* 2007).

The present study evaluated antibacterial and antifungal properties of four selected halophytes, *Suaeda fruticosa, Atriplex leucoclada, Haloxylon salicornicum,* and *Salicornica virginica* of district Mardan (Khyber Pakhton Khawa), Pakistan.

Objectives of the study: During the present investigation, leaf extracts of the selected halophytes were checked for their potential against several virulent microorganisms. *Escherichia coli* causes gastroenteritis, urinary tract infections, and neonatal meningitis. *Bacillus subtilis* may cause endocarditis, pneumonia, bacteremia and septicemia in patients with compromised immune systems. *Pseudomonas aeruginosa* is a causal organism of pulmonary tract, urinary tract, and blood infections, while *Staphylococcus aureus* can cause skin infections, pneumonia, meningitis, and blood infections. *Candida tropicalis* and *Candida albicans* cause oral and genital infections in humans.

Materials and Methods

Assay for antibacterial activity: Antibacterial activity of the methanolic extracts was determined by the agar well diffusion method (Carron *et al*., 1987).

Extraction: Fresh leaves of four known local medicinal plants were selected for the study *viz Suaeda fruticosa, Atriplex leucoclada, Haloxylon salicornicum* and *Salicornica virginica*. Healthy leaves were collected, rinsed with distilled water and air dried for 12 days. The leaves were ground into powder, then soaked in 80% methanol and incubated for two weeks at room temperature (25°C). After 14 days the mixtures were filtered twice, using Whatman-41 filter paper. The extracts were dried by removing the methanol using a rotary film evaporator. Two concentrations (100 mg/ml and 75 mg/ml) of plant extracts were used for assessing antibacterial and antifungal activity.

Preparation of media for bacteria: Nutrient broth medium for growing bacteria was prepared by dissolving 0.4 g of nutrient broth in 50 ml of distilled water; pH was adjusted to 7.0 and the medium was autoclaved. Nutrient agar medium for growing bacteria was prepared by dissolving 2.3 g nutrient agar (Merck) in 100 ml of distilled water; pH was adjusted to 7.0 and the medium was autoclaved at 121°C.

McFarland (0.5) barium sulfate turbidity standard: The standard was prepared by adding 0.5 ml 0.048 M barium chloride to 99.5 ml 0.36 N sulfuric acid. Four to six ml of barium sulfate turbidity standard was compared

to the bacterial suspensions, which were diluted until they matched the colour of the turbidity standard.

Bacterial strains used: Four strains of bacteria were used in the study. Two were Gram-positive (*Staphylococcus aureus* (ATCC 6538) and *Bacillus subtilis* (ATCC 6059)) and two were Gram-negative (*Escherichia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 7221)). The organisms were maintained on nutrient broth medium at 4°C.

Preparation of inocula: Centrifuged pellets of bacteria from 24-hour old cultures in nutrient broth (Sigma) of the selected bacterial strains were mixed with physiologically normal saline solution until they matched a McFarland turbidity standard [10^6 colony forming unit (CFU) ml^{-1}]. These inocula were used for seeding the nutrient agar plates.

Preparation of seeded agar plates: The nutrient agar medium was allowed to cool to 45°C. Petri plates were prepared by pouring 75 ml of seeded nutrient agar. After the agar solidified, four wells per plate were made with a sterile cork borer (5 mm).

Pouring of test solutions; incubation and measurement of zone of inhibitions: Using a micropipette, 100 µl of extract (10 mg/ml, 7.5 mg/ml) was poured in respective wells. These plates were incubated at 37°C. After 24 hours of incubation the diameter of the clear zones of inhibition was measured with a ruler. Antibacterial activity of two dilutions of each plant extract was determined against four bacterial strains. Chloramphenicol (2.0 mg/ml) was used as a control for the assay against four bacterial strains.

Assay for antifungal activity: The agar tube dilution method (Washington & Sutter, 1980) was used for determination of antifungal activity of methanolic extracts of the selected plants.

Microorganisms used: The fungal strains used in this study were *Candida albicans* and *C. tropicalis*, obtained from the Microbiology Department, QAU, Islamabad. Each fungal strain was maintained on Sabouraud dextrose agar (SDA) medium at 4°C.

Assay procedure of antifungal activity: The samples for antifungal assay were prepared from initial stock of 100 mg of extract per ml of dimethyl sulfoxide (DMSO). Medium for fungi was prepared by dissolving 6.5 g of SDA (Merck) per 100 ml of distilled water and adjusting the pH to 5.6. Test tubes were marked at 10 cm. The Sabouraud dextrose agar was dispensed in 4 ml volumes into screw capped tubes or cotton plugged test tubes and was autoclaved at 121°C for 21 minutes. Tubes were allowed to cool to 50°C and non-solidified SDA was loaded with 67 µl from the stock solution, for a final concentration of 200 µg/ml of the pure extract in media. Tubes were then allowed to solidify in a tilted position at room temperature. Three tubes of the extracted sample were prepared for each fungus species. The tubes containing solidified medium and the plant extracts were inoculated with a 4 mm diameter piece of inoculum, taken from a seven-day-old culture of fungus. One tube for each fungus was prepared without extract as a positive control. Terbinafine (0.012 mg/ml in DMSO) was used as a negative control.

The test tubes were incubated at 28°C for seven days. Cultures were examined twice weekly during the incubation. Readings were taken by measuring the linear length of fungal growth (mm) and growth inhibition was calculated with reference to the negative controls.

Percent inhibition of fungal growth for each concentration of extract was determined by the following formula:

$$\text{Percent inhibition of fungal growth} = \frac{\text{100 - Linear growth in test (mm)}}{\text{Linear growth in control (mm)}} \times 100$$

Statistical analysis: The data were analyzed using Analysis of Variance (ANOVA) using COSTATE software (version 8) and comparisons among treatment means were made with Duncan's Multiple Range Test (DMRT).

Results

Antibacterial activity: Results indicated that leaf extracts from all the plants exhibited antibacterial activity against the four pathogenic bacteria tested. Antibacterial activity of *Suaeda fruticosa* varied in different bacterial strains (Fig. 1). Dilution of the plant extract to 75% resulted in a decrease of activity against *E. coli* over that of the undiluted (100%) plant extract. Maximum inhibition was exhibited by chloramphenicol against all four bacterial strains. Plant extracts (75%) showed no inhibition against *B. subtilis* but for *P. aeruginosa* the inhibition was stronger than for the 100% plant extract. The 100% extract showed no inhibition against *S. aureus*, while the 75% plant extract showed considerable inhibition. The degree of inhibition from the 75% extract did not differ significantly between *S. aureus* and *E. coli*.

Antibacterial activity of *Haloxylon salicornicum* against *E. coli, B. subtilis, P. aeruginosa* and *S. aureus* varied (Fig. 2). Only the 100% extract of *Haloxylon salicornicum* showed an equal degree of inhibition against *E. coli*, *B. subtilis* and *S. aureus*. Chloramphenicol was not active against *P. aeruginosa* whereas both 75% and undiluted (100%) extract inhibited the growth of *P. aeruginosa* and *S .aureus*. However, the 75% plant extract showed greater inhibition against *P. aeruginosa* than against *S. aureus*.

Antibacterial activity of *Salicornica virginica* extract (Fig. 3) showed that undiluted extract (100%) was more inhibitory to *B. subtilis* and *P. aeruginosa* than the diluted (75%) extract. The 75% dilution showed no inhibition against *B. subtilis*. Both the 100% and 75% extracts were not very effective against *E. coli*. Maximum inhibition was exhibited by chloramphenicol against all the bacterial strains used except *P. aeruginosa*.

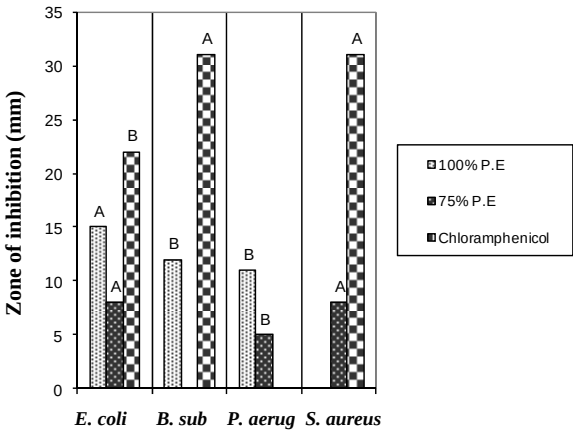

Fig. 1. Antibacterial activity of *Suaeda fruticosa*
P.E: Plant extract, 100% (10 mg/ml) 75% (7.5 mg/ml) *E. coli: Escherichia coli, B. sub: Bacillus subtilis, P. aerug: Pseudomonas aeruginosa, S. aureus: Staphylococcus aureus.*

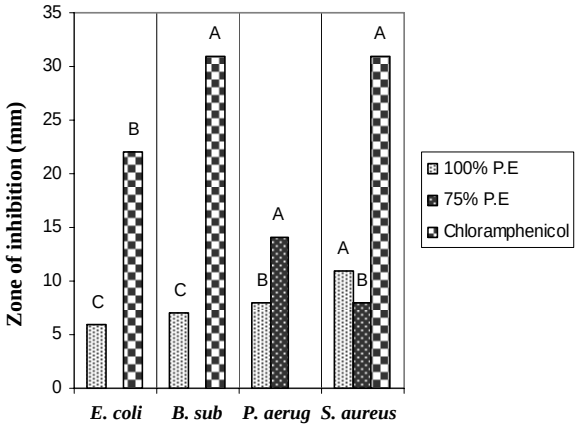

Fig. 2. Antibacterial activity of *Haloxylon salicornicum*
P.E: Plant extract, 100% (10 mg/ml) 75% (7.5 mg/ml) *E. coli: Escherichia coli, B. sub: Bacillus subtilis, P. aerug: Pseudomonas aeruginosa, S. aureus: Staphylococcus aureus.*

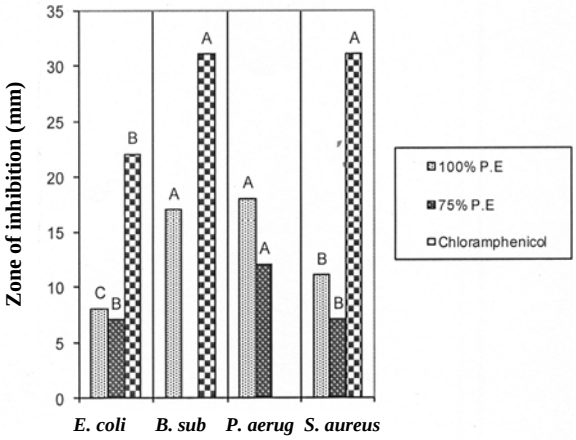

Fig. 3. Antibacterial activity of *Salicornica virginica*
P.E: Plant extract, 100% (10 mg/ml) 75% (7.5 mg/ml) *E. coli: Escherichia coli, B. sub: Bacillus subtilis, P. aerug: Pseudomonas aeruginosa, S. aureus: Staphylococcus aureus.*

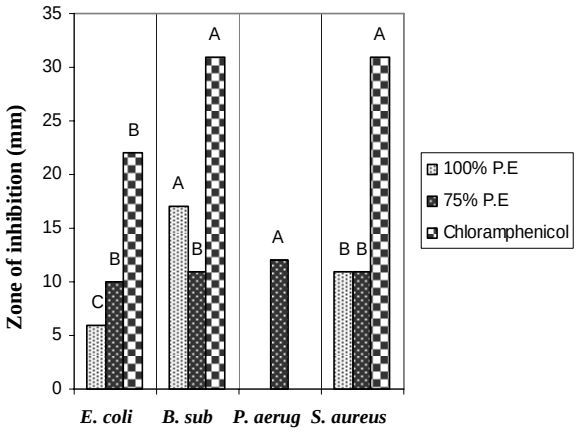

Fig. 4. Antibacterial activity of *Atriplex leucoclada*
P.E: Plant extract, 100% (10 mg/ml) 75% (7.5 mg/ml) *E. coli: Escherichia coli, B. sub: Bacillus subtilis, P. aerug: Pseudomonas aeruginosa, S. aureus: Staphylococcus aureus.*

The 75% extract of *Atriplex leucoclada* showed greater inhibition against *E. coli* than the 100% extract. Chloramphenicol exhibited a higher degree of inhibition than the *A. leucoclada* extracts (Fig. 4). Maximum inhibition against *B. subtilis* was shown by the 100% extract. The 75% extract of *Atriplex leucoclada* was effective against *P. aeruginosa*, while the 100% extract showed no inhibition. The 100% and 75% extracts were equally effective against *S. aureus*, with both exhibiting similar zones of inhibition, but chloramphenicol showed the maximum inhibition against *S. aureus.* DMSO did not show any inhibition against bacterial strains used.

Antifungal activity: Maximum inhibition was shown by *Suaeda fruticosa*, which inhibited 58.4% of the growth of *Candida tropicalis* (Fig. 5), whereas *Atriplex leucoclada* inhibited 52.83%. *Candida albicans* showed greater resistance against *Haloxylon salicornicum* and *Salicornica virginica*, which inhibited fungal growth by 45.2% and 39.6% respectively. Terbinafine inhibited 90% of fungal growth.

Fig. 6 shows antifungal activity of plant extracts against *Candida albicans.* Maximum inhibition was shown by *Haloxylon salicornicum*, which inhibited 84.29% of the fungal growth, while *Suaeda fruticosa* inhibited 64.29%, followed by *Salicornica virginica* (60%) and *Atriplex leucoclada* (54.29%). Terbinafine inhibited 95% of fungal growth. DMSO was not effective against *Candida tropicalis* and *Candida albicans*.

Discussion

Inhibition of microbial growth has already been reported (Puupponen-Pimiä *et al.*, 2001). This mechanism of inhibition has been demonstrated to be from the presence of a strong antioxidant enzyme system. Antibiotic resistance has been reported in the food-borne pathogens *E. coli, S. aureus, and Clostridium perfringens.* Previously acetone, ethanol and ether were used for extraction but they are relatively volatile and rather costly. In the present study we used methanol as a solvent for extraction because of its wide spectrum of solubility of a majority of the chemical compounds having antimicrobial activity (Chandrasekaran & Venkatesalu, 2004).

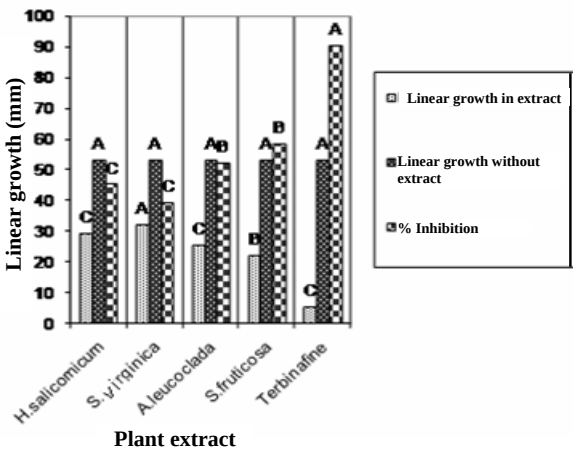

Fig. 5. Antifungal activity against *Candida tropicalis*
H. salicornicum: Haloxylon salicornicum, S. virginica: Salicornica virginica, A. leucoclada: Atriplex leucoclada, S. fruticosa: Suaeda fruticosa, Terbinafine (Fungicide)

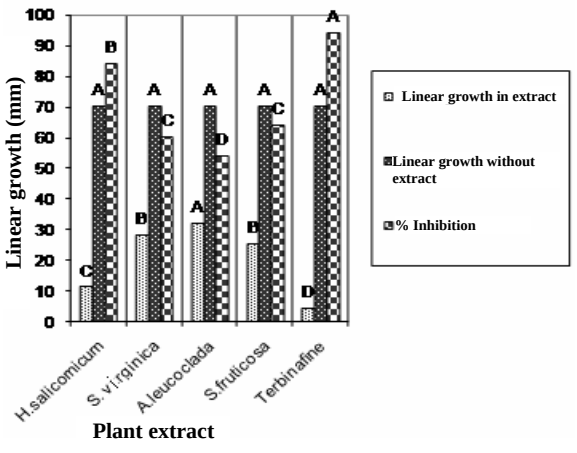

Fig. 6. Antifungal activity against *Candida albicans*
H. salicornicum: Haloxylon salicornicum, S. virginica: Salicornica virginica, A. leucoclada: Atriplex leucoclada, S. fruticosa: Suaeda fruticosa, Terbinafine (Fungicide)

Table 1. Ranking of the plant extract's inhibition potential against bacterial and fungal strains used.

Species studied	Antibacterial activity against				Antifungal activity against	
	E. coli	P. aeru	B. sub	S. aur	C. albicans	C. tropicalis
Suaeda fruticosa	+++	+++	++	+	+++	++
Haloxylon salicornica	+	++	+	++	++	+++
Salicornica virginica	++	++	++	++	+	++
Atriplex leucoclada	++	+	+++	+++	++	+

+ + + (Maximum), + + (Optimum), + (Minimum)

Table 1 represents the inhibition potential of plant extracts against bacterial and fungal strains. In the present study, the antibacterial activity of *Suaeda fruticosa* extracts and of chloramphenicol against *E. coli* can be ranked as chloramphenicol inhibition > plant extract (100%). However, only the 100% extract was effective against *B. subtilis*. For *P. aeruginosa*, the greatest inhibition was shown by chloramphenicol, then the 75% extract followed by 100% extract. In the case of *S. aureus*, only the 75% extract was effective but it had less potency than chloramphenicol. All of the plant extracts evaluated in this research inhibited the growth of the Gram-negative *E. coli and P. aeruginosa*. These results do not correspond with those obtained with methanol extracts from *Artemisia diffusa, Artemisia scoparia* and the ethanol leaf extract of *Pulicaria orientalis* (Ali *et al*., 2001), which did not display any antibacterial activity against *E. coli* and *P. aeruginosa*.

Haloxylon salicornicum exhibited the least antibacterial activity of the plants in this study but had maximum antifungal activity. The undiluted (100%) extract of *Salicornica virginica* showed maximum inhibition against *B. subtilis* and *P. aeruginosa.* Chloramphenicol showed the maximum inhibition against *E. coli* while both 100% and 75% extracts showed similar inhibition to each other. Only the 100% extract was effective against *B. subtilis*; chloramphenicol showed no inhibition. Inhibition against *P. aeruginosa* was ranked as chloramphenicol > 100% extract > 75% extract, while against *S. aureus* it was chloramphenicol >100% extract > 75% extract.

A methanolic extract of *Pogonomyrmex barbatus* displayed a potent antibacterial activity against Gram-positive bacteria including *S. aureus* (Matu *et al.*, 2003) and also exhibited marked antifungal effect against *C. albicans* (Runyoro *et al.*, 2006).

Atriplex leucoclada (75%) extract was effective against *B. subtilis* but did not exhibit inhibitory activity even at 100% concentration against *P. aeruginosa.* Inhibition against *E. coli* ranked as chloramphenicol > 75% extract >100% extract, while for *S. aureus*, the activity was ranked as chloramphenicol > 75% extract = 100% extract.

Antifungal activity of the selected halophytes (2.0 mg/ml DMSO) against *Candida tropicalis* ranked as *H. salicornicum* > *S. fruticosa* > *S. virginica* > *A. leucoclada,* while the % inhibition against *Candida albicans* was *S. fruticosa* > *A. leucoclada* > *H. salicornicum* > *S. virginica.*

Conclusion

The present study demonstrates that plant extracts of the selected halophytes have the potential to inhibit the growth of pathogenic bacteria and fungi. These halophytes could be considered an economical, environmental friendly and sustainable source of antioxidants, biobactericides and biofungicides. In some

cases even diluted plant extracts (75%) were almost or as effective as the 100% extract.

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(Received for publication 14 October 2011)