

Research Article

Antimicrobial and phytochemical screening of *Oligochaeta ramosa* against different pathogenic microbes- An *In vitro* study

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Biologically active compounds obtained from the medicinal plants are the effective chemotherapeutic agents and offering a broad spectrum of activity with greater emphasis on preventive action. The present study was aimed at evaluating the antimicrobial activities of crude methanolic extract of *Oligochaeta ramosa* (Asteraceae) against pathogenic bacteria species of both G +ve strains (*Staphylococcus aureus*, *Bacillus pumilus*, *Streptococcus pneumoniae*), G -ve strains, (*Escherichia coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*) and fungal species (*Candida albicans*, *Aspergillus niger*). *In-vitro* antimicrobial test was performed by disc diffusion method on nutrient agar and sabouraud dextrose agar for bacteria and fungi respectively, in order to analyze the percentage zone of inhibition and phytochemical screening was also performed. Methanolic extract showed significantly high inhibitory effect against G +ve strains, as compared to G -ve strains, whereas, no effect against *C. albicans* and *A. niger*. Modified agar well diffusion method was used to measure the minimum inhibitory concentration (MIC) and MIC values lies within the range of 75 to 150 µg /ml for the G +ve while 300 to 600 µg /ml for G-ve. Or.Cr was found to contain alkaloids, tannins, saponins, flavonoids and antraquinones and these agents may be responsible for antibacterial activity of this plant.

Keywords: *Oligochaeta ramosa*, Methanolic extract, Antimicrobial assay, Nutrient agar

1. INTRODUCTION

The use of plant and its products has a long history that began with folk medicine and through the years has been incorporated into traditional and allopathic medicine (Dubey et al., 2011). Since antiquity, many plants species reported to have pharmacological properties as they are known to possess various secondary metabolites like glycosides, saponins, flavonoids, steroids, tannins, alkaloids, terpenes which is therefore, should be utilized to combat the disease causing pathogens (Kamali and Amir, 2010; Hussain et al., 2011). Side effects and the resistance that pathogenic microorganisms build against antibiotics, recently much attention has been paid to extracts and biologically active compounds isolated from plant species used in herbal medicine (Essawi and Srour, 2000). Plant-based antimicrobials represent a vast untapped source of medicines and further exploration of plant antimicrobials need to occur. Antimicrobials of plants origin have enormous therapeutic potential. They are effective in the

treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials.

Due to the increase of resistance to antibiotics, there is a pressing need to develop new and innovative antimicrobial agents. Among the potential sources of new agents, plants have long been investigated. Because, they contain many bioactive compounds that can be of interest in therapeutic. Because of their low toxicity, there is a long tradition of using dietary plants in the treatment of infectious disease in Pakistani folk medicine.

Oligochaeta ramosa (Roxb.) (Family: Asteraceae) is referred by multiple Synonyms, i.e., *Oligochaeta albispina*, and *Volutarella ramosa* (Roxb.) (Kiritikar and Basu, 1987), and is known by vernacular name of Badaward (Bombay, Gujrati and Hindi), Badavarda (Urdu) and Shaukatelbaida (Arabic). *Oligochaeta ramosa* is widely distributed in all over India in western and south India, Baluchistan, Punjab plains, Karachi, Sindh and Lasbella. It's flowering and

fruiting time is from January - march (Khare, 2007). It is a straggling or erect, stiff, dichotomously branched, annual herb with white-tomentose, striate stem and branches. The leaves are variable, sessile oblong or obovate, entire or usually pinnatifid lobed, whereas lobes are rounded or mucronate. The heads are 1.5-2.0 cm, long, ovoid, homogenous and pale-purple in color. The multiple involucre bracts are seriate, spine-tipped; the outer are spreading or recurved, while the inner are erect. The pappus are multiple, unequal and silvery brown in coloration. The achenes are acutely angled, grooved and punctate between the angles. The base is narrow, top broad, truncate, dull-brown (Vardhana, 2008).

Oligochaeta ramose is tonic and laxative also used in cure of old fever and also for disorders of the liver. It is slightly mucilaginous and used in cough (Khare, 2007; Vardhana, 2008). It is also used as a febrifuge and often prescribed in fever and general debility. The fruit and root are used in Unani medicine in chronic fevers and diseases of liver and intestines. It also contain anti-pyretic activity, purgative and antimicrobial activity and used for the treatment of external swelling and traditionally used in wound healing (Kiritkar and Basu, 1987; Khare, 2007).

Present study was focused to evaluate the *In vitro* antimicrobial activity and phytochemical constituents of methanolic crude extract of *Oligochaeta ramose* against G +ve strains, (*S. aureus*, *Streptococcus pneumoniae*, *B. pumilus*) G -ve strains, (*E. coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*) and two species of fungi (*Candida albicans* and *Aspergillus niger*).

2. MATERIAL AND METHODS

2.1 Identification of plants:

Oligochaeta ramose (collected from the local pansar in Multan) was identified by a taxonomist Dr. Altaf Dasti, Professor and incharge herbarium of Institute of Pure and Applied Biology, Bahauddin Zakariya University, Multan (Pakistan).

2.2 Preparation of plant crude extract:

The plant material was cleaned off adulterants and crushed into coarse powder by an electrical grinder for further procedure. Triple maceration procedure was used for the purpose of extraction of coarse powdered material (Harborne, 1973). One kg of the coarse powdered material was macerated with 70% aqueous- methanol in air tight amber glass bottles at 25 °C, with occasional shaking thrice a day for one week. After maceration, the soaked coarse powdered material was passed through muslin cloth (double

layered), in order to remove vegetative debris and the obtained filtrate was subsequently filtered through a Whatman-1 filter paper. The filtrate was stored in amber glass air-tight container. The previously mentioned extraction procedure was subsequently repeated twice after each two days and filtrates of these three macerations were combined.

Rotary evaporator (Rotavapor, BUCHI labortechnik AG, Model 9230, Switzerland) attached with a vacuum pump and a recirculation chiller was used for evaporation of the filtrate, under reduced pressure at 37 °C to a thick, semi solid paste. The dark green crude extract was lyophilized to remove moisture contents and the approximate yield was 14.5 %. The dried extract was transferred to amber glass jar and stored at -4 °C in a refrigerator.

2.3 Extract solution preparation

In vitro, experiments were performed by dissolving 0.3 gram of the crude extract in 0.1ml (100 µl) of 100% dimethylsulfoxide (DMSO) and volume was made up to 1 ml (1000 µl) with distill water to prepare 0.3 g /ml, w/v stock solution (300 mg/ml), due to its insolubility in distilled water and stored in refrigerator (Hussain et al, 2013). The dimethylsulfoxide alone did not show any biological and physiological activity. Thereafter serial dilution of stock solution (containing 300 mg/ml) was made, to obtain 30 mg/ml concentration which was used for the antimicrobial sensitivity test.

2.4 Determination of phytochemical constituents

Or.Cr was subjected to phytochemical screening for the detection of alkaloids, carbohydrates, tannins, saponins, anthraquinones, steroids and flavonoids as possible important constituents of the plant, according to standard method (Farooq, 2013). Appearance of yellowish brown coloration on mixing of Dragendorff's reagent with HCl treated aqueous plant extract solution, conform the presence of alkaloids in extract. Molisch's, benedict's and fehling's tests were performed for the detection of carbohydrates. Formation of froth on vigorous shaking of the aqueous extract solution, conform the presence of saponin. Development of blue green or dark green coloration on mixing of aqueous FeCl₃ with extract solution indicated presence of phenols and tannins. The appearance of pink, violet or red coloration on exposure to NH₄OH of the mixture of benzene with aqueous solution of plant extract already acidified with 1% HCl was taken as presence of anthraquinones among the plant constituents. The plant material was deemed positive for flavonoids when it gave a yellow color with AlCl₃ reagent.

2.5 Standard drugs (discs) and micro-organisms used:

All standard drug discs i.e., flucloxacillin, vancomycin, ceftriaxone, ciprofloxacin, ceftriaxone, levofloxacin and amphotericin-B, having drug concentration of 15 µg/disc (Oxobid Ltd. Basingstoke, Hampshire, England) were purchased from G.M, Scientific shop, Multan, Pakistan. While, all the microorganisms i.e., *S. aureus*, *Bacillus pumilus*, *Streptococcus pneumoniae*, *C. freundii*, *E. coli*, *Klebsiella pneumoniae*, *Candida albicans* and *Aspergillus niger*, used for the detection of antimicrobial activity were collected from the University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan. All microbes were cultured overnight in a nutrient agar (pH 5) containing agar (1.2%), peptone (0.5%), yeast (0.3%), and NaCl (0.8%), (Cruickshank et al., 1975; Hussain et al., 2013) Inoculums were prepared by transferring microbial colonies from fresh culture plates to tube containing 10 ml of nutrient broth media. The tubes were shaken occasionally for aeration to promote the microbial growth and were incubated overnight at 37 °C.

2.6 Determination of antimicrobial activity

For the determination of the antimicrobial activity, standard disc diffusion method was adopted (Taylor et al., 1995; Hussain et al., 2014^a; Hussain et al., 2014^b) and three types of discs were used, i.e., discs containing standard antibiotics were used as positive control, discs containing plant crude extract or latex were used as sample discs, and discs containing the DMSO were used as negative control. Punch machine was used to prepare the discs having the diameter of 6 mm from the whatman-1 filter. All the glass wares were sterilized by the dry heat of sterilization. Nutrient agar media and sabouraud dextrose agar media prepared in distilled water and sterilized in autoclave at 121 °C for 15 minutes. Pour the media into separate petri dishes and allowed to set as a firm gel on cooling. The thickness of gels layer should range between 2-3 mm. The test petri-dishes were incubated overnight at 37 °C and those showing no growth were selected for further work. The bacterial culture and fungal culture were transferred from inoculums to petri-dishes by using the sterilized aluminum wire loops, which were subsequently spread by streaking method. All the procedure was carried out in the strict aseptic condition using horizontal laminar flow cabinet. Bacterial cultures were incubated at 37 °C in incubator for 24 hrs while fungal cultures at room temperature for 48 hrs. At the end of the incubation period, zone of inhibition (mm) of the each extract was measured in

comparison with the positive and negative control (Andrews, 2001; Khyade and Vaikos, 2011). For the conformation of the results, each test was performed in triplicate as per table.

2.6.1 Calculation of relative percentage inhibition

The relative percentage inhibition of the Or.Cr with respect to positive control was calculated by using the following formula (Ajay et al., 2002; Hussain et al., 2014^b).

$$\text{Relative percentage inhibition of Or.Cr} = 100 \times (a - b) / (c - b)$$

Where,

a: total area of inhibition of the test extract

b: total area of inhibition of the solvent

c: total area of inhibition of the standard drug

The total area of the inhibition was calculated by using

$$\text{Area of zone of inhibition} = \pi r^2$$

Where,

“r” is radius of zone of inhibition

2.6.2 Determination of minimum inhibitory concentration (MIC)

Modified agar well diffusion method was used to determine the MIC of the Or.Cr (Tagg et al., 1976; Ajay et al., 2002). The Or.Cr was dissolved in DMSO to obtain a concentration range of 75, 150, 300, 600, 1200, 5000 and 10000 µg/ml. In each of these plates four wells were cut out using a cork borer. Using a micropipette, 100 µl of each dilution was added in to wells and plates were incubated at 37°C for 24hrs. The minimum concentration of each extract showing a clear zone of inhibition was considered to be MIC.

2.7 Statistical analysis

The results of the antimicrobial activity of crude extract are expressed as mean ± standard deviation of the response of 3 replicates determinations per sample. Statistically significant differences between groups were measured using one-way analysis of variance (ANOVA) followed by two sample t-test of all groups versus their respective control group and *p < 0.05 was considered statistically significant, p > 0.05 was considered as non-significant and **p < 0.01 was considered highly significant. Results were analyzed statically by using “Graph pad Prism” version 6, (Graph Pad Software, San Diego, CA, USA).

3. RESULTS

3.1 Phytochemical screening

Freshly prepared methanolic extracts of *Oligochaeta*

Table 1: Phytochemical evaluation of *Oligochaeta ramose*.

Chemical tests	Or.Cr	Chemical tests	Or.Cr
Test for carbohydrates		Test for tannin	
A. Molisch's test		A. FeCl ₃ test	
B. Fehling's test	Positive	B. Acetic acid test	Positive
C. Benedict's test		C. KmnO ₄ test	
Test for alkaloids		Test for cardiac glycosides	
A. Hager's test		A. Legal test	
B. Wagner's test	Positive	B. Keller-killiani test	Negative
C. Dragendorff's test			
Test for flavonoids		Test for anthraquinone	
A. Lead acetate test	Positive	A. Borntragers's test	Positive
B. Ferric chloride test		B. Modified Borntragers's test	
Test for saponins		Test for steroids	
Foam test	Positive	Lieberman burchard test	Negative
Test for protein		Test for coumarin	
A. Biuret test	Positive	A. Alkaline reagent test	Negative
B. Lead acetate test		B. NaOH soaked paper test	

Where, Or.Cr = Methanolic crude extract of *Oligochaeta ramose*.

3.2 In Vitro antimicrobial activity

Methanolic crude extract of the *Oligochaeta ramose* (Or.Cr) showed the diameter of the zone of inhibition (including diameter of disc 6 mm) of 21.15 mm against *B. pumilus*, 22.55 mm against *S. aureus* and 18.95 mm against *S. pneumoniae* as compared with standard drugs (15 µg/disc) vancomycin (22.29 mm), flucloxacillin (24.65 mm), ceftriaxone (22.50 mm), with relative percentages of inhibition 90.05, 83.68 and 70.93 respectively. Similarly the Or.Cr showed the diameter of the zone of inhibition of 20.20 mm against *E. coli*, 17.45 mm against *C. freundii* and 16.50 mm against *K. pneumoniae* as compared with standard drug (15 µg/disc) ceftriaxone (24.55 mm), ciprofloxacin (22.36 mm) and levofloxacin (21.70 mm) with relative percentages of inhibition 67.69, 60.90 and

ramose was subjected to a preliminary phytochemical screening for various constituents and their results are depicted in **Table 1**.

57.85 respectively. Whereas Or.Cr showed less response against fungal species i.e., *C. albicans* and *A. niger* with relative percentages of inhibition of 36.25 and 25.15 against amphotericin- B respectively. All these results are depicted in **Table 2** and **3**.

As shown in **Table 3**, Or.Cr showed strong inhibition against tested G +ve with MIC value of 75 - 150 µg/ml, whereas, MIC values for the G -ve were ranged from 300-600 µg/ml. MIC values for the fungal species were ranged from 2000 - 5000 µg/ml.

After statistical analysis, P value was determined which was significant for G +ve, i.e., less than 0.05 (P < 0.05). It can be inferred that Or.Cr has significant antibacterial activity against Gram +ve strains as compared to Gram -ve strain of bacteria, while no response against fungal species.

Table 2: Zone of inhibition (mm) of sample (Or.Cr), positive control and negative control against different bacterial and fungal species (values are expressed as mean \pm SEM., n = 3).

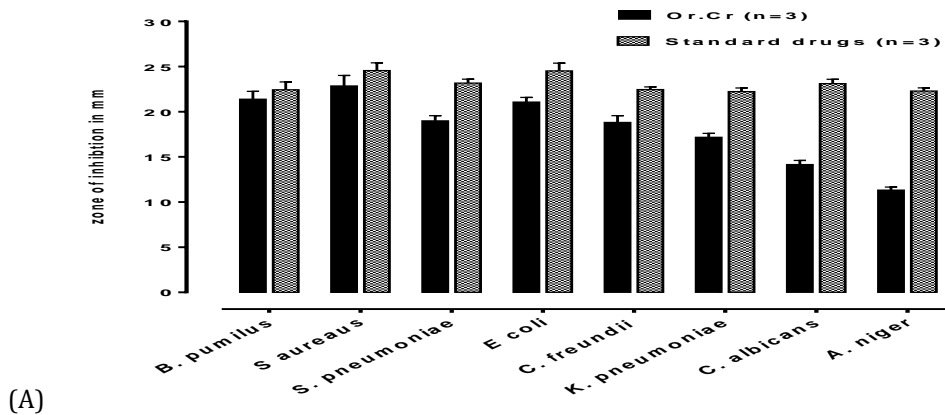
<i>Bacterial Strains</i>	Zone of Inhibition (mm/sensitive strain)			
	Or.Cr ^a	Positive control		Negative control
<i>B. pumilus</i>	21.15	Vancomycin	22.29	NR
<i>S. aureus</i>	22.55	Flucloxacillin	24.65	NR
<i>S. pneumoniae</i>	18.95	Ceftriaxone	22.50	NR
<i>E. coli</i>	20.20	Ceftriaxone	24.55	NR
<i>C. freundii</i>	17.45	Ciprofloxacin	22.36	NR
<i>K. pneumoniae</i>	16.50	Levofloxacin	21.70	NR
<i>C. albicans</i>	13.45	Amphotericin-B	22.35	NR
<i>A. niger</i>	10.95	Amphotericin-B	21.85	NR

Values are presented as mean of triplicate experiments, ^a= Diameter of the zone of inhibition including diameter of disc 6mm, -ve = negative control, i.e., Dimethylsulfoxide, Positive control = Standard drugs; NR = No response

Table 3: Relative percentage inhibition and MIC of Or.Cr against different bacterial and fungal species (values are expressed as mean \pm SEM., n = 3).

Sr. No.	Test Bacteria	RPI (%)	MIC (μ g/ml)
1	<i>B. pumilus</i>	90.05	75
2	<i>S. aureus</i>	83.68	75
3	<i>S. pneumoniae</i>	70.93	150
4	<i>E. coli</i>	67.69	300
5	<i>C. freundii</i>	60.90	600
6	<i>K. pneumoniae</i>	57.85	600
7	<i>C. albicans</i>	36.25	2000
8	<i>A. niger</i>	25.15	5000

RPI= Relative percentage of inhibition; MIC= minimum inhibitory concentration.



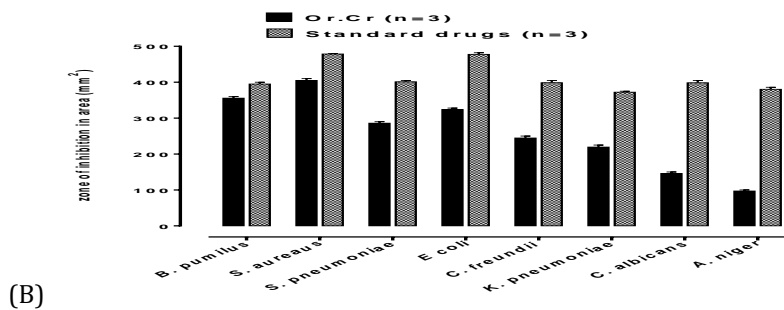


Figure 1: Zone of inhibition of the crude extract of *Oligochaeta ramose* (Or.Cr) in (A) diameter (mm) and (B) area (mm²) against different bacterial and fungal species (values are expressed as mean \pm SEM., n = 3).

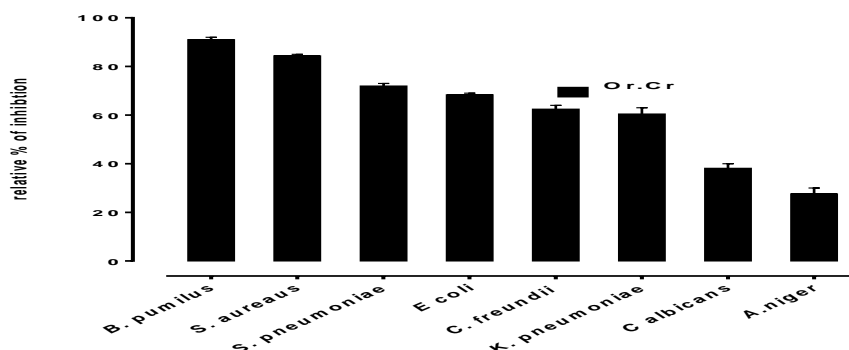


Figure 2: Relative percentage inhibition of crude extracts of *Oligochaeta ramose* (Or.Cr) against different bacterial and fungal species.

4. DISCUSSION

Multi drug resistance is the major hurdle of this era which is leading toward mortality and morbidity so the researchers are trying their best to develop new natural products from medicinal plants against multidrug resistant microbial strains (Braga et al., 2005). Medicinal plants are the major source of the secondary metabolites which have been reported to possess the antimicrobial property (Hussain et al., 2013).

As *Oligochaeta ramose* is known to possess the antimicrobial, antipyretic and anti diarrheal activity in the traditional medicine system, so, in the present study, main focus was on the screening of phytochemical constituents and antimicrobial activity against different pathogenic species of G +ve bacteria, i.e., *Staphylococcus aureus*, *Bacillus pumilus*, *Streptococcus pneumoniae*, G-ve bacteria, i.e., *Citrobacter freundii*, *Escherichia coli* and *Klebsiella pneumoniae* and also against two different fungal species, i.e., *Candida albicans* and *Aspergillus niger*. Antimicrobial results were statistically analyzed by student t-test at P-0.05 confidence limit, Or.Cr showed higher inhibition against studied Gram +ve strains, significantly

low inhibition against G-ve bacteria whereas, very low activity was showed against *Candida albicans* and *Aspergillus niger*. Or.Cr showed the maximum relative percentage inhibition against *Bacillus pumilus* (90.05 %), supporting the view, that medicinal plants might be useful in the development of novel antimicrobial agents (Heinrich and Simon, 2001), whereas, minimum relative percentage inhibition against *Klebsiella pneumoniae* (57.85 %). Phytochemical study of *Oligochaeta ramose* reveals the presence of alkaloids, tannins, saponins flavonoids and anthraquinones that may be responsible for the antibacterial activity.

5. Conclusion

Oligochaeta ramose is believed to possess the strong antibacterial activity due to presence of tannin, alkaloids saponins, flavonoids and anthraquinones, which have been studied (Draughon, 2004). Tannins have important role such as stable and potent antioxidants (Trease and Evans, 1983). Most of the organisms used in the research study were causative agents of diarrhea and dysentery, while *Oligochaeta ramose* inhibit the growth of these microbes, so it

can be used for the treatment of diarrhea and dysentery. Moreover, this study can be used as a tool for the isolation of pure antimicrobial from the plant and more works need to be done with the view of their use for in-vivo studies.

Conflict of Interests

Authors declared no competitive interests for the presented work.

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