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Antimicrobial activity of the methanolic extract of *Phallusia nigra* Sav.

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ABSTRACT

Ascidians exhibit pronounced pharmacological activities which may be of use in the biomedical area. In the present study methanolic extract of the simple ascidian, Phallusia nigra Sav. has been tested against eleven bacterial and three fungal pathogens (hospital isolates) by using disc diffusion method. The extract showed high degree of activity against all the tested bacterial isolates and fungal pathogens in increasing concentrations when compared to that of the standard drugs used. Methanol extract showed the maximum antimicrobial activity. Hence the Minimum Inhibitory Concentration (MIC) for the methanol extract was determined.

Keywords: *Phallusia nigra*, antibacterial, antifungal, zone of inhibition, MIC

INTRODUCTION

Ascidians, or the sea-squirts, are cosmopolitan, exclusively marine chordates, which constitute a rich source of biologically active secondary metabolites [1]. Most of the ascidians are utilized as such as food in various countries and they are known to produce bioactive metabolites which prevent biofouling and this can be considered as a kind of autogenic protection [2]. This mechanism has proved to be timely alternative natural medicine to human beings. From the ascidian, *Tridemnum solidum*, the first marine compound entered human cancer clinical trial as a purified natural product [3], but was unsuccessful in further trials [4]. Halocyamine A, an antimicrobial substance was isolated from haemocytes of the solitary ascidian *Halocynthia roretzi* [5]. Meenakshi reported the antibacterial activity of *Polyclinum madrasensis, Aplidium indicum, Phallusia nigra, Phallusia arabica, Ascidia sydneiensis, Symplegma oceania, Styela canopus, Microcosmus exasperatus* and *Herdmania momus* against twenty bacterial pathogens both MTCC (*Streptococcus pneumoniae, Staphylococcus pyogenes, Bacillus subtilis, Staphylococcus aureus, Nocardia corynobacteriod, Actinomyces humiferus, Enterobacter aerogenes, Shigella flexneri, Klebsiella pneumoniae* and *Pseudomonas putida*) and hospital isolates *(Streptococcus viridans, Corynebacterium diptheriae, Enterococcus faecalis, Staphylococcus epidermis, Salmonella typhi, Vibrio cholera, Shigella species, Nocardia species* and *Actinomyces species* [6]. Bragadeeshwaran described the antibacterial activity of *Polyandrocarpa indica* and *Phallusia Arabica* against ten human bacterial pathogens [7]. Santhanaramasamy and Murugan reported the bacterial epibiosis and fouling deterrent activity of *Eudistoma viride* and *Didemnum psammathodes* [8]. Bala Amutha *et al*., studied the antibacterial activity and antimitotic activities of biofouling marine ascidians, *Polyclinum madrasensis, Aplidium indicum, Phallusia nigra, Phallusia arabica, Ascidia sydneiensis, Symplegma oceania, Styela canopus, Microcosmus exasperatus* and *Herdmania momus* to gastrointestinal, respiratory, urinary tract and wound pathogens [9]. Natarajan and his co-workers reported the antibacterial activity of crude extracts of compound ascidian *Polyclinum madrasensis*[10]. Mohamed Hussain and Ananthan studied the antimicrobial activity of the crude extracts of compound ascidians, *Didemnum candidum* and *Didemnum psammathodes* against eight human pathogenic bacteria and four fungal pathogens [11]. Karthikeyan *et al*., reported the antimicrobial activity of crude extracts of ascidians, *Microcosmus helleri, Microcosmus curvus, Herdmania pallida, Polyclinum madrasensis,*

Didemnum psammathodes and *Didemnum moseleyi* against eight bacterial pathogens and five fungal pathogens [12]. Antibacterial activity of the test and mantle of two marine ascidians, *Phallusia nigra* and *Herdmania pallida* from the Tuticorin coast, India against nine bacterial pathogens, *Bacillus subtilis (BS), Staphylococcus aureus (SA), Entrerobacter aerogenes (EA), Escherichia coli (EC), Klebsiella pneumoniae (KP), Pseudomonas aeruginosa (PA), Salmonella paratyphii (SP), Salmonella typhii (ST)* and *Vibrio cholerae (VC)* was reported [13] by Jaffarali *et al*. Also the antipyretic, analgesic [14] and anaesthetic activities [15] of *Phallusia nigra* has been reported. The potential of ascidians as a source of biologically active product on virulent hospital isolates is largely unexplored. Hence in the present investigation the antimicrobial activity of the methanolic extract of the whole animal of *Phallusia nigra* has been carried out on five gram positive and six gram negative bacteria and three fungal pathogens isolated from hospital samples by using zone of inhibition method. The antibiotics Ofloxacin and Nystatin were used as standard for bacteria and fungi respectively.

MATERIALS AND METHODS

Animal material

Samples of *Phallusia nigra* (Family: Ascidiidae) were collected from Tuticorin coast by SCUBA diving. They were identified and authenticated by Dr. V.K. Meenakshi, Associate Professor, Department of Zoology, A. P. C. Mahalaxmi College for Women, Tuticorin - 628002. A Voucher specimen AS-2083 has been deposited in the National Collections of Ascidians in the Museum of the Department of Zoology, A.P.C. Mahalaxmi College for Women, Tuticorin-628 002.

Preparation of extract

Epibionts adhering to the test were carefully removed, washed with sterile sea water, dried under shade and homogenized to get a coarse powder. The coarse powder was stored in an airtight container and used for further investigations. 100 g of powdered animal material was successively extracted with solvents like petroleum ether (40^0-60^0C), benzene, chloroform, methanol and water using a soxhlet apparatus. The extracts were cooled to room temperature, evaporated in a rotary evaporator under reduced pressure to obtain a brown sticky residue which was used for antimicrobial assay.

Microbial strains used

Antibacterial activity was determined against eleven different bacterial pathogens, five gram positive bacteria, *Bacillus cereus, Bacillus subtilis, Bacillus megaterium, Sarcina lutea* and *Staphylococcus aureus,* six gram negative bacteria, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia, Salmonella typhi*, *Proteus mirabilis* and *Pseudomonas pyocyanus* and three fungi, *Candida albicans*, *Aspergillus niger* and *Saccharomyces cerevisiae.* These clinical strains were obtained from the hospital samples of the Department of Microbiology, P.S.G. Medical and Research Institute, Coimbatore 641 004, Tamilnadu, India.

Preparation of test micro organisms

A loopful of the test organism was transferred to already sterilized 10 ml Nutrient agar and incubated overnight at 37^0C for bacteria and 30^0C for fungi. *Aspergillus niger* was cultured as a slant culture in an acidified PDA (Potato Dextrose Agar) media. 25 ml of sterilized Muller-Hinton Agar (MHA) (Hi Media, Mumbai, India) was poured in petriplates and allowed to solidify at room temperature on which the test organisms were inoculated.

Antimicrobial assay

The antimicrobial activity was measured by Disc Diffusion method [16]. The sterile discs were impregnated with the known concentration of the various extracts (15 µl) and standard drugs. The discs were then placed on the already inoculated petridishes containing the inoculum of test microbes in such a way that there is no overlapping of the zones of inhibition. The seeded plates were then incubated at 37^0C for 24 hours and 48 hours for bacteria and fungi respectively. The antimicrobial activity of the animal extracts was recorded as the mean diameter of the resulting inhibition zone of growth measured in millimetres.

From the results, the Active Index (AI) and Proportion Index (PI) were calculated using the following formulae,

$$\text{Active Index (AI)} = \frac{\text{Inhibition zone of the test sample}}{\text{Inhibition zone of the standard}}$$

$$\text{Proportion Index (PI)} = \frac{\text{Number of positive results obtained for individual extract}}{\text{Total number of tests carried out for each extract}}$$

Minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the methanolic extract of *Phallusia nigra* was determined. The samples of the extract were prepared at three different concentrations, 5 µg/ml, 10 µg/ml and 15 µg/ml The solvent, 90% methanol was used as a solvent control.

RESULTS AND DISCUSSION

Antimicrobial activity of three different concentrations of methanol extract of *Phallusia nigra* has been evaluated *in vitro* against gram positive and gram negative bacteria and fungal pathogens. The antimicrobial effects of animal extract against the different strains are presented in Table 1 and 2. From Table 1 it is clear that the inhibitory effect was proportional to concentration gradient. The minimum inhibitory concentration (MIC) is shown in Table 2. The results were also expressed by means of Active Index (Table 1) and Proportion Index (Fig. 1) and MIC at different concentrations (Fig. 2). From Table 1, it is clear that petroleum ether (40^0-60^0C) and benzene extracts did not show inhibitory effect against any of the pathogens under investigation. Chloroform extract showed low inhibitory effect on *Escherichia coli* and *Salmonella typhi.* Highly significant activity was noticed in methanol and water extracts against both bacteria and fungi. Hence *in vitro* antibacterial screening of the methanolic extract of *Phallusia nigra* against selected clinical isolates were performed and the inhibition zones of the extract against the specific test organisms were given in Table 1. Among the eleven bacteria tested, methanolic extract of *P. nigra* was more sensitive against *Klebsiella pneumonia* and *Aspergillus niger*. On the other hand, antimicrobial activity against *B. megaterium, S. aureus, E. coli, K. pneumonia, S. typhi, P. mirabilis* and *Candida albicans* was considerably higher than the other microbes. Also the methanolic extract showed highly significant activity against all the gram negative bacterial strains except, *P. cyanus*. Fungal strains were found to be highly sensitive to the methanolic extract of *Phallusia nigra* when compared to that of the bacterial strains. This view is contrary with the findings of Abdul Jaffar Ali *et al*., (2008) who reported [13] the maximum antibacterial activity exhibited by the Gram positive bacteria than in Gram negative bacteria of crude methanol extracts of the test and mantle bodies of *Phallusia nigra*. It is clearly evident that the antibacterial activity has been previously reported from extracts of some ascidian extracts caused growth inhibition in gram positive and negative bacteria. From the Table 3, it has been found that MIC lies between 3 µg/ml and 2 µg/ml. Organic substances isolated from the marine plants and animals have been shown to affect bacterial behaviour as reported by Bell and Mitchell [17]. GC-MS study of the methanol extract of *Phallusia nigra* revealed the presence of alcoholic compounds such as dl-3,4 dimethyl-3,4-hexanediol, dl-6-methyl-5-hepten-2-ol and 2-methyl-3-decanol showing antimicrobial activity [18]. Hence it may be concluded that these alcoholic compounds may be responsible for the potent antimicrobial activities of *Phallusia nigra*. It is worthy to note that the product from natural animal source is good for health and devoid of side effects.

Table 1: Antimicrobial activity of the methanol extract of *Phallusia nigra* Sav

Name of the organism	Zone of Inhibition (mm)										
	Petroleum ether (40^0-60^0C)		Benzene		Chloroform		Methanol		Water		Standards
	DIZ *	AI#	DIZ *	AI#	DIZ*	AI#	DIZ*	AI#	DIZ*	AI#	
Bacillus cereus	-	0	-	0	-	0	5	0.42	6	0.50	12[a]
Bacillus subtilis	-	0	-	0	-	0	3	0.19	2	0.13	16[a]
Bacillus megaterium	-	0	-	0	-	0	14	1.08	15	1.15	13[a]
Sarcina lutea	-	0	-	0	-	0	9	0.75	12	1.00	12[a]
Staphylococcus aureus	-	0	-	0	-	0	18	1.06	14	0.82	17[a]
Escherichia coli	-	0	-	0	11	0.64	19	1.12	16	0.94	17[a]
Pseudomonas aeruginosa	-	0	-	0	-	0	16	1.00	17	1.06	16[a]
Klebsiella pneumonia	-	0	-	0	-	0	23	1.21	20	1.05	19[a]
Salmonella typhi	-	0	-	0	9	0.56	19	1.19	22	1.38	16[a]
Proteus mirabilis	-	0	-	0	-	0	16	1.23	18	1.38	13[a]
Pseudomonas pyocyanus	-	0	-	0	-	0	2	0.14	4	0.29	14[a]
Candida albicans	-	0	-	0	-	0	26	1.13	25	1.09	23[b]
Aspergillus niger	-	0	-	0	-	0	25	1.25	28	1.40	20[b]
Saccharomyces cerevisiae	-	0	-	0	-	0	23	1.09	22	1.04	21[b]

**DIZ- Diameter of zone inhibition; #AI- Active Index*
a- Ofloxacin; b- Nystatin; - No inhibitory effect.

Table 2. MIC determination (5 to15 µg/ml) for samples of the methanol extract of *Phallusia nigra*

Name of the organism	Zone of inhibition (mm) Methanol Extract Concentration		
	5 µg/ml	10 µg/ml	15 µg/ml
Bacillus cereus	4.1	5.6	5.9
Bacillus subtilis	-	-	3.0
Bacillus megaterium	7.7	10.5	13.5
Sarcina lutea	6.5	8.5	9.0
Staphylococcus aureus	9.5	13.5	18.6
Escherichia coli	11.5	14.6	19.4
Pseudomonas aeruginosa	7.9	12.4	16.5
Klebsiella pneumonia	12.5	19.5	23.0
Salmonella typhi	11.5	14.5	19.5
Proteus mirabilis	10.5	12.5	16.5
Pseudomonas pyocyanus	-	-	2.5
Candida albicans	20.5	23.6	26.5
Aspergillus niger	16.5	18.5	25.5
Saccharomyces cerevisiae	11.5	13.5	23.5

Table 3. MIC determination (2 to 5 µg/ml) for samples of the methanol extract of *Phallusia nigra*

Name of the organism	Zone of inhibition (mm) Methanol Extract Concentration			
	2 µg/ml	3µg/ml	4 µg/ml	5 µg/ml
Bacillus cereus	-	-	-	4.1
Bacillus subtilis	-	-	-	-
Bacillus megaterium	-	-	-	8
Sarcina lutea	-	-	-	6.5
Staphylococcus aureus	-	-	6	9.5
Escherichia coli	-	-	7	11.5
Pseudomonas aeruginosa	-	-	-	7.9
Klebsiella pneumonia	-	7	9	12.5
Salmonella typhi	-	-	-	10
Proteus mirabilis	-	-	7	10.5
Pseudomonas pyocyanus	-	-	-	-
Candida albicans	-	8	10	20.5
Aspergillus niger	-	6	9	16.5
Saccharomyces cerevisiae	-	-	6	11.5

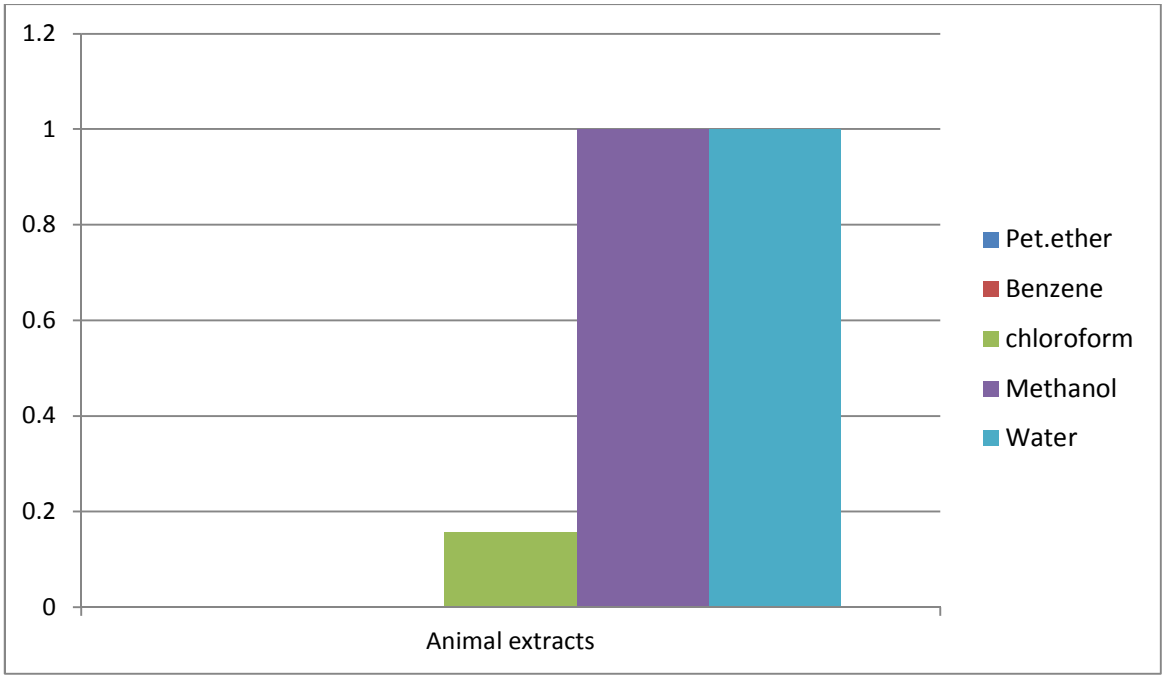

Fig. 1: Proportion index of Antimicrobial activity of the various extracts of *Phallusia nigra* Sav.

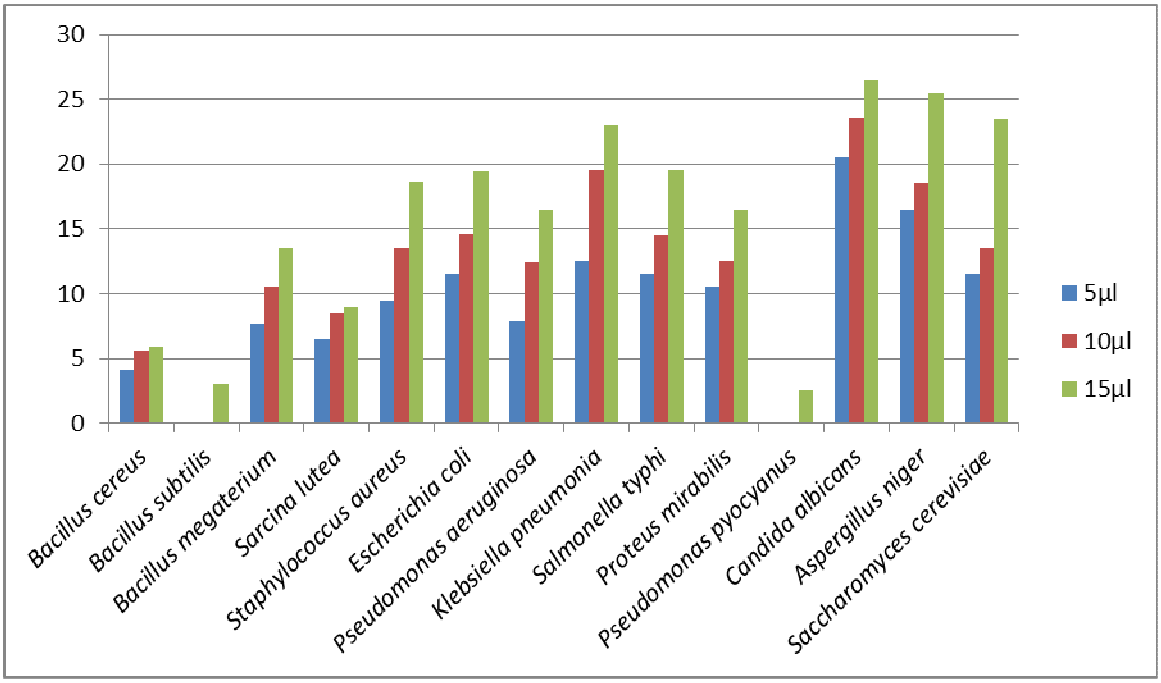

Fig. 2: MIC of the methanolic extract of *Phallusia nigra* Sav. at different concentrations

CONCLUSION

The present investigation reveals that the methanolic extract of *Phallusia nigra* is found to be a good natural antimicrobial agent. Attempts will be made in future to isolate and identify the phytochemical constituents of the methanolic extract responsible for the antimicrobial activity.

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