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Short communication

Antioxidant potential and biochemical evaluation of metabolites from the marine bacteria *Virgibacillus* sp. associated with the sponge *Callyspongia diffusa*

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ABSTRACT

Objective: In this present research, the antioxidant activity of the metabolites produced by the marine bacteria *Virgibacillus* sp. associated with the sponge *Callyspongia diffusa* was evaluated and the crude metabolites was also tested to find out the chemical compounds.
Methods: In view of testing the antioxidant activity, DPPH radical scavenging activity, Hydroxyl radical scavenging activity, Superoxide radical scavenging activity, Nitric oxide radical scavenging activity,, Metal chelating, Ferric reducing antioxidant power were carried out *invitro*.
Results: The DPPH radical scavenging activity was 46.56% at 2000 μg/ml and the IC_{50} value was 857.49 μg/ml, The hydroxyl radical scavenging activity was found significant (60.77% at 2 mg/ml) and the IC_{50} value was found 471.07 μg/ml. Superoxide radical scavenging activity was 25.58% at 2000 μg/ml and the IC_{50} was 1353.28 μg/ml, for nitric oxide, the radical scavenging activity was 25.58% at 2000 μg/ml concentration and in the due course the IC_{50} value was 1353.28 μg/ml. The results of the chemical analysis showed the presence of chemical components such as alkaloids, terpenoids, reducing sugars and anthraquinones.
Conclusion: This research revealed the potential of marine sponge-associated bacterial bioactive compounds in scavenging the free radicals invitro. Separation of individual compounds from the crude metabolites of *Virgibacillus* sp. associated with the marine sponge *C. diffusa* will be resulted in the development of a novel antioxidant molecule.

1. Introduction

Reactive oxygen species (ROS) and free radical mediated reactions are the major reasons for the pathological events such as aging, cancer, coronary heart ailment and Alzheimer's disease.[1] These are the diseases mainly caused in humans and animals due to the problem in our cells that lacks it function to scavenge the formed free radicals. Antioxidants molecules can avert the harmful effects[2,3] The oxidative damage of a tissue is indirectly prevented by increasing cells' natural defenses and/or directly by scavenging the free radical species.[4] Some marine microorganisms have produced biologically active or structurally novel metabolites.[5] The marine fungus, *Keissleriella* sp. YS 4108, produced extracellular polysaccharide which was found with potential antioxidant activity.[6] Extracellular polysaccharides from *Pandora agglomerans* strain KFS-9 showed potential hydroxyl and superoxide radical scavenging activity.[7] The extracellular polysaccharides produced by the marine fungus *Penicillium* sp. F23-2 showed potential antioxidant activity.[8] Two extracellular polysaccharides viz., ETW1 and ETW2 produced by the marine bacterium *Edwardsiella tarda*, are mannans which showed potential antioxidant activities.[9] As bioactive compounds from marine bacteria possess a good antioxidant potential, in this present investigation the metabolites produced by the bacterial strain *Virgibacillus* sp. isolated from the sponge *Callyspongia diffusa* was tested for *invitro* antioxidant activity.

2. Materials and methods

2.1. Sponge collection and identification

The marine sponge was collected off from the Gulf of Mannar region, Hares Island, Tuticorin coast from the net of fisherman. The sponge specimen was rinsed well in sterilized artificial sea water and also treated with 70% alcohol so as to eliminate epiphytic microorganisms present on the surface of the sponge specimen. The sponge specimen was identified by Dr. P.A. Thomas, Retired Scientist, CMFRI, Kerala, India.

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2.2. Isolation of sponge-associated bacteria

Small pieces of (1-cm^2) sponge tissues were excised out from the mesophyll region and homogenated in sterile artificial sea water using a mortar and pestle. The homogenate was subjected to serial dilution technique and the each dilution was plated on Zobell marine agar. The plates were incubated at 37 °C for 24 h. After the incubation time was over, the plates were observed for the presence of various bacterial colonies. The isolated colonies were purified using streak plate technique and stored in Zobell marine agar slants at 4 °C in a refrigerator. From the pure culture, one of the bacterial strains was isolated and identified as *Virgibacillus* sp based on 16 S rRNA sequencing (Fig. 1) and was bioprospected for the production of bioactive metabolites.

2.3. Cultivation of sponge – associated bacterium Virgibacillus sp. for secondary metabolites production

The sponge-associated bacterial strain *Virgibacillus* sp. (CSD-5) was mass cultured using 20 L (10X2L) of Zobell marine broth dissolved in sea water. The culture of the strain *Virgibacillus* sp. were inoculated into the medium and kept for incubation for 72 h at room temperature on a shaker. After the incubation time was over, the cells were separated from the culture medium by centrifuging at 12,000 rpm for 20 min. The cell free supernatant was subjected to ethyl acetate extraction using a separating funnel and the solvent layer was collected. The ethyl acetate extract was condensed with the application of a rotary vacuum evaporator under reduced pressure at 40 °C to collect the crude metabolites of this strain. The crude metabolites obtained was 20 g.

3. Invitro antioxidant activity

3.1. Free radical scavenging activity on DPPH•

The DPPH free radical scavenging activity of the metabolites produced by *Virgibacillus* sp. were tested according to the method of Blois.[10] The metabolites of different concentrations (200–1000 μg) was prepared and was added with freshly prepared 5 ml of 0.1 mM methanolic solution of DPPH. The reaction was carried out at 27 °C for 20 min. After the reaction time was over, the reaction mixture was spectrometrically read at 517 nm. The percentage of DPPH radical scavenging activity was calculated as follows.

DPPH radical scavenging activity (%) = (control OD − sample OD/control OD) × 100. The assay was carried out in triplicate. The IC_{50} value of the metabolites produced by *Virgibacillus* sp. in scavenging DPPH free radical was calculated from the graph of inhibition percentage against sample concentration.

3.2. Hydroxyl radical scavenging activity (HRSA)

The metabolites produced by *Virgibacillus* sp. were tested for hydroxyl radical scavenging activity by following the method of Klein et al[11] The prepared metabolites of different concentrations (200–1000 μg) were added with 1 ml of iron-EDTA solution (0.13% ferrous ammonium sulfate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), and 1 ml of dimethyl sulfoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4). In order to initiate the reaction 0.5 ml of ascorbic acid (0.22%) was added and kept for incubation at 80–90 °C for 15 min in a water bath. After the incubation time was over,1 ml of ice-cold TCA (17.5% w/v) was added so as to stop the reaction. Then, Nash reagent of about three millliliter was added and left at room temperature for 15 min. The reaction mixture was spectrometrically read at 412 nm against reagent blank to find out the intensity of the color formation. The percentage of hydroxyl radical scavenging activity produced by the metabolites of *Virgibacillus* sp. were calculated as follows.

HRSA (%) = (control OD − sample OD/control OD) × 100. The assay was carried out in triplicate. The IC_{50} value of the metabolites produced by *Virgibacillus* sp. in scavenging hydroxyl radical was calculated from the graph of inhibition percentage against sample concentration.

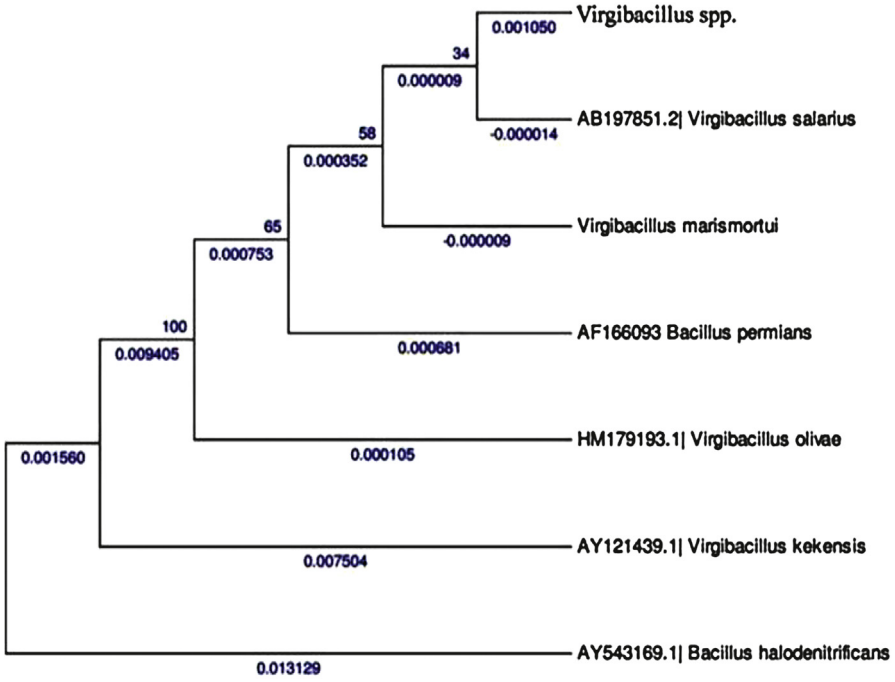

Fig. 1. Phylogenetic tree of the strain CSD-5 associated with the margin sponge *Callyspongia diffusa*.

3.3. Nitric oxide radical scavenging activity (NO)

Sreejayan and Rao method was followed to carryout nitric oxide radical scavenging activity.[12] Different concentrations (200–1000 μg) of metabolites produced by *Virgibacillus* sp. were prepared and treated with three ml of 10 mM sodium nitroprusside in 0.2 M phosphate buffered saline (pH 7.4) and allowed for incubation at room temperature for 150 min. After the incubation time was over, 0.5 ml of Griess reagent was added and the absorbance of the reaction mixture was read at 546 nm. The percentage of radical scavenging activity by the metabolites produced by *Virgibacillus* sp. were calculated as follows.

Nitric oxide radical scavenging activity (%) = (control OD − sample OD/control OD) × 100. The assay was carried out in triplicate. The IC_{50} value of the metabolites produced by *Virgibacillus* sp. in scavenging nitric oxide radical was calculated from the graph of inhibition percentage against sample concentration.

3.4. Superoxide radical scavenging activity

Superoxide radical scavenging activity was carried out as per the method of Beauchamp and Fridovich.[13] Different concentrations (200–1000 μg) of metabolites produced by *Virgibacillus* sp. were prepared and mixed with 50 mM sodium phosphate buffer (pH 7.6), 20 mg riboflavin, 12 mM EDTA, 0.1 mg NBT. The reaction was allowed to stand for 90 s. After the incubation time was over, the absorbance of the reaction mixture was read spectrometrically at 590 nm. The reaction mixture kept in dark place served as blank. The percentage of inhibition of superoxide anion by the metabolites produced by *Virgibacillus* sp. were calculated as follows.

Inhibition (%) = (control OD − sample OD/control OD) × 100. The assay was carried out in triplicate. The IC_{50} value of the metabolites in scavenging superoxide radical was calculated from the graph of inhibition percentage against sample concentration.

3.5. Ferric reducing antioxidant power (FRAP) assay

The ferric reducing capacity of metabolites produced by *Virgibacillus* sp. *were* performed according to the method of Benzie and Strain.[14] Different concentrations (200–1000 μg) of metabolites produced by *Virgibacillus* sp. were prepared. Ten μl of different concentrations of metabolites was mixed with 900 μl of freshly prepared FRAP reagent mixed with 90 μl of water. The reaction mixture was allowed to stand at 37 °C for 30 min and the absorbance was spectrometrically read at 593 nm.

3.6. Phosphomolybdenum reduction assay

Reductions of phosphomolybdenum by the metabolites produced by *Virgibacillus* sp. were carried out by phosphomolybdenum method.[15] From the different concentrations (200–1000 μg) of metabolites, 0.1 ml of sample was taken and mixed with 1 ml of reagent solution and incubated at 95 °C for 90 min in a water bath. After cooling the reaction mixture was spectrometrically read at 765 nm against a blank.

3.7. Metal chelating activity

Dinis et al, method was followed to perform metal chelating activity.[16] Briefly, different concentrations (200–1000 μg) metabolites produced by *Virgibacillus* sp. were mixed well with 50 μl of 2 mM FeCl2 and standard EDTA (50–250 μg). 0.2 ml of 5 mM ferrozine solution was added to initiate the reaction and kept at room temperature for 10 min. The absorbance spectra was read spectrometrically at 562 nm. The assay was carried out in triplicate and the results were expressed as EDTA equivalent.

4. Chemical screening

The crude metabolites of *Virgibacillus* sp. were subjected to various biochemical assessments so as to find out the presence of different kinds of bioactive compounds.[17,18]

5. Results

Marine environment contributes as major resource for the development of potential drugs to cure so many diseases. Particularly, the sponge-associated microorganisms attracted the interest of the researchers across the globe because they produce compounds with potential antibacterial, antifungal, anti tumor and anti malarial activities. But, antioxidant capacity of the metabolites derived from marine sponges associated bacteria is less explored. Hence, the antioxidant potential of the metabolites produced by the bacteria *Virgibacillus* sp. associated with the marine sponge *C. diffusa* was tested for *invitro* antioxidant potential.

The bacterial strain CSD-5 was identified as *Virgibacillus* sp. as per the data of 16S rRNA sequencing analysis. The amplified isolated DNA sequences were compared with the existing sequences of NCBI using the BLASTN program. The results confirmed that the strain CSD-5 was belonged to the genus of *Virgibacillus* sp. with 98% sequence similarity. The phylogenetic tree showed that the strain CSD-5 has an own cluster with *Virgibacillus* sp. Based on BLAST and Phylogenetic results the strain CSD-5 was identified as *Virgibacillus* sp (Fig. 1).

The metabolites isolated from *Virgibacillus* sp. showed enhanced scavenging activity over DPPH free radicals. The scavenging activity was recorded dose dependent. At 1600 μg/ml dose, this activity was 39.61% but at 2000 μg/ml concentration the activity was increased to 46.56%. The IC_{50} value of the metabolites produced by *Virgibacillus* sp. was 857.49 μg/ml (Table 1)

The metabolites produced by *Virgibacillus* sp. showed maximum hydroxyl radical scavenging activity (60.77%) at 2000 μg/ml concentration. The activity was concentration specific. The IC_{50} value of the metabolites in scavenging hydroxyl radical was found 471.07 μg/ml (Table 2)

Superoxide radical scavenging was found to be 25.08% at 2000 μg/ml concentration. The scavenging activity increased when the dose of the metabolites was increased. The IC_{50} value of the metabolites produced by the strain *Virgibacillus* sp. in scavenging superoxide radical was 1.35 mg/ml (Table 3)

Ferric reducing antioxidant potential of the metabolites produced by *Virgibacillus* sp. *were* found to be moderate 208.76 mg/mmol (Fe (II) (Table 4).

Table 1
DPPH radical scavenging activity.

S. no	Samples	Concentrations (μg)	Percentage of DPPH scavenging activity	IC_{50} (μg/ml)
1	Metabolites of *Virgibacillus* sp.	400	11.79 ± 0.35	857.49
		800	17.07 ± 0.50	
		1200	28.94 ± 0.49	
		1600	39.61 ± 0.75	
		2000	46.56 ± 0.51	
2	Ascorbic acid (reference sample)	2.5	49.37 ± 0.31	2.53
		5.0	63.23 ± 1.36	
		7.5	73.05 ± 0.37	
		10	81.39 ± 0.31	
		12.5	91.18 ± 0.63	

Table 2
Hydroxyl radical free radical scavenging activity.

S. no	Samples	Concentrations (μg)	Percentage of hydroxyl radical scavenging activity	IC_{50} (μg/ml)
1	Metabolites of *Virgibacillus* sp.	400	13.01 ± 0.43	471.07 ± 8.87
		800	24.25 ± 1.27	
		1200	36.70 ± 0.43	
		1600	48.60 ± 0.74	
		2000	60.77 ± 0.71	
2	Ascorbic acid (reference sample)	10	29.24 ± 0.08	28.34
		20	40.95 ± 2.83	
		30	52.12 ± 0.08	
		40	57.22 ± 1.45	
		50	65.65 ± 0.51	

Table 3
Superoxide free radical scavenging activity.

S. no	Samples	Concentrations (μg)	Percentage of superoxide scavenging activity	IC_{50} (μg/ml)
1	Metabolites of *Virgibacillus* sp.	400	5.53 ± 0.56	1353.28 ± 61.68
		800	10.67 ± 0.56	
		1200	14.65 ± 0.46	
		1600	20.65 ± 0.71	
		2000	25.58 ± 0.46	
2	Ascorbic acid (reference sample)	10	14.09 ± 2.16	43.55
		20	25.07 ± 0.70	
		30	35.04 ± 0.85	
		40	42.32 ± 0.85	
		50	58.58 ± 1.34	

The metabolites showed minimal scavenging activity over nitric oxide free radicals. The activity was 21.83% at 2000 μg/ml concentration and in the due course the IC_{50} of the metabolites in scavenging nitric oxide radicals was 1939.39 μg/ml (Table 5).

The metabolites produced by Virgibacillus sp. showed no remarkable activity in chelating the ferrous ions (Table 6).

The chemical analysis of the metabolites produced by the strain *Virgibacillus* sp. showed the presence of bioactive components such as alkaloids, terpenoids, reducing sugars and anthroquinones (Table 7).

Statistical analysis: values are means of three independent analyses of the compounds ± standard deviation ($n = 3$).

6. Discussion

The metabolites produced by *Virgibacillus* sp were potential in scavenging DPPH radicals. Invitro as reported for other bacterial metabolites.[19,20] In living cells hydroxyl radicals cause major damages and the elimination of the hydroxyl radicals would result in the protection of living cell systems.[21] As the metabolites

Table 4
Ferric reducing antioxidant power (FRAP) assay.

S. no	Samples	Percentage of ferric reducing antioxidant activity (mmol (Fe(II)/g extract)
1	Metabolites of *Virgibacillus* sp.	208.76 ± 5.84
2	Ascorbic acid (reference sample)	748.7 ± 16.2

Table 5
Nitric oxide free radical scavenging activity.

S. no	Samples	Concentrations (μg)	Percentage of nitric oxide scavenging activity	IC_{50} (μg/ml)
1	Metabolites of *Virgibacillus* sp.	400	4.78 ± 0.61	1939.39 ± 104.97
		800	8.09 ± 0.45	
		1200	13.35 ± 0.45	
		1600	16.76 ± 0.74	
		2000	21.83 ± 1.03	
2	Ascorbic acid (reference sample)	2.5	19.63 ± 1.96	28.32
		5.0	44.00 ± 1.80	
		7.5	59.01 ± 6.70	
		10	74.60 ± 1.63	
		12.5	77.60 ± 0.33	

Table 6
Metal chelating activity.

S. no	Samples	Metal chelating mg EDTA/g extract
1	Metabolites of *Virgibacillus* sp.	0.87 ± 0.07
2	Ascorbic acid (reference sample)	11.2 ± 0.5

produced by the *Virgibacillus* sp. strain showing profound hydroxyl radical scavenging activity, it can be utilized as an effective antioxidant compound to control free radicals induced pathological disorders. This compound can further be investigated to test the *invivo* antioxidant potential in free radicals induced cancer in murine models. In the cells of the living system hydroxyl radical reacts very easily with all biomolecules.[22]As the metabolites showed highly potential hydroxyl radical scavenging activity, it's expected to control the multiplication of cancer cells by its prospective repairing mechanisms on DNA as well as in RNA molecules in the living system. Major free radicals viz., superoxide radical (O_2), hydroxyl radical (OH) and other reactive oxygen species (ROS) are playing a main role in the formation of cancer cells and mutation in the cells of the living system.[23] As the crude extract itself found with potential free radical scavenging activity, testing the individual compounds from the crude metabolites of *Virgibacillus* sp. will ensure the structural elucidation of a novel compound with higher potential of antioxidant activity. The bioactive compounds viz., alkaloids, anthraquinones and terpenoids present in the crude metabolites would have contributed for its antioxidant potential. As the bioactive metabolites showed potential response in scavenging the free radicals, it can be utilized as an effective drug to treat free radicals mediated pathological disorders *viz*. cancer, Alzheimer's disease and heart problems. Terpenoids from the petroleum ether extract of *C. officinalis* flowers showed potential pharmacological activities including anticancer and antioxidant activity.[24] Identification

Table 7
Qualitative screening of chemical components in the crude metabolites of *Virgibacillus* sp.

S. no	Biochemicals	Results
1	Alkaloids	Positive
2	Flavonoids	Negative
3	Steroids	Negative
4	Terpenoids	Positive
5	Anthraquinones	Positive
6	Reducing sugars	Positive
7	Tannins	Negative
8	Saponins	Negative
9	Phenolic compounds	Negative

of potential specific antioxidant bioactive compound would be a very interesting task to study the exact mechanism of free radical scavenging activity in pathological disorders such as cancer, Alzheimer's disease and coronary heart diseases whether it acts directly or indirectly inducing the cellular activity of the affected cells.

7. Conclusion

In the present investigation, the metabolites produced by the marine bacteria *Virgibacillus* sp. associated with the marine sponge *C. diffusa* was evaluated for *invitro* antioxidant and biochemical analysis. This research pointed out the potential of metabolites produced by *Virgibacillus* sp. in scavenging the free radicals such as DPPH, Hydroxyl radical, Superoxide radical and Nitric oxide radicals. The free radical scavenging activity of bacterial metabolites over hydroxyl radical was above 60 percent and in the due course the DPPH radical scavenging activity was recorded 46.56% at a dose of 2 mg/ml. Separation of individual compounds is underway to find out the exact compound responsible for it's potential free radical scavenging activity. As, most of the marine sponge-associated microorganisms derived metabolites are still not utilized in a great level for the development of antioxidant drugs as well as for the pathological disorders caused by the free radicals. The present report on the bioprospecting of the bacteria associated with the marine sponge *C diffusa* will lead to the development of a novel antioxidant molecule.

Conflicts of interest

All authors have none to declare.

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