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Antibacterial and Antifungal Activity in Three Species of *Siphonaria* (Gastropoda: Pulmonata) Collected From Rocky Ledge of Mubarak Village, Karachi

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Abstract.- In the present study hexane, chloroform, methanol, distilled water, hexane:chloroform, chloroform:methanol and methanol:distilled water extracts of three species of *Siphonaria*, *S. ashgar*, *S. belcheri* and *S. kurracheensis* were screened for antibacterial and antifungal activity. The extracts were obtained from the whole body tissue and tested against bacterial strains, *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, *Shigella flexneri*, *Pseudomonas aeruginosa* and *Salmonella typhi*. Pure methanol and chloroform:methanol were the best solvents for extracting antibacterial compounds from *Siphonaria* species, whereas, pure hexane was the weakest solvent for the extraction. The extracts of *S. ashgar* showed antibacterial activity against five pathogenic bacteria, whereas, the extracts of *S. belcheri* and *S. kurracheensis* against two and three pathogenic bacteria, respectively. The only pathogenic bacterium found resistant to the *Siphonaria* extracts was *Bacillus subtilis.* The extracts of *Siphonaria* species showed no antifungal activity against *Candida albicans, C. glabrata, Aspergillus flavus*, *Microsporum canis* and *Fusarium solani*.

Key words: Antibacterial activity, antifungal activity, *Siphonaria* sp., pulmonate gastropods.

INTRODUCTION

In numerable microorganisms inhabit the benthic marine environment and thus these animals particularly the sessile forms are more vulnerable to microbial infections. To combat the microbial pressure these animals have evolved chemical defense mechanisms by producing metabolites. These metabolites have been investigated for their, anti-inflammatory, antimicrobial, cytotoxic and antitumour properties (Burkholder and Burkholder, 1958; McCaffrey and Endean, 1985; Koh, 1997; Anand and Edward, 2001). Like other animals, antimicrobial activity has also been observed in the extracts of various species of gastropods (Fukuyama *et al*., 1998; Prem and Patterson, 2002; Ramasamy and Morugan, 2005). Beside that many marine molluscs and polychaetes have evolved chemical defense mechanism for their eggs and thus produce secondary metabolites which possess antimicrobial activities (Kamiya *et al*., 1984, Matsunaga *et al*., 1986; Yanazaki, 1993; Benkendorff *et al*., 2000, 2001).

Siphonariid (Pulmonata) limpets were found to contain polypropionate metabolites (Faulkner, 1988) that are believed to be used in chemical defense against predators. These metabolites exhibit antimicrobial activity and are of interest to both synthetic and bioorganic chemists (Hochlowski and Faulkner, 1983; Hochlowski *et al*., 1983; Manker *et al*., 1988; Paterson and Perkins, 1992; Garson *et al*., 1994a, b).

Polypropionate metabolites were found in the tissue extracts of *Siphonaria* species, such as, *S. denticulata* (Hochlowski *et al*., 1983), *S. lesson* (Capone and Faulkner, 1984), *S. normalis* (Roll *et al*., 1986), *S. baconi* (Manker and Faulkner, 1989), *S. maura* (Manker and Faulkner, 1989) and *S. capensis* (Beukes and Davies-Colman, 1999). Biskupiak and Ireland (1983) reported the presence of antimicrobial activity in the skin extracts of *S. pectinata* collected from Key Biscayne, Florida. They isolated a new antibiotic, pectinatone from it, which was active against gram (+) bacteria, *Staphlycoccus aureus*, *Bacillus subtilis* and yeast, *Candida albicans* and *Saccromyces cerevisiae*. Paul *et al.* (1997) reported the presence of various compounds in the extracts of *S. pectinata* from the

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intertidal zone of La Caleta (Cadiz, Spain). These polypropionates showed mild cytotoxicity against human tumour cells.

Numerous studies were done on the antibacterial, antifungal, phytotoxic and insecticidal activities of various marine benthic algae found on the coast of Pakistan (Usmanghani *et al.*, 1984; Usmanghani and Shameel, 1986; Sidddiqui *et al.*, 1993; Rizvi and Shameel, 2004, 2005). The other group whose biological activity and antibacterial activity was reported from Pakistan includes the marine bacteria (Uzair *et al*., 2006; Ahmed *et al*., 2008). In the present study three species of gastropod *Siphonaria ashgar, S. belcheri* and *S. kurracheensis* were screened for antibacterial and antifungal activity against human pathogenic bacteria and fungi, respectively.

MATERIALS AND METHODS

The three species of *Siphonaria*, that is, *S. ashgar, S. belcheri* and *S. kurracheensis* were collected from the rocky ledge of Mubarak Village. The animals were brought to the laboratory and the extract was prepared from the tissue.

Extraction of bioactive compounds from Siphonaria *species*

The whole animal was removed from the shell. The whole body tissue was cut into small pieces with sterilized scissors and was grinded using mortar and pestle. Organic extracts were prepared by soaking the grinded tissue in solvents such as hexane, chloroform, methanol, distilled water, hexane:chloroform (1:1, v:v) chloroform:methanol (1:1, v:v) and methanol:distilled water (1:1, v:v). In order to prepare the organic extract, 0.5 gm of grinded tissue was soaked in solvents and left overnight. Next day the crude extract was decanted and fresh solvent was added to the remaining sample and the procedure was repeated until the extract showed no colouration. The extraction was carried out at room temperature in dark. The solvent was evaporated by using rotary evaporation under vacuum. The dried extracts of 10 mg was collected in a pre-weighed vials.

Antibacterial activity of Siphonaria *extracts*

The antibacterial activity was evaluated using the agar well diffusion method (Carron *et al.,* 1987; Kivack *et al.*, 2001; Stepanovic *et al*., 2003).

The strains of bacteria used were *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Shigella flexneri, Pseudomonas aeruginosa* and *Salmonella typhi* to screen the antibacterial activity of Siphonaria extracts. On the first day single colony of each bacterium was inoculated on the nutrient broth and incubated at 30^{o} C for 24 hrs. After 24 hours of incubation, the material was shaken and 10µl was poured on to the plate containing nutrient agar. The plate was rotated to make even distribution of the culture and allowed to solidify. After solidification, numbers of wells were made by using sterile borer of 6 mm diameter. The wells were marked with sample code and 100µl of crude extract was added in their respective wells. Wells supplemented with Dimethyl Sulfoxide (DMSO) and reference antibacterial drug (Imipenum) served as negative and positive control, respectively. The plates were incubated at 30^{o}C for 24 hrs. After incubation the plates were examined for the zones of inhibition and the diameters of these zones were measured in millimeters. The criteria used to show activity was as follows: 0 mm, no activity; 9-12 mm, low activity; 13-15 mm, moderate activity; 16-18 mm, good activity; > 18 mm, significant activity.

Antifungal activity of Siphonaria *extracts*

The antifungal activity was evaluated using the agar tube dilution method (Blank and Rewbell, 1965; Shaukat *et al.*, 1980).

The antifungal activity of crude extracts of species of *Siphonaria* was screened against following microorganism, *Candida albicans* and *C. glabrata* (non-mycelial), *Aspergillus flavus, Microsporum canis* and *Fusarium solani* (mycelia). 24 mg of crude extracts was dissolved in 1 ml Dimethyl Sulfoxide (DMSO) that served as stock solution. 4 ml of Sabouraud Dextrose Agar (SDA) media were poured in each screw capped tubes and were autoclaved at 121^{o}C for 15 minutes. Autoclaved tubes were allowed to cool to 50^{o}C and non-solidified SDA was inoculated with 66.6 µl of

Table I.- Antibacterial activity (mm) of different extracts of *Siphonaria ashgar, S. belcheri* and *S. kurracheensis*.

Species	Solvents	*Escherichia coli*	*Shigella flexneri*	*Pseudomonas aeruginosa*	*Salmonella typhi*	*Bacillus subtilis*	*Staphylococcus aureus*
S.ashgar	Hexane	-	-	-	-	-	-
	Chloroform	-	-	12	-	-	-
	Methanol	9	12	12	12	-	-
	Distilled water	-	-	11.5	-	-	-
	Hexane:chloroform (1:1)	-	-	-	-	-	-
	Chloroform:methanol (1:1)	12	-	12.5	-	-	11
	Methanol:water (1:1)	-	-	14	9	-	-
S. belcheri	Hexane	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	-
	Methanol	-	-	13	-	-	-
	Distilled water	-	-	-	-	-	-
	Hexane:chloroform (1:1)	-	-	10	-	-	-
	Chloroform:methanol (1:1)	-	-	12.5	-	-	11
	Methanol:water (1:1)	-	-	-	-	-	-
S. kurracheensis	Hexane	-	-	-	-	-	-
	Chloroform	-	-	-	-	-	-
	Methanol	-	11	-	-	-	-
	Distilled water	-	-	10	-	-	9
	Hexane:chloroform (1:1)	-	-	-	-	-	-
	Chloroform:methanol (1:1)	-	-	-	-	-	-
	Methanol:water (1:1)	-	-	-	-	-	-

sample (crude extract) which provided a final concentration of 400 µl/ ml of the SDA media with sample. Tubes were then allowed to solidify in slanting position at room temperature for 24 hours. Each tube was inoculated with 4 mm diameter disc of inoculum taken from seven-days-old culture of mycelial fungi and the tubes were incubated for 7-10 days in a relative humidity of 40-50% with an open pan of water in the incubation room. For non-mycelial fungi streaking method was used and the tubes were incubated at 27-29°C for 48 hours. Media was supplemented with DMSO and reference (standard) antifungal drug which served as negative and positive control, respectively. At the end of experiment all plates were examined for the zones of inhibition and the diameters of these zones were measured in millimeters. Percent inhibition was calculated:

100 - linear growth in test (mm)/linear growth in control (mm) x 100

The criteria used to show the inhibition of fungal growth: 0-9%, no activity; 10-39%, low; 40-59%, moderate; 60-69%, good; 70% or above, significant activity.

RESULTS AND DISCUSSION

The study showed that the extract of all the three species of *Siphonaria*, *S. ashgar*, *S. belcheri* and *S. kurracheensis* possessed the antimicrobial activity (Table I). The antibacterial activity in extract of *S. ashgar* was observed against five pathogenic bacteria, whereas this activity in *S. belcheri* and *S. kurracheensis* was noted against two and three pathogenic bacteria, respectively. The only bacterium found resistant to the siphonariid extracts was *Bacillus subtilis.*

The solvents combination of pure methanol and chloroform:methanol was found to be best solvents for extracting antibacterial compounds from *Siphonaria* species, whereas, pure hexane was the weakest solvent (Table I).

The extract of all the three species of *Siphonaria,* showed no antifungal activity against the fungal strains, that is, *Candida albicans, C. glabrata, Aspergillus flavus, Microsporum canis* and *Fusarium solani* during the present study.

The three *Siphonaria* species were found to

exhibit antimicrobial activity against various pathogens, which showed that these species use chemical defense through the production of antibiotic compounds to combat microbial attacks. According to McQuaid *et al.* (1999) the species of *Siphonaria* showed defensive chemical mechanisms against their predators by producing biologically active substances. Earlier different metabolites present in the extract of *Siphonaria* species were found to exhibit antimicrobial activity (Biskupiak and Ireland, 1983; Hochlowski and Faulkner, 1983). The antimicrobial activity has also been found in freshly laid egg masses of 39 molluscs, including *S. denticulata* and *S. zelandica* and 4 species of polychaetes against three human pathogenic bacteria, *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Benkendorff *et al*., 2001). According to Benkendorff *et al.* (2001) the presence of antimicrobial activity in the freshly laid eggs indicated that these animals use chemical defense to protect their early stage embryos against bacterial infection.

In the present study, the antibacterial activity in *Siphonaria* species was determined by Zone of Inhibition (ZI) assay. There is a possibility that the antimicrobial activity in *Siphonaria* species is more widespread than indicated in the present study as has been reported by Benkendorff *et al*. (2001) that extracts from the egg masses of many species of gastropods and polychaetes, which showed negative test in the Zone of Inhibition assay react positively in Fluorescin diacetate assay. The ZI assay has a number of associated limitations (Chand *et al*., 1994; Benkendorff *et al*., 2000). Furthermore according to Jenkins *et al.* (1998) the use of antibiotic disc susceptibility test or disc diffusion assays has ability to identify active metabolites and serve as a mean for the initial screening for antimicrobial activity. Such assays measure only the toxicity (inhibition of cell growth) and thus the absence of antimicrobial activity in laboratory does not necessarily indicate a lack of antimicrobial chemical defense.

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