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ANTIMICROBIAL POTENTIAL OF SECONDARY METABOLITES EXTRACTED FROM *VIBRIO FURNISSII*, A LUMINESCENT BACTERIA ASSOCIATED WITH SQUID, *UROTEUTHIS DUVAUCELI*

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ABSTRACT

Marine biosphere possesses a rich and luxuriant source of various faunal species, especially the invertebrates which are emerging as the novel targets for bioextraction of variety of diverse natural compounds of different unique chemical structures, promising great pharmacological value. In lieu of sparse reports on the antimicrobials produced by bacteria, isolated from Cephalopods, the present study was aimed at investigating the antagonistic activities of Vibrio furnissii, a luminescent bacterium isolated from a squid, Uroteuthis duvauceli. Crude extract of the isolates was tested for antimicrobial activity against 10 clinically isolated human pathogenic strains, viz Shigella dysenteriae, Aeromonas hydrophila, Shigella boydii, Enteropathogenic Escherichia coli serotype, Shigella flexneri, Shiga toxin producing E. coli serotype, Vibrio cholera, Salmonella enteric serovar typhi, Vibrio fluvialis and Shigella sonnei. Furthermore, the ethyl acetate extracts of cell free supernatant analysed for potent biochemical compounds by GC-MS technique, confirmed the presence of various biochemical active entities, which were further validated by disc diffusion method. Maximum range of antibacterial activity was observed in Shigella dysenteriae while the minimum range was observed in Shigella sonnei. Thus, the study clearly outlines the potentiality of marine faunal associated bacterial species as a novel source of diverse unique secondary metabolites against human bacterial pathogenic strains.

KEY WORDS

Uroteuthis duvauceli, Vibrio furnissii, secondary metabolites, antimicrobial activity.

INTRODUCTION

Marine microorganisms are known to be a rich reservoir of various biological and chemical compounds of great pharmaceutical importance. They are used in different clinical applications as they possess antibacterial, antioxidant, antitumor, antiviral and anticancer properties, thereby marking a pivotal and major impact on the development of medical therapeutics (Wright, 1998; Sabu, 2003). Symbiotic bacterial communities consisting of α-proteobacteria, γ-proteobacteria (*Vibrio*, *Pseudomonas*) are residents of various marine invertebrates and are reported to excrete bioactive secondary metabolites into their surrounding environment, which leads to serve the marine biotic diversity in a highly complex environmental habitat of sustenance where competitive rights of space and nutrients exists, is primarily responsible for such great astonishing varieties (Nair *et al*., 2011). One postulate proposes that, these biologically vital chemical species are combative molecules of biochemical origins, offering marine invertebrates a great deal in devising their defence strategies against potential competitors, predators and invaders. During the past few years, findings have reported that these molecules need not to

be the products of biochemical metabolic pathways of marine invertebrates but may be produced by the microbial species associated or inhabiting the host body (Donia and Hamann, 2003; Bernen *et al*., 1997). Various studies have demonstrated that microbial entities associated with marine faunal forms are far exceeding in bioactivity, as against that of the free living sessile forms (Lu *et al*., 2009). *Vibrio* sp are reported to produce novel secondary metabolites with potential biological effects (Al-Zereini *et al*.,2010). In the current investigation, an antibacterial activity of *Vibrio furnissii*, a luminescent bacterium against human pathogen bacterial strains has been carried out in detail to furnish a novel source of antimicrobial agents.

MATERIALS AND METHODS

Squid *Uroteuthis duvacelli* was collected from the Panighat jetty ($11^0$41'N, $92^0$43' E), transferred to the laboratory in ice box and rinsed with sterile filtered seawater to remove debris. The surface of the body was scrapped with a sterile cotton swab and transferred to 9 ml of sterile seawater. From this, 0.1 ml was plated on Zobell Marine agar plate and incubated at 25°C for 24 hours. After incubation, single and discrete isolated colonies were restreaked on TCBS agar plate for purity confirmation. Initially, all cultures were tested for antimicrobial activity by cross streaking method followed by disc diffusion method to assay the activity against human pathogenic strains (Lemos *et al* 1985).

Preparation of bacterial culture crude extract

The bacterial strains were inoculated into 200 ml Zobell marine broth and incubated for 3 days at 25 ^{0}C in an orbital shaker. After incubation, the culture was centrifuged at 10000 rpm for 15 min and the resulting supernatant was extracted using equal volume of ethyl acetate and agitated overnight on a magnetic stirrer. The extract was concentrated using rotary evaporator (Buchi, Essen Germany) at 45 ^{0}C under vacuum and was dissolved in methanol to a final concentration of 50 mg/ml. This extract was used for antimicrobial assay following disc diffusion method.

Gas chromatography-Mass spectrometry

GC–MS analysis was performed to identify the bioactive compounds present in the methanolic crude extract analysed in a GC-7890 A (Agilent Technologies) fused silica capillary column (Length 30 m, I.D. 0.25 mm., film thickness 0.25 µm) coupled with MS-5975 C Inert XL MSD with Triple –Axis Detector with 7693 autosampler. Helium was used as a carrier gas at a constant flow rate of 1ml/min. Sample of 1µl was injected into the capillary column of 5 MS DB with a temperature gradient program of 60°C for 5 min, then an increase from 60°C to 280°C at a rate of 5°C per min and finally 5 min at 325°C. The chemical compounds were identified and characterized based on its retention time (RT). This analysis was carried out at Analytical Laboratory Facility, CARe-KERALAM Ltd., Koratty, Kerala and the interpretation of chromatogram was done using database of National Institute Standard and Technology (NIST, U.S.).

Microbial cultures

Ten human bacterial pathogens *Shigella dysenteriae* type 5 (NK2440), *Aeromonas hydrophila* (IDH1585), *Shigella boydii* type 1 (NK2379), Enteropathogenic *Escherichia coli* serotype (0115), *Shigella flexneri* (MTCC 1457), Shiga toxin producing *E.coli* serotype (0157: H7 VT3), *Vibrio cholera* (0139), *Salmonella enteric serovar typhi* (C6953), *Vibrio fluvialis* (IDH 02036) and *Shigella sonnei* (NK4010) maintained in our laboratory, were tested. All isolates were regularly maintained on Mueller Hinton agar plates and stored as slants and glycerol cultures.

Antimicrobial assay

The antimicrobial activity was carried out by disc diffusion method as described by Bauer and Kirby (1966). All pathogenic bacterial strains were plated on Mueller Hinton agar plates and sterile discs impregnated with 50 µl and 100µl of test compound were placed on the surface with 15 mg/ml of Gentamycin disc used as positive control while 50 µl of methanol as negative control. Plates were incubated at 37 ^{0}C for 24 hrs and the diameter of growth inhibition zones (mm) were measured around each disc. All the experiments were performed in triplicates.

Results and Discussion:

Genus *Vibrio* consists of more than 100 species grouped into 14 clades found mostly associated with marine organisms such as corals, finfishes, shellfishes, sea urchins, seahorses and crustaceans (Widder 2010; Zarubin *et al*., 2012). Bioactive compounds of diverse chemically varied structures have been reported from marine *Vibrio* associated with sponges (Kobayashi *et al*., 1994; Dobler *et al*., 2002). Similar report regarding production of bioactive compounds by marine luminescent *Vibrio* spp was reported by Halldorson and Duran (2003) and Hastings *et al*. (1986). In the present

study, 5 bioluminescent *Vibrio* spp. were isolated from the squid *Uroteuthis duvauceli*. Among these 5, only *Vibrio furnissii* demonstrated antibacterial activity and no activity was observed in the remaining four. The methanolic extract of luminescent bacteria showed variable sizes of inhibition zone against all tested human pathogenic strains. It was observed that 50 µl extract (50 mg/ml concentration) shows moderate inhibition zone of 21.66 mm against *Shigella dysetriae* type 5 whereas an inhibition zone of 10.66 mm was observed against Shigella sonnei. The 100 µl extract (concentration 50 mg/ml) shows maximum inhibition zone of 26.33 mm against Shigella dysenteriae type 5 and the minimum inhibition zone of 11 mm against Shigella sonnei. No inhibition zone was observed with the negative control pure methanol (Figure 1 and 2). Our results corroborate with the earlier findings of Molina et al. (2016).

Fig 1: Antibacterial activity of methanolic extract of *Vibrio furnissii* against tested human pathogens

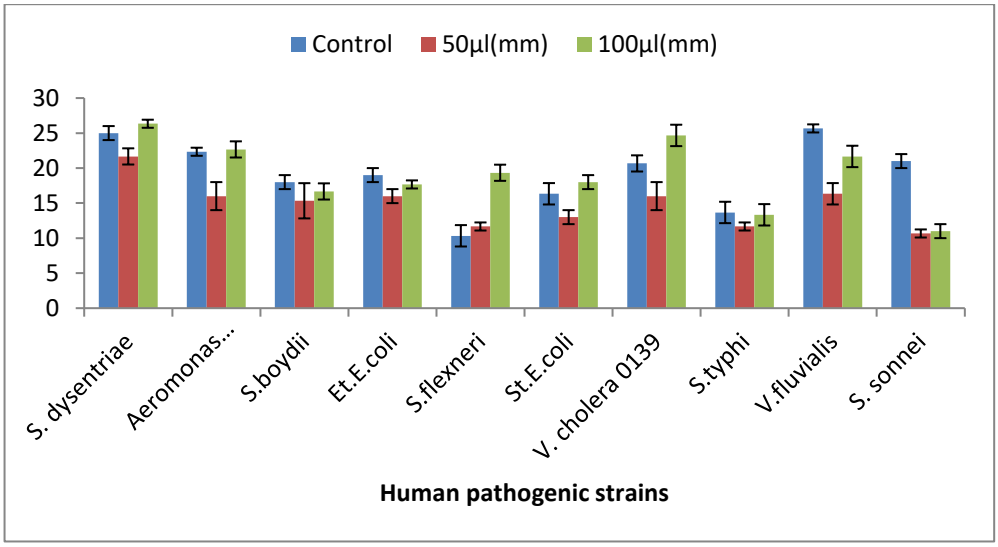
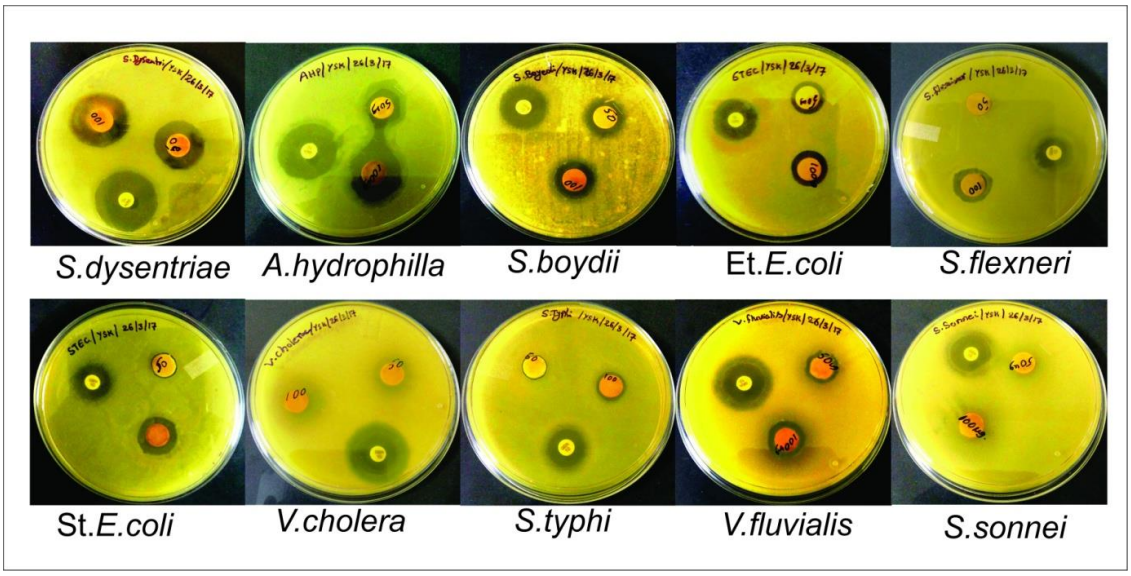


Fig 2: Antibacterial activity against 10 different pathogens



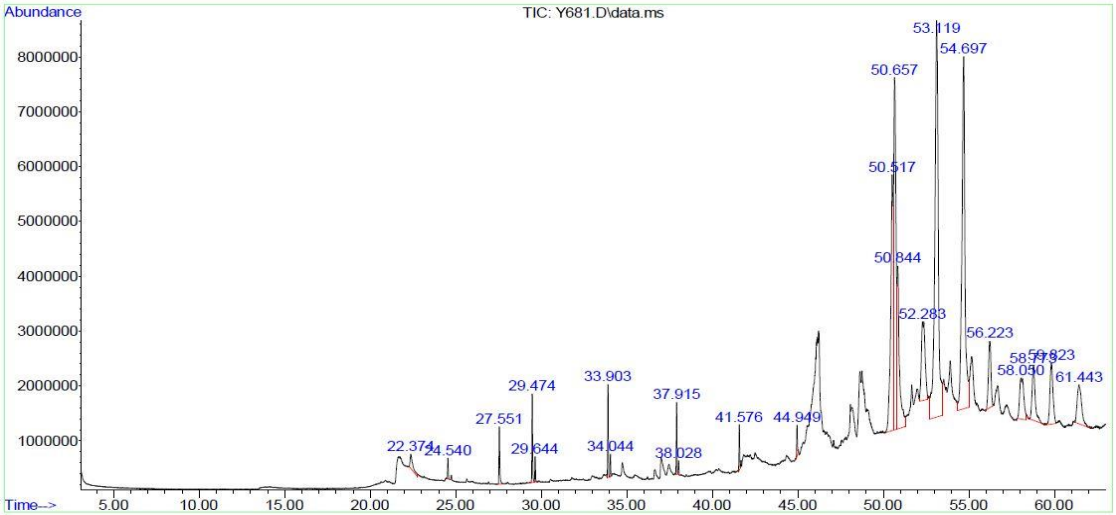
The GC-MS chromatogram suggested that 20 compounds (Table 1 and Figure 3) are present in the methanolic bacterial extract. The major peak (retention time 53.119, peak area 24.111%) suggested the presence of Urs-12-en-28-al followed by Urs-12-en-28-al,3-(acetyloxy)-, (3β)- next higher in concentration at the retention times 54.697 and 50.517 with peak areas 18.445% and 10.096%, respectively. Lup-20(29)-en-3-one (retention time 50.844, peak

area 6.493%) was the next other major compound detected in the chromatogram. Apart from these four major peaks, the other minor peaks denoting the specific compounds are also present, thereby, suggesting a wide variety of diverse chemically defined structural bioactive compounds present in the extract which may exert a combined bactericidal effect. Strains producing antimicrobial compounds exhibits an ecological role by serving as a defensive mechanism to sustain and protect their ecological niche from the invasion and interference of alien invasive microbial species. Thus, marine invertebrates represent a fascinating hotspot nurturing a quantum of unknown microbial communities with unexploited potent sources of diverse secondary metabolites. Further studies are needed for the evaluation of specific compounds which paves a new route for drug designing with a safer way out to clinical applications.

Table 1: GC – MS analysis of methanolic extract of *Vibrio furnissii*

S.No.	Compound Name	Retention time	Molecular formula	Molecular weight	Peak Area
1	Propanedioic acid, phenyl-	22.374	$C_9H_8O_4$	180.159	0.681
2	1-Tetradecene	24.540	$C_{14}H_{28}$	196.372	0.229
3	Phenol, 2,4-bis(1,1-dimethylethyl)-	27.551	$C_{14}H_{22}O$	206.329	0.812
4	1-Hexadecanol	29.474	$C_{16}H_{34}O$	242.447	1.015
5	Hexadecane	29.644	$C_{16}H_{34}$	226.448	0.279
6	1-Nonadecene	33.903	$C_{19}H_{38}$	266.513	0.998
7	Octadecane	34.044	$C_{18}H_{38}$	254.502	0.217
8	1-Nonadecene	37.915	$C_{19}H_{38}$	266.513	0.763
9	Eicosane	38.028	$C_{20}H_{42}$	282.556	0.132
10	1-Docosene	41.576	$C_{22}H_{44}$	308.594	0.505
11	17-Pentatriacontene	44.949	$C_{35}H_{70}$	490.945	0.357
12	Urs-12-en-28-al, 3-(acetyloxy)-, (3β)-	50.517	$C_{32}H_{50}O_3$	482.7376	10.096
13	Lup-20(29)-en-3-one	50.844	$C_{30}H_{48}$	424.702	6.493
14	Lupeol	52.283	$C_{30}H_{50}O$	426.729	5.145
15	Urs-12-en-28-al	53.119	$C_{30}H_{48}O$	424.713	24.111
16	Urs-12-en-28-al, 3-(acetyloxy)-, (3β)-	54.697	$C_{32}H_{50}O_3$	482.7376	18.445
17	Urs-12-en-28-al, 3-(acetyloxy)-, (3β)-	56.223	$C_{32}H_{50}O_3$	482.7376	2.894
18	(14β)12,13-Epoxyolean-3-ol, acetate	58.050	$C_{32}H_{52}$	484.754	2.977
19	Urs-12-en-28-al, 3-(acetyloxy)-, (3β)-	58.773	$C_{32}H_{50}O_3$	482.7376	2.894
20	Cholestane, 3-iodo-	59.823	$C_{29}H_{49}IO_2S_2$	620.733	3.246

Fig 3: GC-MS chromatogram of methanolic extract of *Vibrio furnissii*



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