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ANTIBACTERIAL ACTIVITY AND BRINE SHRIMP TOXICITY OF *ARTEMISIA DUBIA* EXTRACT

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Abstract

The present investigation deals with bioassays of methanol extract of leaves (ML), methanol extract of flowers (MF), chloroform extract of leaves (CL) and chloroform extract of flowers (CF) of *Artemisia dubia*. Antibacterial activity of these extracts was examined, *In vitro*, using agar well diffusion method, against 3 Gram-positive bacteria (*Bacillus subtillis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Micrococcus luteus* ATCC 10240), and five Gram-negative bacteria (*Escherichia coli* ATCC 15224, *Pseudomonas picketti* ATCC 49129, *Bordetella bronchiseptica* ATCC 4617, *Enterobacter aerogens* ATCC 13048, *Salmonella setubal* ATCC 19196). Brine shrimp lethality assay was carried out to check the cytotoxicity of these extracts. The antibacterial assay results showed ML was effective against *Bacillus subtillis, Staphylococcus aureus, Micrococcus luteus and Escherichia coli*; CL against *Staphylococcus aureus* and *Micrococcus luteus*; MF against *Escherichia* coli, while CF was not effective against any of the strains. Brine shrimp toxicity assay results showed toxicity of MF against newly hatched nauplii; ML, CL and CF showed toxicity with LC_{50} value higher than 1000 ppm.

Introduction

Medicinal plants existed long before the first human appeared on earth. No one knows how long it took for humans to discover the curative power of medicinal plants. Now a day, plants are a valuable source of natural products for maintaining human health, with more intensive studies for natural therapies. In developed countries, about 80% individuals use traditional medicines, derived from medicinal plants. There is a need to investigate such plants sources to better understand their pharmacological properties with safety and efficacy (Verpoorte, 2000). *Artemisia* is one of the diverse genera of Asteraceae family with many important medicinally valuable essential oils and secondary metabolites (Zia *et al*., 2007). Essential oils of *Artemisia* spp. have been widely used for a variety of medicinal purposes for many years (Ahameethunisa & Hopper, 2010). The genus *Artemisia* contains more than 400 species and most of its known species are found predominantly in Asia, Europe and North America (Mucciarelli & Maffei, 2002). The genus is widely used in many parts of the world either alone or in combination with other plants as herbal remedies for a variety of human diseases (Endalkachew & Michael, 2010). About 30 species of the genus are growing naturally throughout Pakistan (Qureshi *et al*., 2002). This genus is naturalized and widely distributed in Northern Areas of Pakistan, Kohat, Kashmir and in many other places of Baluchistan (Ghafoor *et al*., 2002).

Different species of *Artemisia* have been used to treat fever and malaria (Ashraf *et al*., 2010), anthelmentic stomachache, high blood pressure and diabetes (Ahmad *et al*., 2006), diarrhea, cough and common cold (Hayat *et al*., 2009). Phyto-pharmacological evaluation of *Artemisia* shows the presence of antibacterial (Setzer *et al*., 2004), antifertlity (Rao *et al*., 1988), antifungal (Maruzzella *et al*., 1960), antidiadetic (Nofal *et al*., 2009), anti-inflammatory (Mino *et al*., 2004), antipyretic (Derakhshanfar *et al*., 2006), anthelmintic (Jan *et al*., 2009), antihepatitic (Gilani & Janbaz, 1995), antimalarial (Zafar *et al*., 1990), antiamoebic (Tahir *et al*., 1991) and antimoluscs (Joshi *et al*., 2005) activities. The present investigation was undertaken to evaluate the antibacterial activity and brine shrimp toxicity of *Artemisia dubia.*

Materials and Methods

Plant material: Plant sample was collected in summer 2007 from Donga Gali, Distt. Abbotabad, NWFP, Pakistan, The plant sample was identified by a (Taxonomist) and voucher specimens was deposited at the Herbarium of Department of Biochemistry, Quaid-i-Azam University, Islamabad, Pakistan.

Extraction: Flowers (300 grams) and leaves (500 grams) of fresh plant (*Artemisia dubia*) were ground in 2.0 liters and 3.0 liters of methanol respectively using kitchen blender. Poorly homogenized mixtures were macerated for 4 weeks in extraction bottle. After 4 weeks, maximum amount of methanol was separated from the plant material by pressing the plant material in white colored cotton cloth, which was thoroughly cleaned and rinsed with distilled water and methanol respectively. Filtrate was filtered twice, first using ordinary filter paper and then Whatman-41 filter paper. Methanol was evaporated at low temperature and reduced pressure. 10.0 grams methanolic extract of flowers (MF) and 11.50 grams methanolic extract of leaves (ML) was obtained. After methanolic extraction residual material of flowers and leaves was used again for successive extraction in 1.5 liters and 2.0 liters of chloroform respectively for 4 weeks. Again it was filtered and evaporated as above and 5.3 grams chloroformic extract of flowers (CF) and 9.0 grams chloroformic extract of leaves (CL) was obtained. Extraction of flowers and leaves was carried out separately and all necessary precautions were adopted to avoid cross contamination. All the extracts were stored at –20ºC until further analysis was carried out.

Antibacterial assay

Preparation of test solutions: A volume of 10ml of stock solution with a concentration of 15mg/ml was prepared in dimethyl sulfoxide (DMSO). Its further dilutions of 12.5, 10, 7.5, 5, 3, 2 and 1mg/ml were prepared to determine the antibacterial activity of extracts at different concentrations. Solution of Roxithromycin and Cefixime-USP, 2mg/ml in DMSO, were used as positive control while pure DMSO was used as negative control.

Microorganisms used: Eight strains of bacteria were used, including three gram positive strains, *Staphylococcus aureus* (ATCC 6538), *Bacillus subtilis* (ATCC 6633) and *Micrococcus luteus* (ATCC 10240), and 5 gram negative strains, *Escherichia coli* (ATCC 15224), *Salmonella setubal* (ATCC 19196), *Pseudomonas picketii* (ATCC 49129), *Bordetella bronchiseptica* (ATCC 4617) and *Enterobacter aerogens* (ATCC 13048). All the organisms were cultured and maintained on nutrient agar medium at 4°C.

Preparation of inocula: Centrifuged pallets of bacteria from 24 hours old culture in nutrient broth (Sigma) of selected bacterial strain was mixed with physiological saline and turbidity was corrected by adding sterile physiological saline until a Mac Farland turbidity standard of 0.5 [10^6 colony forming unit (CFU) per ml]. The standardized suspension of bacteria was used for seeding the nutrient agar.

Preparation of seeded agar plates: Nutrient agar (Merck) at 2% w/v concentration and 7.0 ± 0.2 pH at 25°C was prepared and autoclaved for 15 min. at 121°C and 20lbs pressure. It was cooled up to 45°C, and inoculated with the standardized bacterial culture to have 10^4 CFU per ml. Petri plates (14 cm) were prepared by pouring 75ml of seeded nutrient agar and allowed to solidify. Wells (11 per plate) were made with sterile cork borer (8mm) and were sealed.

Pouring of test solutions, Incubation and measurement of zone of inhibition: Using Micropipette (Socorex), 100µl of test solution was poured in the respective wells. Eight solutions of extract with different concentrations, two solutions for positive control (Roxithromycin and Cefixime-USP, one for each) and one for negative control DMSO were applied to each Petri plate. These plates were incubated at 37°C in incubator (Yamato IC83). After 24h and 48h of incubation, diameter of the clear zones with no bacterial growth around each well was measured. Triplicate plates were prepared for each extract. Then average of these three plates was taken and standard deviation was calculated. Antibacterial activity of all dilutions of extracts (ML, MF, CL and CF) was determined against all eight strains of bacteria.

Brine shrimp toxicity assay

Sample preparation: Samples were prepared by dissolving 60mg each of methanolic extract and chloroformic extract of leaves and flowers (ML, MF, CL and CF) in methanol (solvent) and chloroform (solvent) respectively to make 6ml (10mg/ml or 10,000 ppm) stock solution. From the stock solution, further diluted concentrations (7,500 ppm, 5,000 ppm, 2,500 ppm, 1000 ppm and 100 ppm) were prepared.

Hatching shrimps: Brine shrimp (*Artemia salina*) eggs (Sera, Heidelberg, Germany) were hatched in artificial sea water present in shallow rectangular dish (22x32 cm). Sea water was prepared with commercial salt mixture (Harvest CO.H.K.) with distilled water. A plastic divider with several holes of 2mm size was clamped in the dish to make two unequal compartments i.e. one was large compartment while other was small compartment. Brine shrimp eggs (about 25mg) were sprinkled in the larger, dark compartment, (covered with aluminium foil), while the smaller compartment was illuminated. After 24 h of starting hatching, phototropic nauplii (brine shrimp larvae) were collected by pipette from the lightened side having been separated by the divider from their shells.

Bioassay: Two-dram vials were used for this bioassay. To prepare final concentration of 1000ppm, 750ppm, 500ppm, 250ppm, 100ppm, 10ppm, 0.5ml of the each solution 10,000ppm, 7,500ppm, 5,000ppm, 2,500ppm, 1000ppm, 100ppm was taken in the vial respectively. For control, 0.5ml of methanol and 0.5ml of chloroform was taken. Solvent was allowed to evaporate overnight and then it was put in high vacuum for 3 h to ensure complete evaporation of the solvent. Three replicates were prepared for each concentration.

Ten shrimps were transferred to each vial using Pasteur pipette, and artificial sea water was added to make 5ml. The nauplii can be counted macroscopically in the stem of pipette against a light background. The vials were maintained under illumination at room temperature 25°C to 28°C. Survivors were counted with the aid of 3x magnifying glass after 24h. In case, where control death occurred, the data was corrected using Abbott's formula (Abbot, 1925).

% Death = [(Test – Control) / Survivors of control] X 100

LC_{50} Determination: Using Abbott's formula % death was calculated. Then LC_{50} was calculated by graphical calculation.

Results and discussion

Antibacterial assay: Our results show methanolic extract of leaves (ML) is effective against *Escherichia coli, Bacillus subtillis*, *Staphylococcus aureus* and *Micrococcus luteus* (Table 1), while chloroformic extract of leaves (CL) have relatively low activity against *Staphylococcus aureus* and *Micrococcus luteus* only (Table 2). Methanolic extract of flowers (MF) is mildly effective against *Escherichia coli* only (Table 3), while chloroform extract of flowers (CF) has no antibacterial activity against all eight strains of bacteria. In the present study, two solvents of different polarities were used so, that maximum number of compounds could be extracted and tested for their biological activities but the methanolic extract were more antibacterially active than the

chloroformic extracts. Initial screening of plants for possible antimicrobial activities typically begins by using crude aqueous or alcohol extraction and can be followed by various organic extraction methods. Since nearly all of the identified components from plants are active against microorganism are aromatic or saturated organic compounds, they are often obtained through initial ethanol or methanol extraction (Vileges *et al*., 1997). Antibacterial activity of methanol and hexane extracts of 18 plants was studied, in which methanol extract showed significant activity against tested organisms (Duraipandiyan *et al*., 2006). Results also showed that leaves may have some important medicinal constituents, which could further be investigated to get some novel antibacterial compounds from plants. As Gupta *et al*., (1998) concluded that secondary metabolites derived from the plant sources are of remarkable interest as they are generally safer in their usage. Pharmacologically these secondary metabolites are more effective than synthetic and semisynthetic antimicrobial agents.

Table 1. Antibacterial activity of methanolic extract of leaves (ML) of *Artemisia dubia.*

Conc. mg/ml	Zone of Inhibition (mm)							
	E. coli		*B. sub.*		*S. aureus*		*M. luteus*	
	24 h	48 h	24 h	48 h	24 h	48 h	24 h	48 h
15	9.5 ± 0.03	9.5 ± 0.03	11.5 ± 0.07	11.5 ± 0.07	12.0 ± 0.10	12.0 ± 0.10	9.5 ± 0.03	9.5 ± 0.03
12.5	9.0 ± 0.01	9.0 ± 0.01	11.0 ± 0.06	11.0 ± 0.06	11.5 ± 0.07	11.5 ± 0.07	8.5 ± 0.05	8.5 ± 0.05
10	9.0 ± 0.01	9.0 ± 0.01	11.0 ± 0.06	11.0 ± 0.06	11.5 ± 0.07	11.0 ± 0.06	8.3 ± 0.04	8.3 ± 0.04
7.5	9.0 ± 0.01	-	10.5 ± 0.10	11.0 ± 0.06	11.0 ± 0.06	10.0 ± 0.05	8.2 ± 0.02	8.2 ± 0.02
5	9.0 ± 0.01	-	10.0 ± 0.10	10.0 ± 0.10	10.5 ± 0.10	10.0 ± 0.05	-	-
3	-	-	10.0 ± 0.10	10.0 ± 0.10	10.5 ± 0.1	10.0 ± 0.05	-	-
2	-	-	9.5 ± 0.03	9.5 ± 0.03	10.0 ± 0.05	10.0± 0.05	-	-
1	-	-	-	-	10.0 ± 0.05	10.0 ± 0.05	-	-
Cef. 2	38.7 ± 1.1	38.50 ± 1.0	17.0 ± 0.8	17.0 ± 0.9	33.0 ± 0.5	32.0 ± 0.7	19 ± 0.7	19 ± 0. 8
Rox. 2	15.0 ± 0.4	15.0 ± 0.5	27.0 ± 0.5	26.0 ± 0.5	26.0 ± 0.10	27.5 ± 0.15	43 ± 1.2	43 ± 1.3
DMSO	-	-	-	-	-	-	-	-

Table 2. Antibacterial activity of chloroform extract of leaves (CL) of *Artemisia dubia*.

Conc. mg/ml	Zone of inhibition (mm)			
	S. aureus		*M. luteus*	
	24 h	48 h	24 h	48 h
15	10.5 ± 0.10	10.5 ± 0.10	9.0 ± 0.05	9.0 ± 0.05
12.5	10.5 ± 0.10	10.0 ± 0.05	8.5 ± 0.01	8.5 ± 0.01
10	10.0 ± 0.05	10.0 ± 0.05	8.4 ± 0.01	8.4 ± 0.01
7.5	10.0 ± 0.05	10.0 ± 0.05	8.2 ± 0.01	8.2 ± 0.01
5	9.5 ± 0.03	9.5 ± 0.03	8.1 ± 0.01	8.1 ± 0.01
3	9.0 ± 0.01	9.0 ± 0.01	-	-
2	9.0 ± 0.01	9.0 ± 0.01	-	-
1	9.0 ± 0.01	8.5 ± 0.20	-	-
Cef. 2	32.0 ± 1.2	29.0 ± 1.2	19.0 ± 2.2	19.0 ± 2.0
Rox. 2	22.0 ± 0.7	27.5 ± 0.8	43.0 ± 0.20	43.0 ± 0.20
DMSO	-	-	-	-

Table 3. Antibacterial activity of methanolic extract of flowers (MF) of *Artemisia dubia.*

Conc. mg/ml	Zone of inhibition of *E. coli*	
	24 h	48 h
15	9 ± 0.01	10 ± 0.10
12.5	9 ± 0.01	9.5 ± 0.03
10	-	-
7.5	-	-
5	-	-
3	-	-
2	-	-
1	-	-
Cef. 2	38.5 ± 0.6	38.5 ± 0.6
Rox. 2	15.5 ± 0.5	15.5 ± 0.6
DMSO	-	-

Brine shrimp toxicity assay: Results of the brine shrimp lethality assay are shown in Table 4. The MF has LC_{50} value at 266 ppm and 7% lethality at concentration of 10 ppm. It is apparent that MF has cytotoxic activity, which can further be evaluated for pharmacological activity because brine shrimps have been previously utilized in various bio-systems. Among these are included the analysis of pesticides (Fatope *et al*., 1993), plants having ethnomedical uses related to cancer (Mongelli *et al*., 1996), and tropical plants used medicinally (together with screening for larvicidal, fungicidal, and molluscicidal activity by (Cepleanu *et al*., 1994). Brine shrimp is now considered as a suitable probe for the pharmacological activities in plant extracts which may be showing toxicity towards the newly hatched nauplii (Meyer *et al*., 1982).

ML has 37% lethality at 1000 ppm, CL has 13.4% lethality at 1000 ppm and CF has 10% lethality at 1000 ppm. So, all these have LC_{50} value higher than 1000 ppm, which is not significant.

Table 4. Percent death and LC_{50} values for Brine shrimp assay of crude extract of *Artemisia dubia.*

Extracts	Percent death at 24 hour						LC_{50}
	10 ppm	100 ppm	250 ppm	500 ppm	750 ppm	1000 ppm	
MF	7	16.7	46.7	96.7	100	100	266ppm
ML	0	13.4	23.4	33.4	30	37	>1000ppm
CL	0	0	0	6.7	10	13.4	>1000ppm
CF	0	10	10	10	13.4	10	>1000ppm

ML is methanolic extract of leaves; CL is chloroform extract of leaves; MF is methanolic extract of flowers
CF is chloroform extract of flowers

Conclusions

Results conclude that *Artemisia dubia* extract has valuable antimicrobial activity against *Staphylococcus aureus* and *Bacillus subtilis* and the methanolic extract of flowers has brine shrimp cytotoxic effect.

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