ANTIMICROBIAL PROFILE OF EXTREMOPHILES FROM AQUA TO TERRESTRIAL HABITATS.

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Summary

The emergence of antibiotic resistance among pathogenic and commensal bacteria has become a serious problem worldwide. Use and overuse of antibiotics in a number of settings are contributing to the development of antibiotic-resistant microorganisms (1). Recently, the rate of discovery of new compounds from existing genera obtained from terrestrial sources has decreased, while the rate of re-isolation of known compounds has increased. Moreover, rise in the number of drug-resistant pathogens and limited success of strategies such as combinatorial chemistry in providing new agents indicates an uncertain forecast for future antimicrobial therapy (2, 3). Thus, it is critical that new groups of microbes from unexplored habitats be pursued as sources of novel antibiotics and other small-molecule therapeutic agents(4). In the present study 36 bacterial and 24 fungal isolates from different aqua to terrestrial sources were grown in broth culture for production of metabolites. The intracellular metabolites were extracted with methanol and extracellular metabolites were extracted with ethyl acetate. The crude intra and extracellular extracts were evaluated for antimicrobial activity by agar diffusion method using various bacterial and fungal test organisms. Ciprofloxacin (100 µg/ml) was used as standard for antibacterial studies and Nystatin disc (10 µg/disc) was used for antifungal studies. It was found that the isolate UG-3 (extracellular extract) showed both antibacterial and antifungal activity against all the test organisms employed. UG-6 was also equally promising, except that *E.coli* was resistant to the extracellular extract of UG-6. Intracellular extracts of MC-1, E-1, LS-4 , PSA-2 and extracellular extracts of M-4. LS-3F, GS-4, VHSS-2, LS-3XY, VHSP-5, PSA-3 were found to be active against all test organisms except *Candida albicans.* Extracellular extract of GS-1 and intracellular extracts of G-1, G-5, and K2A were found to be active against all test bacteria employed.

Keywords: Extremophiles, antimicrobial, microbial metabolites, Agar diffusion assay.

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Introduction

The success of penicillin in treating infection led to an expansion in the area of drug discovery from microorganisms. Microorganisms remain the major source of antimicrobial compounds. The compounds discovered from these microorganisms differ widely both in structure and activity. There is definitely still a growing demand for development of new specialized antimicrobial compounds (5). The most urging reason is the rapidly growing number of antibiotic resistant strains of bacterial and fungal pathogens. The generous use of traditional antibiotics for the last half a century has created a selective pressure for increased resistance and the resistant strains are no longer limited to hospitals. One of the biggest health threats facing the global population remains the multidrug resistant strains of the tuberculosis bacteria - *Mycobacterium tuberculosis* (6, 7). Methicillin resistant *S. aureus* (MRSA), vancomycin resistant *Enterococci* (VRE) and β-lactam resistant *Streptococcus pneumoniae* are already creating huge medical problems and a few years ago a vancomycin resistant MRSA appeared (8). Furthermore, there are also possibilities that previously non-virulent bacteria are turning into pathogens by picking up virulent genes from other strains in their environment, which makes the situation even more complicated (9). Moreover the most recent estimates suggest that by now we only know approx. 5% of the total species of fungi and may be as little as 0.1 % of the bacteria(10). And among the ones already described, only a small fraction has been examined for metabolite profile.

This observation prompted us to search for novel antimicrobial compounds from microbial sources. Therefore anticipating potent novel compounds, antimicrobial activity of extracts of various microorganisms isolated from aqua to terrestrial sources were planned. In the present study various bacterial and fungal isolates from different aqua to terrestrial sources were grown in liquid culture for production of metabolites. The intra and the extracellular metabolites were extracted with various solvents and the crude intra and extracellular extracts were evaluated for antimicrobial activity by agar diffusion method using various bacterial and fungal test organism.

Materials and methods

Test organisms and drugs used

Ciprofloxacin was purchased from Ranbaxy Laboratories Ltd., Delhi. Nystatin Disc and all the media required were obtained from Hi-media lab Pvt Ltd, Mumbai. Ethyl acetate and Dimethyl sulphoxide were purchased from Qualigens Fine chemicals, Mumbai and Methanol from Merck India Ltd, Mumbai. Test Organisms used for the study were *Bacillus subtilis* (NCIM No-2063), *Escherichia coli (*NCIM No-2345), *Staphylococcus aureus (*NCIM No-2079), *Pseudomonas aeruginosa* (NCIM No-2036), *Candida albicans (*NCIM No-3100) and *Rhizopus oligosporus (*NCIM No-1215).

Isolation of bacterial and fungal isolates

Sixty different microbial isolates which were deposited in the Department of Pharmaceutical Biotechnology, Manipal College of Pharmaceutical Sciences, and Manipal were used for the study. Of these 36 were bacterial and 24 were fungal isolates. They were isolated from various sources like Garden soil, Manipal End Point Soil, Paddy field soil, Hussain Sagar Lake soil, Manipal lake water, Pickle Sample and Kudremukh soil by applying unusual conditions like high temperature(50^0 C), high salt concentration, low sugar concentration etc according to Bergey's directions (11), (Table-1)

Preparation of culture media
All the culture media were formulated according to the manufacturer's specifications. They were weighed, dissolved in the required amount of water with the aid of heat, distributed to conical flasks or test tubes, and then sterilized in an autoclave at 121 °C for specified period. Nutrient broth was used for bacterial cultures and fungal broth containing 1% peptone and 2% dextrose was used for fungal cultures.

Broth culturing of isolates
The inoculum was prepared by transferring a single colony of the isolates into 5 ml of the corresponding medium. The fungal isolates were incubated at 27^0C for 72-96 hours and bacterial isolates were incubated at 37^0C for 2 -3 days. This inoculum was transferred to 95 ml of the corresponding broth and incubated at 37^0C for 2 -3 days in case of bacterial cultures and 27^0C for 4-5 days in case of fungal cultures, on a rotary shaker incubator at 120 rpm for the production of metabolites. These broths were used for the extraction of extracellular and intracellular metabolites.

Extraction of extracellular metabolites
The broth cultures were transferred to 50 ml falcon tubes and centrifuged at 4000 rpm for 15 minutes to get a clear supernatant. The supernatant was taken into a 500 ml separating funnel for extraction with three equal volumes of ethyl acetate (50 ml). The combined extracts were evaporated under reduced pressure at a temperature of 40^0C. The weight of crude extract of each sample was noted (Table- 2).

Extraction of intracellular metabolites
The cell sediment obtained after centrifugation was redispersed in a small quantity of methanol (25 ml) and probe sonicated with 60% amplitude for 30 minutes to rupture the cells and release the intracellular principles. The samples were immersed in ice bath to compensate for the heat produced during sonication. The samples were again centrifuged at 4000 rpm for 15 minutes to get a clear supernatant. The solvent was evaporated off at reduced pressure and extract was freeze dried. The weight of crude extract of each sample was noted.

Sample preparation for biological studies
The extracellular crude extracts were dissolved in 1 ml DMSO and transferred to sterile vials of 2 ml capacity. Intracellular crude extracts were dissolved in 1 ml sterile water and transferred to 2ml sterile vials. These samples were stored at room temperature, protected from light.

Antimicrobial activity study
Intra and extracellular extracts of all isolates were screened for their antimicrobial activities by agar diffusion method (12), using standard inoculum of test organisms as described in Indian Pharmacopoeia (13). Antibacterial studies were carried out using Mueller Hinton Agar (MHA) medium and Sabouraud's Dextrose Agar (SDA) medium was used for screening antifungal activity Cups of diameter 8 mm were made in the seeded agar plate with a sterile cork borer, individual wells were loaded with 30µl of extract separately and it was allowed to diffuse for 30 minutes. 30 µl of Ciprofloxacin (100 µg/ml) was used as standard for antibacterial studies and Nystatin disc (10 µg/disc) was used for antifungal studies. The plates were incubated in specified conditions and zone of inhibition were measured (Table- 3, 4, 5, 6).
Microscopy(Figure-1)and biochemical tests were also performed with the view of partial characterization of some promising isolates (14) (Tables- 7 to 12).

Table-1: Details of Isolation

Working code [a]	Isolate code	Source & Conditions for isolation
B1	G1	Garden soil; 1% Glucose; 50^0C
B2	G2	Garden soil; 1% Glucose; 50^0C
B3	G3	Garden soil; 1% Glucose; 50^0C
B4	G4	Garden soil; 1% Glucose; 50^0C
B5	G5	Garden soil; 1% Glucose; 50^0C
B6	M1	Manipal end point soil; 5% Salt
B7	M3	Manipal end point soil; 5% Salt
B8	MC1	MCOPS soil; 5% salt
B9	E1	Manipal end point soil; Normal
B10	E2	Manipal end point soil; Normal
B11	P1	Paddy field soil
B12	LS-1	Hussain sagar lake soil; 7% salt
B13	LS-3	Hussain sagar lake soil; 7% salt
B14	LS-3B	Hussain sagar lake soil; 7% salt
B15	LS-4	Hussain sagar lake soil; 7% salt
B16	LS-5	Hussain sagar lake soil; 7% salt
B17	SS-2	Hyderabad soil; 50^0C
B18	SS-5	Hyderabad soil; 50^0C
B19	GS-1	Garden soil; 1% Glucose; 5% salt
B20	GS-5	Garden soil; 1% Glucose; 5% salt
B21	UG-3	Manipal end point soil; Normal
B22	UG-4	Manipal end point soil; Normal
B23	UG-6	Manipal end point soil; Normal
B24	UG-SS	Manipal lake water; Normal
B25	VHSP-3W	Home soil; 50^0C
B26	VHSP-3Y	Home soil; 50^0C
B27	PSA-1	Home made pickle
B28	K2A	Kudremukh soil; 12% salt
B29	VHS2	Home soil; 50^0C
B30	VHS1	Home soil; 50^0C
B31	VHS5	Home soil; 50^0C
B32	VHS5A	Home soil; 50^0C
B33	VHSS1	Hyderabad soil; 50^0C
B34	VHSP6	Home soil; 50^0C
B35	PSTN1	Paddy field soil; 50^0C
B36	LS-5Y	Hussain sagar lake soil; 7% salt
F1	M2	Manipal end point soil; 5% Salt
F2	M4	Manipal end point soil; 5% Salt
F3	LS3F	Hussain sagar lake soil; 7% salt
F4	LS3XY	Hussain sagar lake soil; 7% salt
F5	LS5F	Hussain sagar lake soil; 7% salt
F6	GS4	Garden soil; 1% Glucose; 5% salt
F7	GS6	Garden soil; 1% Glucose; 5% salt

F8	VHSS2	Hyderabad soil; 50^0C
F9	VHSP5	Home soil; 50^0C
F10	PSA3	Home made pickle
F11	MC2	MCOPS soil; 5% salt
F12	LS-2	Hussain sagar lake soil; 7% salt
F13	PSA2	Home made pickle
F14	VHS3	Home soil; 50^0C
F15	VHS4	Home soil; 50^0C
F16	VHSP1W	Home soil; 50^0C
F17	VHSP1B	Home soil; 50^0C
F18	VHSP2	Home soil; 50^0C
F19	VHSP4Y	Home soil; 50^0C
F20	VHSP4G	Home soil; 50^0C
F21	VHSS3	Hyderabad soil; 50^0C
F22	PSTN2	Paddy field soil; 50^0C
F23	PSTN3G	Paddy field soil; 50^0C
F24	PSTN3B	Paddy field soil; 50^0C

[a] B-Bacterial; F-Fungal isolates

Table-2: Yield of Bacterial and Fungal Extracts

Working code	Isolate code	Weight of extracellular extract(mg)	Weight of intracellular extract(mg)
B1	G1	11	33
B2	G2	21	39
B3	G3	9	15
B4	G4	11	89
B5	G5	9	34
B6	M1	10	109
B7	M3	4	38
B8	MC1	23	63
B9	E1	13	24
B10	E2	10	19
B11	P1	15	65
B12	LS-1	22	33
B13	LS-3	50	56
B14	LS-3B	26	123
B15	LS-4	8	37
B16	LS-5	23	11
B17	SS-2	56	144
B18	SS-5	10	174
B19	GS-1	15	52
B20	GS-5	12	123
B21	UG-3	8	63
B22	UG-4	5	38

B23	UG-6	11	47
B24	UG-SS	9	27
B25	VHSP-3W	2	49
B26	VHSP-3Y	4	389
B27	PSA-1	12	49
B28	K2A	8	154
B29	VHS2	9	98
B30	VHS1	10	357
B31	VHS5	3	75
B32	VHS5A	5	41
B33	VHSS1	4	38
B34	VHSP6	7	105
B35	PSTN1	7	91
B36	LS-5Y	17	29
F1	M2	93	76
F2	M4	123	89
F3	LS3F	43	127
F4	LS3XY	193	98
F5	LS5F	85	67
F6	GS4	15	59
F7	GS6	22	70
F8	VHSS2	80	63
F9	VHSP5	187	45
F10	PSA3	65	29
F11	MC2	20	58
F12	LS-2	25	97
F13	PSA2	36	167
F14	VHS3	9	13
F15	VHS4	24	31
F16	VHSP1W	23	46
F17	VHSP1B	26	78
F18	VHSP2	27	60
F19	VHSP4Y	85	187
F20	VHSP4G	30	88
F21	VHSS3	36	95
F22	PSTN2	62	53
F23	PSTN3G	56	115
F24	PSTN3B	33	74

Table-3: Antifungal and antibacterial activity of bacterial extracellular (ethyl acetate) extracts

Working code	Isolate code	Diameter of Zone of Inhibition (mm)					
		C. albicans	*R.oligo-sporus*	*B. subtilis*	*S. aureus*	*E.coli*	*P. aeruginosa*
B1-E	G1	-	13	11	-	-	-
B2-E	G2	-	-	19	-	-	-
B3-E	G3	-	-	12	10	-	-
B5-E	G5	-	-	13	-	-	13
B6-E	M1	-	-	24	-	-	-
B7-E	M3	-	-	11	15	-	25
B8-E	MC1	-	-	16	-	-	15
B9-E	E1	-	11	11	-	-	-
B10-E	E2	-	-	12	-	-	10
B11-E	P1	-	-	16	-	-	-
B12-E	LS-1	-	-	10	-	-	-
B13-E	LS-3	11	-	10	-	11	-
B14-E	LS-3B	10	-	11	-	-	-
B15-E	LS-4	-	-	11	-	-	-
B16-E	LS-5	-	-	11	-	-	13
B17-E	SS-2	-	-	13	-	-	-
B18-E	SS-5	13	-	-	-	-	-
B19-E	GS-1	-	-	19	14	18	26
B20-E	GS-5	-	-	-	-	15	25
B21-E	UG-3	21	11	12	16	17	20
B22-E	UG-4	-	-	12	-	-	15
B23-E	UG-6	13	9	13	14	-	13
B24-E	UG-SS	-	-	14	-	-	16
B28-E	K2A	-	-	12	-	-	14
B29-E	VHS2	-	-	-	-	12	-
B30-E	VHS1	-	12	-	-	-	-
B31-E	VHS5	-	12	15	-	-	25
B32-E	VHS5A	-	11	23	-	-	25
B33-E	VHSS1	-	-	-	-	-	-
B34-E	VHSP6	-	15	24	-	18	21
B35-E	PSTN1	-	12	26	-	12	-
B36-E	LS-5Y	-	-	10	-	-	-
Ciprofloxacin (100µg/ml)		NA	NA	35	30	30	34
Nystatin Disc		16	10	NA	NA	NA	NA

'-' Extract not active. NA- Not Applicable

Table-4: Antifungal and antibacterial activity of bacterial intracellular (methanolic) extracts

Working code	Isolate code	Diameter of Zone of Inhibition (mm)					
		C. albicans	*R.oligo-sporus*	*B. subtilis*	*S. aureus*	*E.coli*	*P. aeruginosa*
B1-M	G1	-	-	12	12	09	12
B4-M	G4	-	-	10	10	10	-
B5-M	G5	-	-	14	20	15	15
B6-M	M1	-	-	10	11	-	-
B7-M	M3	-	13	10	-	14	-
B8-M	MC1	-	12	12	12	12	15
B9-M	E1	-	11	14	14	09	15
B10-M	E2	-	-	11	-	-	-
B13-M	LS-3	-	-	12	-	-	13
B14-M	LS-3B	-	-	09	-	-	-
B15-M	LS-4	-	12	13	13	13	14
B16-M	LS-5	11	13	12	-	-	-
B17-M	SS-2	-	-	13	-	-	-
B18-M	SS-5	-	-	10	-	-	-
B19-M	GS-1	-	-	12	-	12	-
B20-M	GS-5	12	-	14	15	16	-
B21-M	UG-3	12	-	12	21	17	-
B22-M	UG-4	-	-	11	-	-	-
B23-M	UG-6	-	-	10	-	-	-
B25-M	VHSP-3W	-	-	11	-	-	-
B26-M	VHSP-3Y	-	-	16	-	-	14
B27-M	PSA-1	-	-	13	-	-	-
B28-M	K2A	-	-	11	11	13	11
B29-M	VHS2	-	-	11	-	-	-
B30-M	VHS1	-	-	12	-	-	-
B31-M	VHS5	-	-	15	17	15	-
B32-M	VHS5A	-	-	14	15	14	-
B33-M	VHSS1	-	-	13	12	13	-
B34-M	VHSP6	-	-	12	15	14	-
B35-M	PSTN1	-	-	13	-	12	-
B36-M	LS-5Y	-	-	10	-	-	14
Ciprofloxacin (100µg/ml)		NA	NA	35	30	30	34
Nystatin disc		16	10	NA	NA	NA	NA

'-' Extract not active, NA- Not applicable

Table-5: Antifungal and antibacterial activity of fungal extracellular (ethyl acetate) extracts

Working code	Isolate code	Diameter of Zone of Inhibition (mm)					
		C. albicans	*R.oligo-sporus*	*B. subtilis*	*S. aureus*	*E.coli*	*P. aeruginosa*
F2-E	M4	-	22	15	17	17	15
F3-E	LS3F	-	13	13	13	11	11
F4-E	LS3XY	-	-	20	13	09	14
F5-E	LS5F	-	12	-	13	12	15
F6-E	GS4	-	16	20	22	13	26
F7-E	GS6	-	-	-	-	-	-
F8-E	VHSS2	-	13	12	15	15	20
F9-E	VHSP5	-	-	19	11	16	14
F10-E	PSA3	-	-	19	14	17	12
F12-E	LS-2	10	15	-	10	12	15
F13-E	PSA2	13	15	-	13	15	17
F15-E	VHS4	-	-	11	12	-	-
F16-E	VHSP1W	-	-	-	-	-	13
F18-E	VHSP2	-	15	-	10	-	-
F19-E	VHSP4Y	-	-	-	12	-	-
F20-E	VHSP4G	-	-	-	-	16	-
F21-E	VHSS3	-	15	11	11	13	-
F22-E	PSTN2	-	12	10	10	11	-
F23-E	PSTN3G	-	-	13	12	13	-
Ciprofloxacin(100µg/ml)		NA	NA	35	30	30	34
Nystatin disc		15	11	NA	NA	NA	NA

'-'Extract not active, NA- Not applicable

Table-6: Antifungal and antibacterial activity of fungal intracellular (methanol) extracts

Working code	Isolate code	Diameter of Zone of Inhibition (mm)					
		C. albicans	*R.oligo-sporus*	*B. subtilis*	*S. aureus*	*E.coli*	*P. aeruginosa*
F9-M	VHSP5	-	13	-	-	-	-
F13-M	PSA2	-	13	16	16	17	17
F14-M	VHS3	-	11	-	-	-	-
F16-M	VHSP1W	-	12	-	-	-	-
F22-M	PSTN2	-	-	-	-	12	-
Ciprofloxacin (100µg/ml)		NA	NA	33	32	30	32
Nystatin disc		15	12	NA	NA	NA	NA

'-' Extract not active, NA- Not applicable

Table-7: Biochemical test of selected isolates

Isolate code	Catalase test	Indole test	MR test	VP test	Citrate Utilization	TSI Agar Test	Urease test	Gelatin Liquifaction	Starch Utilization		Casein Utilization	
									Growth	Hydrolysis	Growth	Hydrolysis
G-2	+	-	-	+ +	-	Glucose	-	-	+	+	+	+
G-4	+	-	+	+	-	Glucose	+	+ +	+	+	+	+
LS-3	+	-	-	+ +	+	Glucose	+	+	+	+	+	-
LS-4	+	-	+	+	-	Glucose	-	+	+	+	+	+
LS-2	+	-	+	+	+	-	+	-	-	-	-	-
PSA-2	+	-	+	-	+	-	+	-	+	-	+	-
VHS-3	+	-	-	-	-	-	-	-	+	-	-	-
VHS-4	+	-	-	+	+	Glucose	-	+ +	+	-	+	-
VHSP-4Y	+	-	-	+	+	Glucose	+ +	+	+	+	+	+
VHSS-3	+	-	+	-	+	Lactose/ sucrose	-	+	+	-	+	+
GS-4	+	-	-	-	+ +	Glucose	+	-	+	+	+	F
VHSS-2	+	-	-	-	+ +	Glucose	+ +	+	+	+	+	+
UG-3	+	-	+	+	-	Glucose	-	+	+	-	+	+
UG-6	+	-	+	+	-	Glucose	-	-	+	-	+	+

'+' Positive reaction '+ +' Strongly positive '-' Negative reaction 'F' Faint

Table -8: Carbohydrate Utilization Test

Isolate code	Arabinose	Raffinose	Sucrose	Inositol	Inulin	Xylose
G-2	F	F	+	-	-	F
G-4	-	-	+	F	+	F
LS-3	-	F	+ +	F	+	-
LS-4	F	+	+	+	+	-
LS-2	+	-	+	+ +	+ +	+
PSA-2	+	+ +	+ +	+ +	+ +	+ +
VHS-3	F	-	F	-	F	-
VHS-4	+	+ +	+ +	+ +	+ +	+ +
VHSP-4Y	-	+ +	+ +	+	+	+ +
VHSS-3	+	+ +	+ +	+ +	+ +	+ +
GS-4	+ +	+ +	+ +	+ +	+ +	+ +
VHSS-2	+ +	+ +	+ +	+ +	+ +	+ +

'+'- Positive result '+ +'- Strongly positive '-'- Negative reaction 'F'- Faint

Table -9: Litmus Milk Test

Working code	Supernatant	Medium
G-2	Pale yellow	Pale ,Coagulated
G-4	Straw coloured	White, coagulated
LS-3	Pale yellow, pink ring on the top	Pale, coagulated
LS-4	Pink supernatant	Yellow-white, coagulated, purple ring at the interface
LS-2	Straw coloured, purple ring on the top	Pale yellow coagulated
PSA-2	Dark pink, pink ring on the top	Unchanged
VHS-3	No change	No change
VHS-4	Purple	Uncoagulated, Unchanged
VHSP-4Y	Straw coloured	Uncoagulated, Unchanged
VHSS-3	Pink, coagulated	Uncoagulated, Unchanged
GS-4	Yellow	White, coagulated
VHSS-2	Yellow	White, coagulated

Table-10: Growth of Bacterial Isolates on Selective Medium

Working code	EMB agar		Mannitol Salt agar		SIM medium		
	Colony	Medium	Colony	Medium	Growth on surface	Growth along stab line	Diffusion in stab line
G-2	-	Dark	Yellow	Yellow	+ +	+	-
G-4	Pink	Yellow	-	Red	+ +	+ +	+ +
LS-3	-	Dark	Pink	Pink	+ +	-	-
LS-4	Small, white with pink centre	Yellow	-	Red	+ +	+ +	+ +

‘+’ Positive reaction
‘+ +’ Strongly positive
‘-’ Negative reaction

Table-11: Microscopy of selected isolates

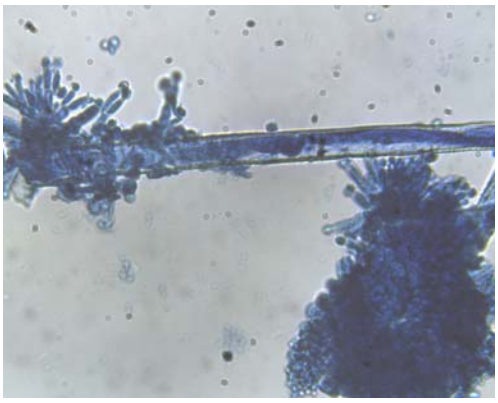
Isolate code	Gram's Reaction	Shape	Cell Arrangement	Motility	pH of culture broth
G-2	+	Rods	Bacilli	Non-motile	9.15
G-4	+	Rods	Two or three cells in chain	Motile	9.12
LS-3	+	Rods	Bacilli	Non-motile	9.92
LS-4	+	Rods	No characteristic arrangement	Motile	9.61
UG-3	+	Rods	No characteristic arrangement	Motile	6.56
UG-6	-	Rods	No characteristic arrangement	Motile	9.32

'+' Positive reaction

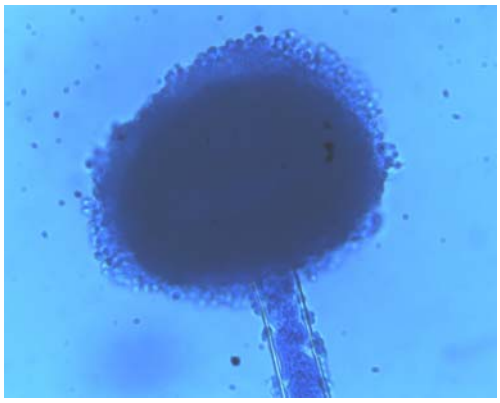
Table-12: Morphology of Selected Fungal Isolates

Isolate code	Growth on solid medium		Growth in liquid medium	
	Growth pattern	Spore colour	Pellet nature	pH
LS-2	Small colony, Curled	Bluish green	Very small & uniform	5.26
PSA-2	Big colony, white mycelium	Yellow-black	Medium size	2.29
VHS-3	Very small white colony	No spores seen	Few large tubes & pellets	6.29
VHS-4	Big colony, filamentous/erose	Gray spores towards periphery	Small – medium even pellets	4.29
VHSP-4Y	White , small , wrinkled colony	Yellowish green, scanty	Few large tubes & pellets	6.62
VHSS-3	Big colony, filamentous/erose	Yellowish black/ darker center	Small uniform pellets	3.35
GS-4	Pulvinate/undulate	Yellow spores	Irregular medium shaped pellets	3.44
VHSS-2	Small colony-yellow white wrinkels	Very few yellow	Small, smooth donnet shaped	4.18

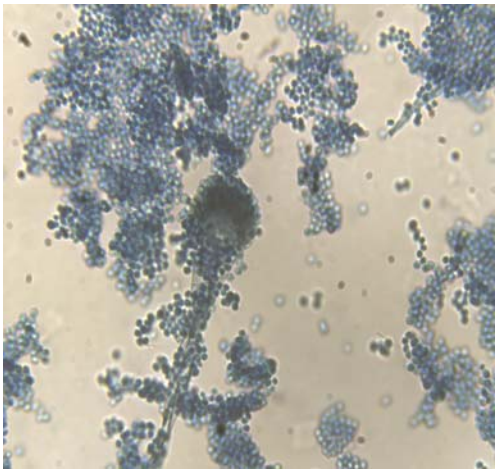
Figure-1: Lactophenol Cotton Blue Staining of Selected Fungal Isolates



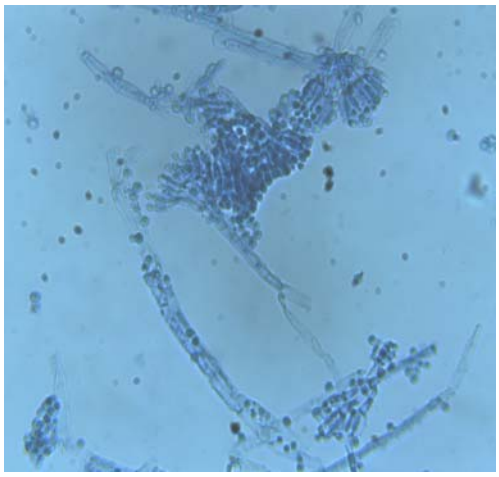
LS-2



VHSS-3

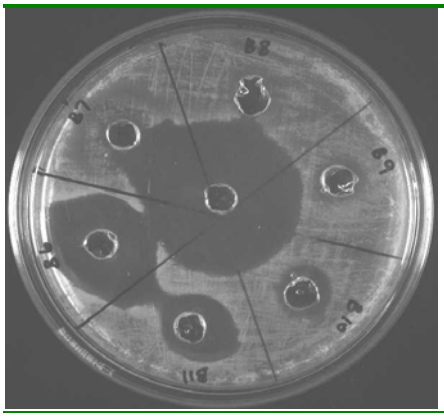


VHS-4

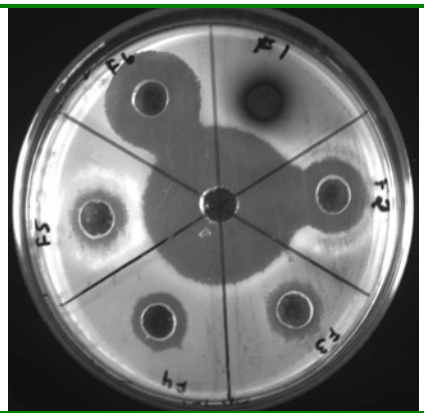


VHSS-2

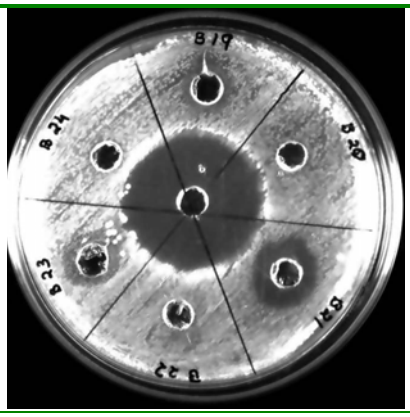
Figure-2: Antimicrobial Activity of some bacterial and fungal extracts.



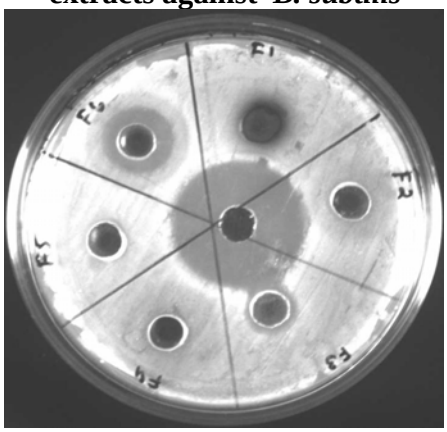
Zone of Inhibition of bacterial extracts against *B. subtilis*



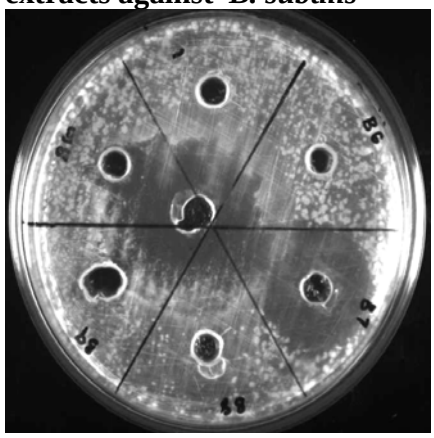
Zone of Inhibition of fungal extracts against *B. subtilis*



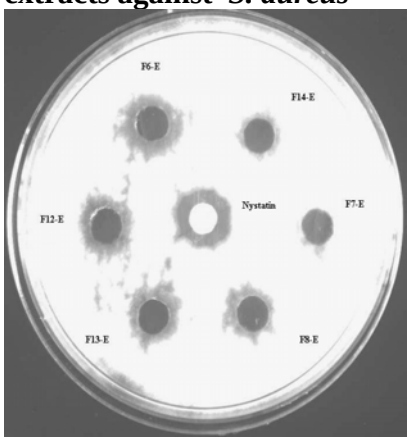
Zone of Inhibition of bacterial extracts against *S. aureus*



Zone of Inhibition of bacterial extracts against *P. aeruginosa*



Zone of Inhibition of bacterial extracts against *P. aeruginosa*



Zone of Inhibition of fungal extracts against *R. oligosporus.*

Results and Discussion

Intracellular and Extracellular extracts of each isolate were screened for antimicrobial activity by agar diffusion method. Antibacterial activity was studied using two Gram positive (*B. subtilis and S. aureus)* and two Gram negative (*E. coli* and *P. aeruginosa*) organisms. Antifungal activity studies were carried out using *Candida albicans* and *Rhizopus oligosporus* as test organisms. The results are shown in Tables-3 to 6 and Figure-2.

The extracellular extracts of LS-3, LS-3B, SS-5, UG-3, UG-6, LS-2, PSA-2 and the intracellular extracts of LS-5, GS-5 and UG-3 were found to be active against *Candida albicans* and the zones are comparable with that of Nystatin. The bacterial extracellular extracts of G-1, E-1, UG-3, UG-6, VHS-1, VHS-5, VHS-5A, VHSP-6, PSTN-1 and the fungal extracellular extracts of M-4, LS-3F, LS-5F, GS-4, VHSS-2, LS-2, PSA-2, VHSP-2, VHSS-3, and PSTN-2 were found to be equally or more active than Nystatin against *Rhizopus oligosporus* . The intracellular extracts of VHSP-5, PSA-2, VHS-3 and VHSP-1W are also found to be equally active as nystatin against *Rhizopus oligosporus*.

All the bacterial extracellular extracts except those of G4, SS-5, GS-5, VHSP-3W, VHSP-3Y, PSA-1, VHS-2, VHS-1, VHSS-1 and all intracellular except those of G-2, G-3, P-1, LS-1 and UGSS were found to be active against *B. subtilis* with diameter of zone of inhibition ranging from 11-26 mm in case of extracellular extracts and 10-15 mm in case of intracellular extracts, while reference Ciprofloxacin (3µg) showed a zone of 35 mm. Fungal extracellular extracts of M-4, LS-3F, LS-3XY, GS-4, VHSS-2, VHSP-5, PSA-3, VHS-4, VHSS-3, PSTN-2 and PSTN-3G and intracellular extracts of PSA-2 were found to be active against *B. subtilis* with zones in the range of 11-20 mm where as Ciprofloxacin showed a value of 33 mm.

In case of *S. aureus,* bacterial extracellular extracts of G-3, M-3, GS-1, UG-3, UG-6 and intracellular extracts of G-1, G-4, G-5, M-1, MC-1, E-1, LS-4, GS-5, UG-3, K2A, VHS-5, VHS5A, VHSS-1 and VHSP-6 were found to be active in a range of 10-21 mm when the reference value is 30 mm. All the fungal extracellular extracts except those of GS-6, MC-2, VHS-3, VHSP-1W, VHSP-1B, VHSP-4G, PSTN-3B were found to be active showing a zone of 10-22 mm when the standard gave 30 mm. The only active fungal intracellular extract against *S. aureus* was that of PSA-2 with a zone of 16 mm when ciprofloxacin gave a zone of 32 mm.

The bacterial extracellular extracts of LS-3, GS-1, GS-5, UG-3, VHS-2, VHSP-6,PSTN-1 and intracellular extracts of G-1, G-4, G-5, M-3, MC-1, E-1, LS-4, GS-1, GS-5, UG-3, K2A, VHS-5, VHS-5A, VHSS-1, VHSP-6 and PSTN-1 were found to show mild activity against *E. coli* . The fungal extracellular extracts of M-2, GS-6, MC-2, VHS-3, VHS-4, VHSP-1W, VHSP-1B, VHSP-2, VHSP-4Y and PSTN-3 were found to have no activity against *E. coli*. Only two fungal intracellular extracts such as PSA-2 and PSTN-2 showed activity against *E. coli.*
In case of *P. aeruginosa,* bacterial extracellular extracts of G-5, M-3, MC-1,E-2, LS-5, GS-1,GS-5, UG-3, UG-4, UG-6, UGSS, K2A, VHS-5 and VHS-5A were found to be active showing inhibition zone diameters in a range of 10-26 mm, while the reference value is 34 mm. Bacterial intracellular extracts of G-1, G-5, MC-1, E-1, LS-3, LS-4, VHSP-3Y, K2A and LS-5Y showed mild activity. Fungal extracellular extracts such as M-4, LS-3F, LS-3XY, LS-5F, GS-4, VHSS-2, VHSP-5, PSA-3, LS-2, PSA-2 and VHSP-1W and intracellular extract of PSA-2 showed significant activity against *P. aeruginosa.*

From the results of antibacterial and antifungal studies, it was found that the isolate UG-3 (extracellular extract) showed both antibacterial and antifungal activity against all the test organisms employed. UG-6 was also equally promising, except that *E.coli* was resistant to the extracellular extract of UG-6. Intracellular extracts of MC-1, E-1, LS-4 , PSA-2 and extracellular extracts of M-4. LS-3F, GS-4, VHSS-2, LS-3XY, VHSP-5, PSA-3 were found to be active against all test organisms except *Candida albicans.* Extracellular extract of GS-1 and intracellular extracts of G-1, G-5, and K2A were found to be active against all test bacteria employed.
Microscopic examination and biochemical tests were also performed in the case of some of the most promising isolates

Conclusion

The need for discovery of new isolates from uncommon habitats like marine and testing for their biological activity profiles are gaining prominence in the light of fact that these sources are underexploited. The present work lead to some promising isolates with good antimicrobial profile. Taxonomical studies for identification of isolates, characterization of compounds and optimization of production parameters are in progress.

Acknowledgements

The authors wish to acknowledge the Management, Manipal University, DST-FIST authorities and All India Council for Technical Education, for providing necessary infrastructure and financial support for carrying out this study.

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