REPORT

Antimicrobial activities of rhizomes of *Polygonatum verticillatum*: Attributed to its total flavonoidal and phenolic contents

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Abstract: The current study was undertaken to evaluate the rhizomes of *Polygonatum verticillatum* against various pathogenic bacteria and fungi. Broad spectrum antibacterial activity was demonstrated by the crude extract of the plant and its subsequent solvent fractions; predominantly against Gram-negative bacteria. MICs of the extracts against *Escherchia coli*, *Salmonella typhi* and *Shigella flexeneri* were in the range of 1.5-40 µg/ml, 03-06 µg/ml and 03-40 µg/ml, respectively. The only sensitive Gram-positive bacterium was *Staphylococcus aureus* with MICs in the range of 75-80 µg/ml. The fungicidal activity was limited to *Microspoum canis* and *Fusarium solani* and the MICs were in the range of 350-360 µg/mL and 190-290 µg/ml respectively. The various fractions of rhizomes contained significant concentration of total flavonoidal and total phenolic contents that could be responsible for the current findings.

Keywords: *Polygonatum verticillatum*, Rhizomes, antibacterial, antifungal, total flavonoidal, phenolic contents.

INTRODUCTION

Over the years, medicinal plants have been materialized by the different civilizations of the world as a powerful heeling tool against various human sicknesses (Khan *et al*., 2009; Sengul *et al*., 2009). Faith of the clinical microbiologist in medicinal plant is strengthened by the fact that even the commonly used food staff possessed antimicrobial secondary metabolites that prevents them from deterioration. However, the scientific examination of the medicinal plants and plant based compounds for the healing of various infectious disorders was started in the late 19th century (Khan *et al*., 2007).

Even in the present era of medical engineering, the irrational approach towards the clinical use of different available antibiotics is accountable for the proliferation of pathogenic bacteria that induced resistance to various classes of antibiotics. Eventually this fabricated a progressive loss in the therapeutic effectiveness of antibiotic (Woodford and Ellington, 2007; Khan *et al*., 2011). Many new antibiotics have been introduced to combat with such bacterial infections but multiple resistance genes have appeared rapidly among bacteria which developed resistance to those antibiotics. Nature has been a foundation of therapeutic components for many decades. Larger number of recent drugs has been derived from medicinal plants including antimicrobial, with possibly novel mechanism of action and thus reducing antimicrobial resistance.

P. verticillatum [L.] All. (Nooreallam) is a member of genus *Polygonatum* consist of approximately 57 species of family *Convallariaceae* (Khan *et al*., 2010). The syrup of fresh rhizomes of *Polygonatum verticillatum* is used in the treatment of pain, pyrexia, burning sensation and for phthisis (Singh, 2006). Rhizomes are also used in combination with other herbs to promote urine discharge (diuretic) and urinary infections (Ballabh *et al*., 2008). Other ethnobotanical uses of the rhizomes include as emollient, aphrodisiac, vitiated condition of pitta and vata, appetizer and tonic, glactagogue (increases milk release), weakness (Alam, 2004). Recently we have rationalized scientifically the analgesic profile of rhizomes and aerial parts of the plant in different pain models (Khan *et al*., 2010a; Khan *et al*., 2011) while the aerial parts of the plant exhibited significant phytotoxicity and nutritional value (Saeed *et al*., 2010a; Saeed *et al*., 2010b). The current study was designed to validate the ethnobotanical uses of the rhizomes of *Polygonatum* in infectious disorders.

MATERIALS AND METHODS

Plant material

The whole plant, *P. verticillatum* [L.] All. was collected from District Swat, Khyber Pukhtonkhawa, Pakistan in July-Aug 2007. The botanical identity of the plant

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material was done by the Taxonomy Department of PCSIR Laboratories, Peshawar and a specimen with catalogue No: 9970 (PES) was deposited in the herbarium of PCSIR Laboratories, Peshawar.

Plant extraction and fractionation

The air-shade dried rhizomes of the *Polygonatum verticillatum* (8 kg) were ground to fine powder. The powder material was extracted by maceration with methanol at room temperature for 14 days with occasional shaking (Khan *et al*., 2009). The methanol extracted residue was filtered off with a muslin cloth and the filtrate was concentrated under vacuum at low temperature (40°C) using rotary evaporator, yielded a dark greenish semisolid material (2200 g, 27.50% w/w). The crude methanol extract (1.6 kg) was dissolved in distilled water and sequentially fractionated with hexane, chloroform, ethyl acetate and butanol, yielding hexane (258 g, 16.13% w/w), chloroform (219 g, 13.69% w/w), ethyl acetate (226 g, 14.13% w/w), butanol (265 g, 16.56% w/w), and aqueous (501 g, 31.31% w/w). These fractions were then screened for various pharmacological and phytochemical studies.

Antibacterial bioassay

The crude extract and its subsequent solvent fractions of rhizomes were evaluated against a variety of human pathogens by agar well diffusion method as described perversely (Khan *et al*., 2008). Briefly, test material (3 mg/ml) was dissolve in dimethyl sulfoxide (DMSO). Approximately 45 ml of molten nutrient agar was dispensed on sterilized petri-plates, and was permitted to harden. Bacteria were dispersed on these nutrient agar plates by preparing sterile soft agar accumulating 100 µl of bacterial culture. 6 mm long sterile metallic borer was used for wells digging at suitable distance and spotted for identification. Sample (100 µl) was poured into each well, and the plates were incubated at 37^{o}C for 24 h. The antibacterial activity was estimated in terms of inhibition zone. The drug, Imipenem (a broad-spectrum β-lactam antibiotic) and DMSO were used as positive and negative control respectively.

Antifungal bioassay

Agar tube dilution method was employed to evaluate antifungal activity (Khan *et al*., 2008). The extracts (24 mg/ml) were dissolved in dimethyl sulfoxide (DMSO) for the preparation of stock solution. Sabouraud dextrose agar (SDA) 4 ml was distributed into screw cap tubes, which were autoclaved at 120^{o}C for 15 min and then cooled to 15^{o}C. The non-solidified SDA media was poisoned with stock solution (66.6 µl) giving the final concentration of 400 µg of the extract per ml of SDA. Solidification of tubes was occurred at room temperature in slanted position. Each tube was inoculated with a piece (4 mm diameter) of inoculums removed from a seven days old culture of fungi for non-mycelial growth; an agar surface streak was employed. DMSO was used as control while Miconazol and Amphotericin-B as standard drug. After incubation period (7 days) at 28 ± 1^{o}C w relative humidity (40-50%), inhibition of fungal growth was observed in test tubes.

MIC determination by macrodilution method

Crude extract and its solvent fractions (10 mg/ml) were dissolved in DMSO. Sterile water was used for dilution, consecutively in microplates, kept in a laminar flow cabinet. Similar concentration of an actively growing culture of the test microbes was added to different wells and cultures were grown for 12h in 100% relative humidity at 37ºC. Later on, wells were felt with tetrazolium violet. Violet color of the culture signifies growth. MIC was rated as the least drug concentrations accountable for the prevention of growth. Acetone was used as negative control while Imipenem, Amphotericin B and miconazole were positive controls in the assay (Atta-ur-Rehman *et al*., 2001).

Bacterial and fungal strains

Assays were executed on different bacterial and fungal strains. Bacterial strains were *E. coli* ATCC 25922*, B. subtilis* ATCC 6633*, S. flexeneri (clinical isolate), S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 *and S. typhi* ATCC 19430. Fungal strains include *T. longifusus* (clinical isolate), *C. albicans* ATCC 2091*, A. flavus* ATCC 32611*, M. canis* ATCC 11622*, F. solani* 11712 and *C. glaberata* ATCC 90030. The strains were kept on agar slant at 4^{o}C. Before any test, the activation of strains were occurred at 37^{o}C for 24 h on nutrient agar (NA) or Sabouraud glucose agar (SGA) respectively for bacteria and fungi.

Total flavonoidal contents

To determine the flavonoid contents, continue extraction of the samples (10 g) i.e. crude extract and solvent fractions of the plant with 10 ml of the 80% aqueous methanol at ambient temperature. The resulting solutions were filtered through Whatman filter paper No.42 (125 mm). The filtered was then taken in a beaker and evaporated at water bath to get flavonoid contents (Boham and Kocipai, 1994).

Total phenolic contents

The total phenol concentration of extracts and its subsequent solvent fractions were determined by method described previously (Khan *et al.*, 2008). Briefly, 10 mg of extracts were and introduced to Folin-Denis reagent (5 ml), Na_2CO_3 (20%, 10 ml). Distilled water was used for dilution (factor 100). After filtration, it was kept at room temperature for 10 min. Using Spectronic 20D (Milton Roy), absorbance was taken at 770 nm against blank. The total phenolic contents were calculated by comparing with a standard curve of tannic acid.

STATISTICAL ANALYSIS

Data are presented as mean of three different experimental findings.

RESULTS

The results of antibacterial assay are presented in tables 1-2 while antifungal in 3−4. *S. flexeneri* was the most sensitive Gram positive bacterium (MIC: 3−30 µg/ml). Except *P. aeruginosa*, all tested Gram negative bacteria showed marked antibacterial activity. Nevertheless, the antifungal activity was limited to *M.canis* and *F. solani.*

DISCUSSION

Broad spectrum antibacterial activity was observed for the crude extract and its subsequent solvent fractions. Of the tested Gram-Positive bacteria, *S. flexeneri* was the most sensitive strain to all fractions of the plant and the MIC was in the range of 3-30 µg/ml. *S. aureus* was susceptible to chloroform, ethyl acetate and butanol fractions and the MIC was ranges from 75-80 µg/ml. The crude extract and its solvent fractions were ineffective against *B. subtilis*. Among the tested Gram negative bacteria, *E. coli* demonstrated marked sensitivity to crude drug and its fractions. The MIC was estimated in the range of 1.5-40 µg/ml. *S. typhi* exhibited notable sensitivity to extracts and the MIC calculated in the range of 3-6 µg/ml.

As shown in table 3 and 4, the plant exhibited antifungal activity only against *M. canis* and *F. solani*. Similarly, only the chloroform and butanol fractions were effective against *M. canis* and the MIC was 350 and 360 µg/ml respectively. For *F. solani*, only the crude extract and hexane fraction registered antifungal activity with MIC 190 and 290 µg/ml respectively. The rest of tested fungi were unsusceptible to the extract and fractions.

Food borne infections represent a foremost health problem for the health care professional's world wide. In humans, *Shigella* species are the contributory microbes of bacillary dysentery (Sansontti, 2001). *Salmonella* is one of the essential pathogenic genera involved in food borne bacterial infections (Cetinkaya *et al*., 2008). Marked antibacterial activity was estimated for the crude extract of the rhizomes of *P. verticillatum* and its solvent fractions against the *S. flexeneri* (clinical isolated). The sensitive of *S. flexeneri* was notable to all fractions of the plant and the MICs were in the range of 03-30 µg/ml. Comparable trend of sensitive was observed for *S. typhi* and the calculated MICs were in the range of 3-6 µg/ml.

Table 1: Antibacterial activity of the crude methanol extract of rhizomes of *Polygonatum verticillatum* and its subsequent solvent fractions

Bacterial strains	Zones of inhibition (in mm)						
	Std	Crude	Hexane	$CHCl_3$	EtOAc	Butanol	Aqueous
Gram-Positive							
Bacillus subtilis	23	-	-	-	-	-	-
Staphylococcus aureus	27	-	-	10	10	11	-
Gram-Negative							
Escherchia coli	20	14	20	20	18	-	-
Pseudomonas aeruginosa	29	-	-	-	-	-	-
Salmonella typhi	26	-	11	10	10	-	-
Shigella flexeneri	28	16	14	10	15	14	20

Standard drug (Std) = Imipenem, Control = DMSO, Sample= 3 µg/ml. Data represent mean of three different experiments.

Table 2: Antibacterial activity of crude extract and the fractions of rhizomes of *Polygonatum verticillatum* represented as minimum inhibitory concentration (MIC)

Bacterial strains	MIC (µg/ml)						
	Std	Crude	Hexane	$CHCl_3$	EtOAc	Butanol	Aqueous
Gram-Positive							
Bacillus subtilis	0.22	-	-	-	-	-	-
Staphylococcus aureus	0.17	-	-	75	80	75	-
Gram-Negative							
Escherchia coli	0.19	40	05	06	1.5	-	-
Pseudomonas aeruginosa	0.31	-	-	-	-		-
Salmonella typhi	0.17	-	03	05	06	-	-
Shigella flexeneri	0.13	30	50	16	40	05	03

Standard drug (Std) = Imipenem

Table 3: Anti-fungal activities of the crude extract of rhizomes of *Polygonatum verticillatum* and its subsequent solvent fractions

Fungal strains	% Inhibition of Fungal Growth						
	Std	Crude	Hexane	$CHCl_3$	EtOAc	Butanol	Aqueous
Trichophyton longifusus	70 [1]	-	-	-	-	-	-
Candida albicans	110 [1]	-	-	-	-	-	-
Aspergillus flavus	20 [2]	-	-	-	-	-	-
Microspoum canis	98.4 [1]	-	-	10	-	10	-
Fusarium solani	73.26 [1]	40	70	-	-	-	-
Candida glaberata	110.8 [1]	-	-	-	-	-	-

[1]Standandard Drug = Miconazole, [2]Standandard Drug = Amphotericin B.

Table 4: The minimum inhibitory concentration (MIC) of the crude extract of rhizomes of *Polygonatum verticillatum* and its subsequent solvent fractions in antifungal assay

Fungal strains	MIC (µg/ml)						
	Std	Crude	Hexane	$CHCl_3$	EtOAc	Butanol	Aqueous
T. longifusus	1.4[1]	-	-	-	-	-	-
C. albicans	1.8[1]	-	-	-	-	-	-
A. flavus	2.3[2]	-	-	-	-	-	-
M. canis	1.6	-	-	360	-	350	-
F. solani	1.1[1]	290	190	-	-	-	-
C. glaberata	0.5[1]	-	-	-	-	-	-

Standard drug (std)= [1]Miconazole, [2]Amphotericin-B.

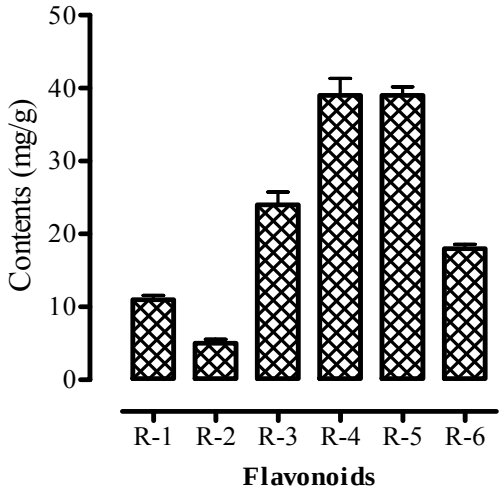

Fig. 1: The total flavonoids content in various fractions of the rhizomes of the plant. R-1=Crude extract, R-2= Hexane, R-3=Chloroform, R-4=Ethyl acetate, R-5=Butanol, R-6=Aqueous

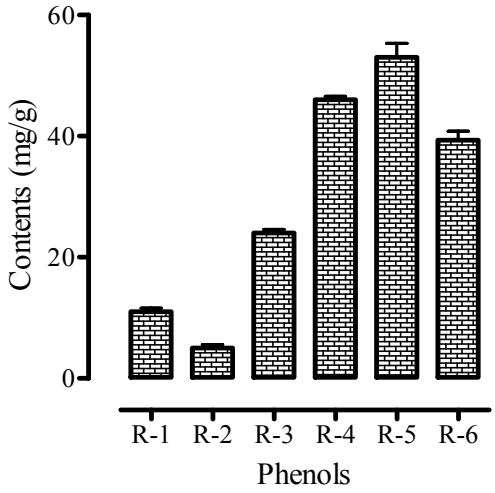

Fig. 2: The total phenols content in various fractions of the rhizomes of the plant. R-1=Crude extract, R-2= Hexane, R-3=Chloroform, R-4=Ethyl acetate, R-5=Butanol, R-6=Aqueous.

Therefore, the results of the preliminary evaluation validated the use of the plant for the treatment of feverish conditions and dysentery.

E. coli is the major causative agent in urinary tract infections (UTIs). It has been estimated that around 50-80% of women will be the victim of UTI at least once in their lifetime and 20-50% of women will faced repeated incidents (U-Syn and Young-Hyun, 2008). The crude extract and its solvent fractions demonstrated profound antibacterial activity against *E. coli*. The MICs were estimated in the range of 1.5-40 µg/ml. *S. aureus* can be a major causative agent in a wide verity of infections ranging from minor skin infections to postoperative wound infections. The wide spread usage of antibiotics is responsible for the proliferation of genes with increasing resistance to many strains of *S. aureus* (Cermelli *et al*., 2008). Among the tested fractions of plant, *S. aureus* was

sensitive to chloroform, ethyl acetate and butanol fractions and the MICs were ranges from 75-80 µg/ml. Based on the results of total content of flavonoids (fig. 1) and phenols (fig. 2), the current findings can be attributed to these pharmacological active classes. The antimicrobial potential of these groups are well documented (Kuete *et al*., 2007; Oliveira *et al*., 2008).

In conclusion, our study registered broad spectrum antibacterial activity of the various extracts of rhizomes of *P. verticillatum* against various pathogenic bacteria. In this regard, we have provided a scientific rationale for the traditional practice of the plant in the treatment of various infectious diseases and also as an alternate natural heeling agent to synthetic drugs. Moreover, further evaluation of the antibacterial properties of various fractions and elucidation of the secondary metabolites responsible for the activities is warranted for lead compounds of clinical utility.

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