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Marine natural product, Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- ($C_7H_{10}N_2O_2$) of antioxidant properties from *Bacillus* species at Lakshadweep archipelago

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PEER REVIEW

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Comments

The present manuscript is interesting and shows the potential antioxidant effects of a new compound isolated from a *Bacillus* sp.
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ABSTRACT

Objective: To investigate the antioxidant property of purified bioactive compound from sponge associated bacteria *Bacillus* species.
Methods: The potential active compound was subjected for assay of total antioxidant activity, α,α-diphenyl-α-picrylhydrazyl (DPPH) radical scavenging activity, nitric oxide radical scavenging activity, hydrogen peroxide (H_2O_2) scavenging activity and total reducing power. Further, the 16S rRNA gene sequence was carried out to identify the sponge symbiotic bacteria.
Results: The results showed linear increase of total antioxidant activity, DPPH radical scavenging activity, nitric oxide radical scavenging activity, H_2O_2 scavenging activity and total reducing power. IC_{50} value of the active compound for DPPH activity, H_2O_2 scavenging activity, nitric oxide scavenging activity was recorded as 15.025 μg/mL, 23.73 μg/mL, and 41.70 μg/mL respectively. The potential strain was identified as *Bacillus* species from GenBank database (GenBank Accession number JX985748).
Conclusions: The present study has reported the antioxidant property of purified bioactive compound from sponge associated *Bacillus* species. The report will be helpful to pharmaceutical and antioxidant researchers for further studies.

KEYWORDS

Secondary metabolites, Antioxidant activity, *Bacillus* species, Lakshadweep archipelago

1. Introduction

The biological cells produce free radicals generally by electron transfer reactions, which can be generated by enzymatic reactions or non-enzymatic reactions mediated metabolic function. Free radicals can be defined as molecules or molecular fragments containing one or more unpaired electrons in atomic or molecular orbital[1]. Oxidation reactions can produce free radicals, which start chain reactions and damage the cells. Uncontrolled production of free radicals which attack macromolecules such as membrane lipids, proteins and DNA may lead to many health problems such as cancer, diabetes mellitus, neurodegenerative and inflammatory diseases with severe tissue injuries[2]. Antioxidants terminate these chain reactions by removing free radical intermediates and inhibit other oxidation reactions by oxidizing themselves. Major oxidation reactions, which are capable of stabilizing free radicals, cause damage to the cells, resulting in the cells' nutritional defects and non-infectious disease of vertebrates and invertebrates.

Broadly defined, an antioxidant is a compound that inhibits

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or delays the oxidation of substrates even if the compound is present in a significantly lower concentration than the oxidized substrate[3]. The scavenging of reactive oxygen species (ROS) is one of possible mechanism of action. Others include the prevention of ROS formation by metal binding or enzyme inhibition. Chain breaking antioxidants prevent damage by interfering with the free radical propagation cascades. The antioxidant compounds can be recycled in the cell or irreversibly damaged, but their oxidation products are less harmful or can be further converted to harmless substances[4]. At the cellular and organism level the antioxidant actions are provided by numerous enzymes, ascorbic acid, uric acid glutathione, tocopherols and several others. Many compounds are of antioxidant activity in addition to their specialized physiological function[5].

ROS are continuously produced during normal physiologic events, and removed by antioxidant defense mechanisms[6]. There is a balance between ROS and antioxidant system in organisms. In unfavorable condition, ROS are overproduced, resulting in lipid peroxidation and oxidative stress[7]. The imbalance between ROS and antioxidant defense mechanisms leads to oxidative modification in cellular membrane or intracellular molecules. Various endogenous antioxidant defense mechanisms play an important role in the elimination of ROS and lipid peroxidation, and protect the cells against toxic effects of ROS and lipid peroxidation[4]. Therefore, the exploration and utilization of more effective antioxidant compounds from natural sources are desired.

The antioxidant activity of secondary metabolites has also been widely established in *in vitro* systems and involves several of the above mentioned mechanisms of action. Some antioxidant compounds are extracted easily from available sources, such as marine resources, agricultural and horticultural crops (fish meal, cephalopods, seaweeds, alga, spirulina, maize, buckwheat, grapevine, carrots, beetroot, citrus and tea leaves, *etc*)[6,8]. Pharmaceutical industries produced many synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, tert-butylhydroquinone and propyl gallate to inhibit peroxidation processes[9]. However, the use of these synthetic antioxidants must be strictly controlled due to potential health issues[10].

Marine sponges are known to be important sources of novel marine natural products. Sponge-associated microorganisms represent a treasure house of biodiversity for the discovery of marine natural products with biotechnological potential. A total of 18 *Dysidea* species have been analyzed to date, more than 400 originate from about thirty as yet un-described *Dysidea* species. Increasing importance of sponge's species diversity is reflected in the diversity of produced secondary metabolites in recent times.

Metabolites are derived through fermentation and purified by series of chromatography techniques and characterized for antioxidant properties by *in vitro* analysis. The pyrrole (which contains pyrrole ring) containing metabolites derived from sponges have shown activity as growth inhibitors of various tumor cell lines, in particular against the human prostate cancer cell line LNcaP, larvicidal activity and inhibits bacterial fish pathogens and clinical pathogens. Hence, natural antioxidants are safe alternatives to synthetic products for use in the food industry. Therefore, the natural sources have been recognized as safe and effective antioxidants in the context of their efficiency and non-toxicity. Earlier, the sponges were collected at various depths in Agatti Island, Lakshadweep archipelago. The Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- ($C_7H_{10}N_2O_2$) was separated from the bacteria by standard method and it was published already. The present study is aimed to investigate the antioxidant property of purified bioactive compound from sponge associated bacteria *Bacillus* sp.

2. Materials and methods

2.1. Total antioxidant activity

Total antioxidant activity of the active compound Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- (20, 40, 60, 80, 100 μg/mL) and ascorbic acid was determined according to the method of Foon *et al*[11]. Briefly, 0.3 mL of sample was mixed with 3.0 mL of reagent solution (0.6 mol/L sulphuric acid, 28 mmol/L sodium phosphate and 4 mmol/L ammonium molybdate). Reaction mixture was incubated at 95 °C for 90 min under water bath. Absorbance of all the sample mixtures was measured at 695 nm. Ascorbic acid (20, 40, 60, 80, 100 μg/mL) was used as positive control.

2.2. α,α-diphenyl-α-picrylhydrazyl (DPPH) radical scavenging

Antioxidant potential of the active compound was determined on the basis of its scavenging activity of the stable DPPH free radical based on the method of Zakaria *et al*[12], where 100 μL of various concentrations of the active compound (25, 50, 75 and 100 μg/mL) were mixed with 290 μL DPPH solution (120 μmol/L) in ethyl acetate and incubated in darkness at 37 °C for 30 min. Absorbance was recorded at 517 nm. Inhibition of free radical by DPPH in percentage of inhibition (%) was calculated with the following equation:

Percentage of Inhibition (%)=(A_{blank}-A_{sample})/A_{blank}×100, where, A_{blank} is the absorbance of the control reaction and A_{sample} is the absorbance of the test compound. The values of inhibition were calculated for the various concentrations of the active compound obtained from potential *Bacillus* species. Ascorbic acid (25, 50, 75 and 100 μg/mL) were used as positive controls and all the tests were carried out in triplicate. The IC_{50} concentration (providing with 50% inhibition) values were calculated by using the dose inhibition curve in linear range by plotting the compound concentration versus the corresponding scavenging effect.

2.3. Scavenging of hydrogen peroxide (H_2O_2)

Ability of the active compound and standard (ascorbic acid) to scavenge H_2O_2 was determined according to the method of Gulcin *et al*[13]. Briefly, 40 mmol/L H_2O_2 was prepared in phosphate buffer (pH 7.4) and the H_2O_2 concentration was determined using spectrophotometer by measuring the absorption with the extinction coefficient for H_2O_2 of 81 L/mol·cm. The active compound (25, 50, 75 and 100 μg/mL) and standard (25, 50, 75 and 100 μg/mL) was added to 0.6 mL of 40 mmol/L H_2O_2 solution and the absorbance was read at 230 nm after 10 min incubation against a blank solution containing phosphate buffer without H_2O_2. The percentage of scavenging of H_2O_2 was calculated as follows:

Scavenging effect (%)=$(A_{cont}-A_{test})/A_{cont}\times100$, the IC_{50} concentration (providing with 50% inhibition) values were calculated by using the dose inhibition curve in linear range by plotting the extract concentration versus the corresponding scavenging effect.

2.4. Nitric oxide radical (NO) scavenging activity

NO generated from sodium nitroprusside in aqueous solution at physiological pH interacted with oxygen to produce nitrite ions, which were measured by the Griess reaction[14]. About 3 mL of the reaction mixture containing 10 mmol/L sodium nitroprusside and the active compound (25, 50, 75 and 100 μg/mL) in ethyl acetate were incubated at 25 °C for 150 min. After incubation, 0.5 mL of the reaction mixture was mixed with 1 mL of sulfanilic acid reagent (0.33% sulfanilic acid in 20% glacial acetic acid) and allowed to stand for 5 min for complete diazotization. Then, 1 mL of naphthyl ethylene diamine dihydrochloride (0.1%) was added with the solution mixed. The mixture was allowed to stand for 30 min at 25 °C. A pink coloured chromophore developed to red at 540 nm against the corresponding blank solution. Ascorbic acid (25, 50, 75 and 100 μg/mL) was used as positive control. The NO scavenging activity of the purified compound was reported as % of scavenging and IC_{50} was calculated as above.

2.5. Total reducing power

Total reducing capacity of active compound and standard (ascorbic acid) was determined according to the method of Prochazkova *et al*[15]. The purified compound (20, 40, 60, 80 and 100 μg/mL) in ethyl acetate (0.2 mol/L, pH 6.6) was mixed with 1% potassium ferric cyanide and the mixture was incubated at 50 °C for 20 min; 2.5 mL of 10% trichloroacetic acid was added to the mixture and centrifuged at 5000 r/min for 10 min. The upper layer of solution (2.5 mL) was mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$ and the colour developed was measured at 700 nm. Ascorbic acid (20, 40, 60, 80 and 100 μg/mL) was used as positive control. The higher the absorbance of the reaction mixture was, the greater the reducing power was.

3. Results

3.1. Total antioxidant activity

Total antioxidant activity was calculated from the formation of green phosphate complex at acidic pH, which was measured using spectrophotometer at 695 nm. Interaction of free radicals with the active compound and standard ascorbic acid terminated the free radical cations and the results have been expressed in ascorbic acid equivalent (μg/mL). Total antioxidant value of the active compound at 20, 40, 60, 80 and 100 μg/mL was estimated at (0.60±0.08), (1.01±0.01), (1.60±0.01), (2.07±0.01) and (2.62±0.01) absorbance respectively at 695 nm (Figure 1).

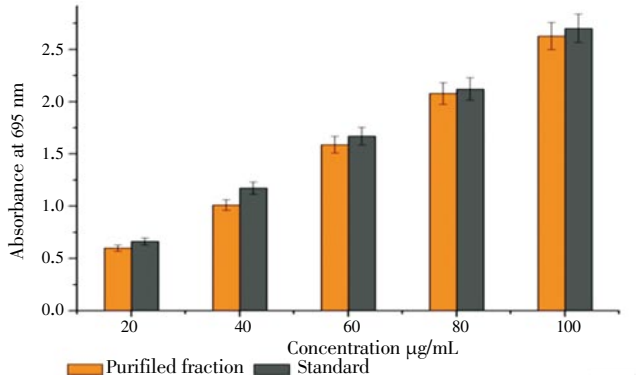

Figure 1. Total antioxidant activity of purified compund of *Bacillus* species and standard ascorbic acid.

3.2. DPPH free radical scavenging activity

DPPH scavenging activity of the active compound at 25, 50, 75 and 100 μg/mL was found to be (20.83±0.34)%, (42.66±0.36)%, (63.45±0.37)% and (85.43±0.38)% respectively (Figure 2). IC_{50} of the active compound was 15.025 μg/mL, indicating its higher inhibiting and potential activity against free radicals than others. The ability of active compound to scavenge DPPH could also reflect its ability to inhibit the formation of free radicals. A linear increase in free radical scavenging ability of purified compound was observed with its increasing concentration.

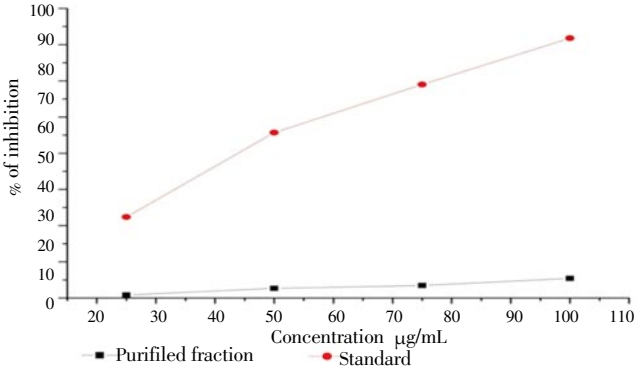

Figure 2. DPHH radical-scavenging activity of purified compound of *Bacillus* species and standard ascorbic acid.

3.3. H_2O_2 scavenging activity

Ability of the purified compound to scavenge H_2O_2 was determined and compared with that of ascorbic acid as standards (Figure 3). Active compound exhibited 15.96%, 32.64%, 45.75% and 63.27% scavenging effect on H_2O_2 at 25, 50, 75 and 100 μg/mL concentrations respectively. IC_{50} of the H_2O_2 scavenging activity of the compund was 23.73 μg/mL. The active compound was capable of scavenging H_2O_2 in an amount dependent manner.

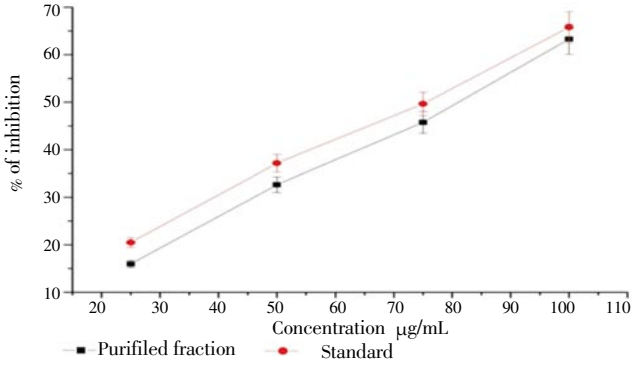

Figure 3. H_2O_2 scavenging activity of purified compound of *Bacillus* species and standard ascorbic acid.

3.4. NO scavenging activity

Active compound decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro*. NO scavenging ability of the active compound at 25, 50, 75 and 100 μg/mL was (35.26±0.19)%, (38.17±0.20)%, (40.67 ±0.20)% and (41.81±0.23)% respectively and it was lower than that of ascorbic acid (Figure 4). IC_{50} of NO scavenging activity of the active compound was 41.70 μg/mL.

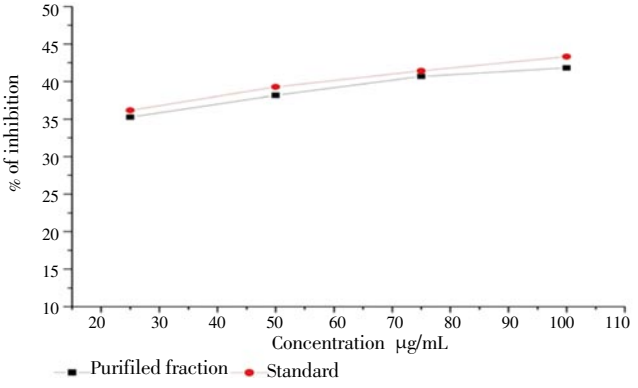

Figure 4. NO radical scavenging effect of purified compound of *Bacillus* species and standard ascorbic acid.

3.5. Total reducing power

Reducing activity of the active compound increased with increasing concentration of the samples (Figure 5). Reducing power of the active compund was weaker than that of the standard compound (ascorbic acid). The reducing capacity of a compound from Fe^{3+}/ferric cyanide complex to the ferrous form may serve as a significant indicator of its antioxidant capacity. A linear increase in reducing power with increasing concentration of active compound was observed. Purified ethyl acetate compound showed significant reducing power compared to standard ascorbic acid.

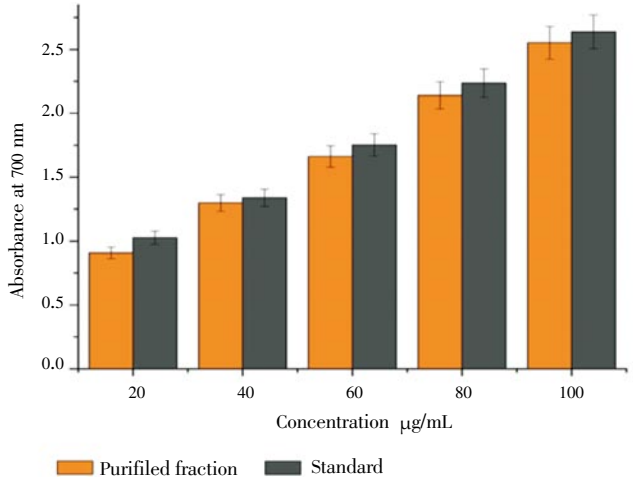

Figure 5. Total reducing power of purified compound of *Bacillus* species and standard ascorbic acid.

4. Discussion

The natural resources of both potential and established antioxidants are vast. Some antioxidant compounds are extracted from easily available sources, such as marine resources, agricultural and horticultural crops (fish meal, cephalopods, seaweeds, algae, spirulina, maize, buckwheat, grapevine, carrots, beetroot, citrus, tea leaves, *etc*.), or medicinal plants. The trend started find out bioactive molecules produced by bacteria, fungi, algae and many others[16]. The complex and rather expensive *in vitro* technology needed for products may be obtained from abundant and cheap sources. The necessary conditions that make using biotechnological methods for the production of secondary metabolites through bacteria economically viable have been firmly established: high economic value, sufficient production can be made, long-lasting availability and some natural sources of antioxidant (rare/endangered/ overexploited species), and difficult cultivation[17]. The media composition has to be optimized for both an intensive biomass increase and the production of the desired metabolite.

The present study revealed total antioxidant activity of active compound and the standard ascorbic acid (20, 40, 60, 80, 100 μg/mL) at 695 nm. The active compound exhibited effective antioxidant activity at all concentrations. At 100 μg/mL, the total antioxidant ability of active molecule from sponge isolate on free radicals was similar to that of

standard. The antioxidant activity of purified ethyl acetate compound increased with increasing concentration, which was similar to that of standard ascorbic acid. Our results are in accordance with the report of Kumaqai *et al*[18]. A compound PC-766 B isolated from marine actinomycetes *Nocardia brasiliensis* exhibited dose dependent antioxidant activity. Takamatsu *et al.*[19] reported that Cymopo and avrainvilleol, a meromonoterpene compound isolated from marine sponges, exhibited antioxidant activity with the IC_{50} value of 4.0-6.1 μmol/L.

In this study, spectrum value gives the reductive capability of purified compound compared with ascorbic acid. A linear increase in reducing power with increasing concentration of active compound was observed, and the reducing power of the active compound was weaker than that of ascorbic acid. The purified compound was capable of scavenging H_2O_2 in an amount dependent manner. The scavenging activity of purified compound with standard ascorbic acid [(25-100) μg/mL] shows purified compound (100 μg/mL) exhibits a IC_{50} of scavenging activity of 23.73 μg/mL. The prior study also reported that sponge extract superoxide scavenging inhibition and H_2O_2 induced DNA strand scission protection was observed with the compound strobilin-felixinin, sesterterpene isolated from marine sponges[10].

Other examples of recently reported antioxidant activities of *in vitro* cultured, polyphenol rich plants include: *Curcuma longa* mass propagation on a thin film rocker system where significant and clone dependent DPPH scavenging were noted[20]; *Cistanche deserticola* cell suspensions producing antioxidant phenylethanoid glycosides were also assayed by a DPPH test[21]; *Stevia rebaudiana*, the herbal sweetener, callus extracted with water and methanol had higher antioxidant activity than the leaves of field grown plants, which correlated with the higher levels of flavonoids and total polyphenols[22].

The present study observed total phenolic content of the purified compound obtained by using Folin-ciocalteu reagent and the results are expressed as ascorbic acid. The phenol content of purified compound (100 μg/mL) was observed. A linear increase in free radical scavenging ability of purified compound was observed with increasing concentration I(%) value 15.025 mg/mL. DPPH radical scavenging by antioxidants has been attributed to their hydrogen donating ability of -OH and -CH3 groups. The activity of the compounds corresponds to the number of hydrogen atoms available for donation by hydroxyl groups[23]. The DPPH method is based on the reduction of DPPH, a stable free radical. With the odd electron, the free radical DPPH gives a maximum absorption at 517 nm by visible spectroscopy (purple color). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, *e.g.*, a free radical scavenging antioxidant, the absorption strength is decreased, and the resulting de-colorization (yellow color) is stoichiometric with respect to the number of electrons captured[24]. This reaction has been widely used to investigate the free radical scavenging ability of pure compounds or act as hydrogen donors.

The present result suggested that the compound "Pyrrol" and the structure Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- isolated from sponge associated *Bacillus* species could be beneficial to help reduce oxidative damages in cell induced by oxygen radicals, and effectively used as potential antioxidants. The antioxidant activity of *Bacillus* species compound may be attributed to connecting radicals and the radical chain reaction. The antioxidant mechanisms of this purified compound are complex and required further investigation.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

There is growing interest in analyzing compounds from natural sources with potential antioxidant and anti-inflammatory properties. The present study aimed to investigate the antioxidant capabilities of a purified and bioactive compound isolated from the bacteria *Bacillus* sp. associated to a sponge.

Research frontiers

The present research work evaluates the antioxidant activity of Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- extracted from the bacteria *Bacillus* sp. associated to the sponge *Dysidea fragilis*. These antioxidant capabilities were assessed by estimating different biochemical parameters such as total antioxidant activity, radical and hydrogen peroxide scavenging and total reducing power.

Related reports

Many natural compounds obtained from aquatic organisms

have demonstrated an antioxidant capability that could be useful against stressful situations. Between these compounds it can be found terpenes or polyphenols.

Innovations and breakthroughs

The present results evidenced that Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro- isolated from an sponge associated *Bacillus* sp. has *in vitro* antioxidant properties that could be beneficial to help reduce oxidative damages in cells.

Applications

This scientific study suggests the potential role of this compound as an adjuvant in therapies against several diseases in which oxidative stress could be implicated. However, the antioxidant mechanisms of this purified compound are complex and required further investigation.

Peer review

The present manuscript is interesting and shows the potential antioxidant effects of a new compound isolated from a *Bacillus* sp.

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