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ANTIBACTERIAL ACTIVITY OF THE CRUDE EXTRACTS OF *B. SUBTILIS* FROM SML

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ABSTRACT: The rise of antibiotic-resistant pathogens leads to the development of new antibiotics and drugs. Natural products capture the attention of the researchers for such drug discovery. Apart from medicinal plants, many microorganisms also play a great role in the discovery of antibiotics. In this context, bacteria from Sea surface microlayer (SML) are also investigated nowadays. The secondary metabolites of bacteria from such extreme environment are expected to produce some effective metabolites which may have potential activity against pathogens. In the present study, *B. subtilis* was isolated from SML and different crude extracts of the bacteria was tested against human pathogens. The results declared that ethyl acetate extract has efficient activity rather than methanolic extracts. The common opportunistic pathogen *E. coli* was found highly susceptible to the extract followed by the other pathogens. The present study will pave the way for further studies on purification of crude extract and testify them for other biomedical applications like anticancer activity.

INTRODUCTION: The story of antibiotics had started more than half a century ago. The increase in the antibiotic resistant pathogens leads to the study of organisms in the environment and the discovery of the chemical compounds present in some of them could be beneficial for treating illness. Several of these organisms produce secondary metabolites, which are part of a wide variety of natural compounds used by humans to combat diseases. Secondary metabolites are defined as organic compounds formed as bioproducts in organisms, not directly related to growth, development, and normal reproduction.

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The importance of finding and using these secondary metabolites can be justified to know the natural substances that can be beneficial for man and to identify the organisms that produce these substances to make rational exploitation of them, because they may be the only carriers of useful compounds to combat pathogenic microbes [1].

Marine organisms possess an inexhaustible source of useful chemical substances for the development of new drugs. Microorganisms residing in the extreme environment are expected to alternate their metabolism and may produce new metabolites which may possess antibiotic activity. Such an extreme environment is sea surface microlayer (SML). SML is the topmost 1-1000 µm of the sea surface which is said to be gelatinous and involved in biogeochemical cycles [2]. It is an interface between the atmosphere and sea surface. The air-water interface and associated surface microlayers of marine and inland waters represent an important

marine ecosystem [3, 4], with biological activity at this interface being greater than in the bulk water [5]. Collectively, organisms within the microlayer are known as the neuston [6,] and the community of bacteria present within this neuston layer are named the bacterioneuston. Compared with the water, only a few centimeters below the sea surface, the microlayer has been demonstrated to contain an abundance of bacteria [7, 8, 9, 10, 11, 12].

For example, some estimates commonly state that bacteria in the sea surface microlayer are 10^3 - 10^5 times more abundant than the bacterioplankton of the water column [13]. In this study, this bacterioneuston is investigated for bioactive metabolites.

MATERIALS AND METHODS:

Preparation of Bacterial Extracts: The bacteria *Bacillus subtilis* was isolated from SML using standard membrane method [14, 15]. The identity was confirmed by biochemical and 16 srRNA sequencings. About 10 liters of Zobell marine broth was inoculated with *B. subtilis* culture. After 3-4 days of growth, the culture was centrifuged at 8,000 rpm for 10 minutes, and the pellet was discarded.

The collected supernatant was shaken vigorously in a separating funnel along with the solvents ethyl acetate and methanol separately. After shaking, the solvent alone was collected and using rotary vacuum evaporator, the solvent was dried out, and the crude extract of the *B. subtilis* was obtained. Herewith, the crude extract of *B. subtilis* was obtained using two solvents ethyl acetate and methanol separately.

Selection of Pathogens: Seven bacterial pathogens were selected for this study. Gram-negative bacteria such as *Klebsiella pneumonia* (MTCC-7028), *Pseudomonas aeuroginosa* (MTCC-1934), *Escherichia coli* (MTCC-1698), *Salmonella typhi* (MTCC-733) and gram-positive bacteria such as *Staphylococcus aureus* (MTCC-902) and *Streptomyces pneumonia* (MTCC-4734). The pathogens were maintained by subculturing them in fresh media periodically.

Disc Diffusion Assay: The test organisms were inoculated in nutrient broth and incubated for 24 hrs, and these pathogens were swabbed in Mueller Hinton agar plates. Then, the crude extracts were loaded in sterile discs, and these loaded discs were placed over the swabbed surface of the plates. A sterile disc loaded with only respective solvent was used as control. The antibiotic Gentamycin, which is an aminoglycoside antibacterial agent, was used as standard. The assay was done in triplicates.

RESULTS AND DISCUSSION: The antibacterial activities of ethyl acetate and methanol extract of *B. subtilis* were analyzed *in-vitro* by disc diffusion method. The growth inhibitory effect of crude extracts of *B. subtilis* was tested against gram positive and gram negative bacteria *viz., K. pneumoniae, P. aeuroginosa, E. coli, S. typhi, S. aureus,* and *S. pneumoniae*. The antibacterial activity of both the solvent extracts was calculated by measuring the inhibition zone formed around the crude extract loaded a disc in millimeter (mm).

The antibacterial activities of *B. subtilis* against gram-negative pathogenic bacteria were presented in **Table 1.**

TABLE 1: ANTIBACTERIAL ACTIVITY OF DIFFERENT EXTRACTS OF *B. SUBTILIS* AGAINST GRAM NEGATIVE PATHOGENS

S. no.	Pathogens	Crude extract	The diameter of zone of inhibition (mm)			
			Extract concentrations (µg/ml)		Gentamycin (25µg)	Control (100% solvent)
			50%	100%		
1	*Klebsiella pneumoniae*	Ethyl acetate	8	17	24	-
		Methanol	-	8		
2	*Pseudomonas aeruginosa*	Ethyl acetate	6	15	25	-
		Methanol	5	11		
3	*Escherichia coli*	Ethyl acetate	9	20	21	-
		Methanol	7	13		
4	*Salmonella typhii*	Ethyl acetate	5	11	24	-
		Methanol	-	9		

The maximum antibacterial activity of ethyl acetate extract was observed against the pathogen *E. coli* at 100% concentration followed by *K. pneumonia, P. aeruginosa,* and *S. typhi.* The methanol extract

showed maximum antibacterial activity against *E. coli* at 100% concentration followed by *P. aeruginosa, S. typhi,* and *K. pneumonia*. The 50% concentration of ethyl acetate extract showed some weak activity against all the pathogens, but the 50% concentration of methanol extract showed no activity against *K. pneumonia* and *S. typhi*. The antibacterial activities of *B. subtilis* against gram-positive pathogenic bacteria were presented in **Table 2.**

The maximum antibacterial activity of ethyl acetate extract was observed against the pathogen *S. aureus* at 100% concentration, followed by *S. pneumoniae*. The methanol extract showed maximum antibacterial activity against *S. aureus* at 100% concentration, followed by *S. pneumoniae*. At 50% concentration, ethyl acetate extract showed weak activity against both the pathogens but the methanol extract showed no activity against *S. pneumoniae.*

TABLE 2: ANTIBACTERIAL ACTIVITY OF DIFFERENT EXTRACTS OF *B. SUBTILIS* AGAINST GRAM POSITIVE PATHOGENS

S. no.	Pathogens	Crude extract	The diameter of zone of inhibition (mm)			
			Extract concentrations (µg/ml)		Ciprofloxacin	Control
			50%	100%	(25µg)	(100% solvent)
1	*Staphylococcus aureus*	Ethyl acetate	7	19	28	-
		Methanol	5	12		
2	*Streptomyces pneumonia*	Ethyl acetate	3	13	22	-
		Methanol	-	9		

The activity was observed high in ethyl acetate extract rather than methanolic extract against both grams positive and gram negative pathogens. This leads to the fact that ethyl acetate is a better solvent for making the bacterial extract.

The activity of the crude extract was observed lesser than the standard Gentamycin and Ciprofloxacin. The extract showed increasing inhibitory activity with an increase in concentration (50% -100%). These combined antibacterial activities increase the chance of the utilization of bacterioneuston metabolites for biomedical applications. The metabolites have a potential antibacterial activity which may have anticancer property too.

Bacillus species have been found to possess chemical compounds with anticancer activity. Although this type of bacteria can grow in almost any substrate, it is possible to suggest that this species seems to have acquired the skill to synthesize compounds capable of inhibiting HCT-116 colorectal cancer cells [16]. Also, the *Bacillus* sp. isolated from SML may have some additional properties. Selective response mechanisms exist against certain organisms, as shown in marine biofilms of *Bacteriodetes, Planctomycetes, a, c-* and *d Proteobacteria,* where the production of chemical substances such as violacein has been observed. This compound works as a defense mechanism against certain specific predators like the protozoa consuming bacteria. This allows the successful persistence of the bacterial biofilm in several marine environments [17].

CONCLUSION: The present study of testing antibacterial activity of *Bacillus* sp. crude extract using different solvent showed that ethyl acetate is the better choice to make microbial extract than methanol. The ethyl acetate extract showed moderate activity at 50% concentration and strong activity at 100% concentration, whereas methanol extract showed weak activity at 50% concentration and moderate activity at 100% concentration against pathogens. This study reveals that crude extract of *B. subtilis* from SML possesses antibacterial activity which could be purified and further studied for the anticancer property.

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CONFLICT OF INTEREST: Nil

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