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RESEARCH ARTICLE

Marine *Streptomyces* Sp. VITMK1 Derived Pyrrolo [1, 2-A] Pyrazine-1, 4-Dione, Hexahydro-3-(2-Methylpropyl) and Its Free Radical Scavenging Activity

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Abstract:

Background:

Free radical generation has been proved to be responsible for various cellular diseases. It is necessary to combat free radicals using antioxidants derived from natural sources.

Objective:

The objective of this study is to evaluate the antioxidant activity of the diketopiperazine compound extracted from *Streptomyces* sp. VITMK1 isolated from mangrove sediment soil collected from Pichavaram, Tamil Nadu, India.

Methods:

The antioxidant potential of pyrrolo [1, 2-A] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl) (diketopiperazine) extracted from *Streptomyces* sp. VITMK1 was studied using reducing power assay. The scavenging of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical and nitric oxide (NO) radical by the compound was also studied. The cytotoxic activity of the compound on RAW 264.7 macrophage cell line was studied using MTT cell viability assay.

Results:

The compound exhibited strong DPPH radical scavenging activity (72.48±0.32% at 500 µg/mL) and NO radical scavenging activity (73.03±1.02% at 500 µg/mL). MTT cell viability assay revealed that the compound exhibited concentration-dependent cell viability and was observed to be 92% at 125 µg/mL concentration.

Conclusion:

The antioxidant activity of the diketopiperazine compound extracted from *Streptomyces* sp. VITMK1 can be probed further to establish its radical scavenging activity.

Keywords: DPPH radical scavenging assay, Nitric oxide scavenging activity, Pyrrolo [1, 2-A] pyrazine-1, 4-dione, Hexahydro-3-(2-methylpropyl)–, Reducing power assay.

1. INTRODUCTION

Reactive oxygen species (ROS) are a group of free radicals that are derived from oxygen. ROS are produced as a result of cellular metabolism and excessive accumulation of ROS leads to oxidative stress which plays an important role in the pathogenesis of various diseases like cardiovascular diseases, inflammatory diseases, atherosclerosis, cancer [1]

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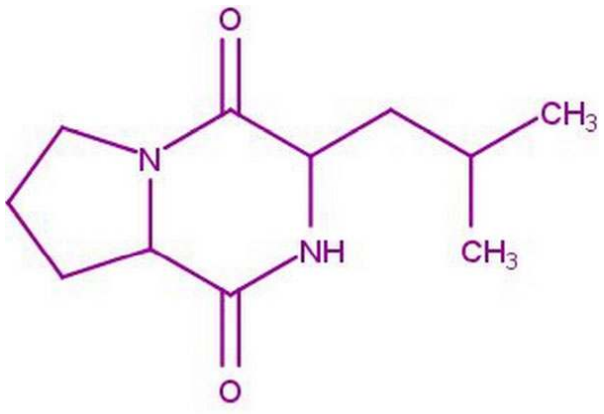
and in many pathological progression in the central nervous system [2]. Mitochondrial electron transport chain, enzymatic reactions, respiratory burst during phagocytosis and xenobiotic metabolisms (redox cycling) are endogenous sources of free radicals. Exogenous sources include ionizing radiation, UV radiation, ultrasound and chemicals *etc*. Antioxidants prevent oxidation by donating electrons and thereby combat free radicals and neutralize them. Antioxidants provide protection against various infections and degenerative diseases [3]. Human cells have a natural mechanism aided by enzymes superoxide dismutase, catalase and glutathione peroxidase to neutralize the free radicals thereby protecting the cells [4]. Micronutrients from food such as vitamins A, E and β-carotene, selenium and zinc and proteins albumin, ceruloplasmin and transferrin can provide additional protection of cells against cellular damage [5]. Due to the changing life style and growing population, there is a need to use synthetic antioxidants. Butylated hydroxyanisole (BHA), butylated hydroxyltoluene (BHT) and propyl gallate (PG) have been used to control the oxidation process. Their use must be strictly regulated since they cause deleterious effects to human health [6]. This leads to search for novel antioxidant compounds from natural sources. A lot of research is done on natural antioxidants from plant and microbial sources which serve as safe therapeutics [7]. Appreciable quantity of seaweed are consumed on a daily basis globally through food and the seaweed components such as sulfated polysaccharides, phenolic compounds (phlorotannins and bromophenols), and fucoxanthins are reported to have antioxidant properties [8]. Microorganisms live in varied environmental conditions and thereby are adapted to them by evolutionary process. They would have developed specific defense mechanism to combat oxidative stress in their extreme conditions [9]. They could serve as the natural source for novel antioxidants. Among microorganisms, actinomycetes along with marine bacteria have the ability to produce secondary metabolites with diverse chemical structures. Actinomycetes are a group of gram positive unicellular bacteria that have a high G+C content. They are known for their ability to produce secondary metabolites with a broad spectrum of biological activity such as antimicrobial, antitumor, immunosuppressive, antioxidant, enzyme inhibitory and others [10]. Each actinomycetes strain has the potential to produce 10-20 secondary metabolites [11]. Among actinomycetes, *Streptomyces* have been reported to contribute 70% of the secondary metabolites described under actinomycetes [12]. The response of the antioxidant system of *Streptomyces* growth under oxidative condition is now studied for their antioxidant potential [13]. This study reports the antioxidant activity of a diketopiperazine compound, Pyrrolo [1, 2-A] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)- extracted from *Streptomyces* sp. VITMK1 isolated from mangrove soil sediment collected from Pichavaram, Tamil Nadu, India.

2. MATERIALS AND METHODS

2.1. Isolation of Streptomyces sp. VITMK1 and Extraction of a Diketopiperazine Compound

The strain *Streptomyces* sp. VITMK1 was isolated from mangrove soil sediment collected from Pichavaram, Tamil Nadu, India. The isolate was subjected to submerged fermentation in tryptone yeast extract medium for 7 days followed by extraction using ethyl acetate (EA). The EA extract was purified using column chromatography resulting in a diketopiperazine compound, Pyrrolo [1, 2-A] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)- [14]. The chemical structure of the compound is given in Fig. (**1**). The compound was studied for its antioxidant activity.



Pyrrolo [1,2-A] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)

Fig. (1). Chemical structure of pyrrolo [1, 2-A] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl).

2.2. Reducing Power Assay

The reducing power assay was carried out according to the method described by Oyaizu with minor modifications [15]. 2 ml of the compound at various concentrations (62.5-500 µg/ml) was mixed with 2.5 ml of 200 mM/l of sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 minutes. 2.5 ml of 10% trichloroacetic acid (w/v) was added and the mixture was centrifuged at 650 rpm for 10 minutes. The upper layer was separated and equal volume of deionised water was added with 1 ml of 0.1% ferric chloride and the absorbance was measured at 700 nm. Ascorbic acid was used as a standard.

2.3. Nitric Oxide (NO) Scavenging Assay

The NO scavenging activity was determined according to the method described by Marcocci [16]. 1 ml of the compound at various concentrations (62.5-500 µg/ml) was mixed with 1 ml of 5 mM sodium nitroprusside and the mixture was incubated at 25°C for 3 hours. After incubation, 50 µl of griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% napthylene diaminedihydrochloride) was added and the absorbance was measured at 540 nm. The NO scavenging activity was calculated by the following equation:

$$NO\ scavenging\ activity\ (\%) = \left[\frac{A_c - A_t}{A_c}\right] X100$$

where A_c is the absorbance of the control and A_t is the absorbance of the test sample. Ascorbic acid was used as a standard.

2.4. DPPH Free Radical Scavenging Assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a purple colored crystalline powder composed of stable free-radical molecules. The ability to scavenge the free radical (DPPH) was done according to the method described by Hatano [17]. 2 ml of the compound at various concentrations (62.5-500 µg/ml) was mixed with 2 ml of DPPH (0.002% w/v in methanol) and was incubated for 20 minutes in the dark. After incubation, the absorbance was measured at 517 nm. The DPPH scavenging activity was calculated using the same equation that was used to calculate NO scavenging activity. Ascorbic acid was used as a standard.

2.5. MTT Cell Viability Assay

The cytotoxic effect of Pyrrolo [1, 2-A] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)- was assessed on RAW macrophages 264.7 cell line as described by Mosmann [18]. RAW cells (5×10^5 per well) were seeded onto a 96-well plate and was incubated at 37°C 5% CO_2 incubator for 24 hours. Following incubation, the cells were treated with various concentration of the compound (15.625 -250 µg/ml) and incubated for 24 hours. Then, 20 µl of MTT (5mg/ml PBS) was added to each well and incubated for 4 hours. The medium was removed and the cells were dissolved in DMSO. The plate was covered in tinfoil and was agitated in a plate rocker for 15 minutes. Optical density at 590 nm was measured using a microplate reader. The percentage of viable cells at each concentration was calculated by the following equation:

$$Percentage\ of\ viable\ cells = \frac{Optical\ density\ of\ treated\ cells}{Optical\ density\ of\ untreated\ cells} X100$$

2.6. Statistical Analysis

All experiments were carried out in triplicates. The data represented in the figures are mean ± Standard Error (SE) calculated using Microsoft Excel 2007.

3. RESULTS

3.1. Reducing Power Assay

Reducing power is an indication of the compound's antioxidant potential. The potassium ferricyanide gets reduced to potassium ferrocyanide which then reacts with ferric chloride to form a ferrous ferric complex. The complex had absorption maxima at 700 nm. Increased concentration resulted in increased absorbance values as shown in Fig. (**2**). This indicated the reducing potential of Pyrrolo [1, 2-A] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)-.

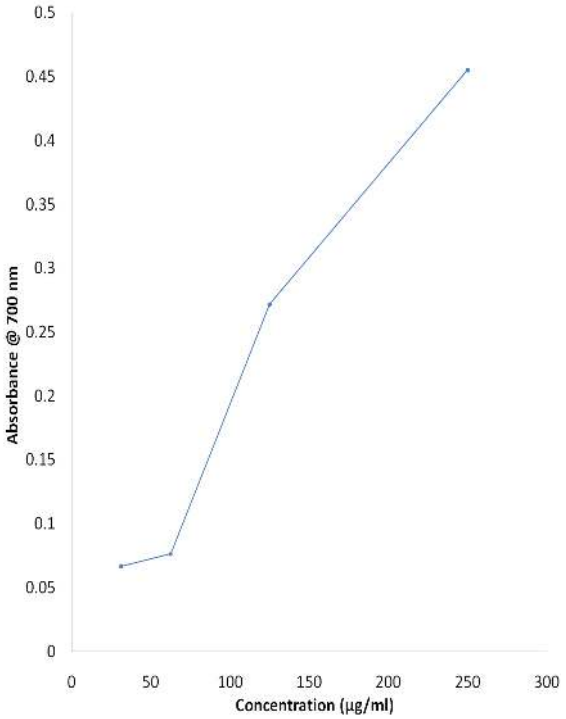


Fig. (2). Reducing power assay of the diketopiperazine compound extracted from *Streptomyces* sp. VITMK1.

3.2. Nitric Oxide (NO) Scavenging Assay

Sodium nitroprusside in aqueous solution at biological pH spontaneously generate nitrite oxide which reacts with oxygen forming nitrite ions. Nitrite ions in the presence of griess reagent will have absorption maxima at 550 nm. As can be seen from Fig. (**3**), the compound at increasing concentration showed higher NO scavenging activity as follows: 12.90±1.58% at 31.25 µg/mL, 12.22±0.77% at 62.5 µg/mL, 41.72±0.97% at 125 µg/mL. 57.48±0.74% at 250 µg/mL and 73.03±1.02% at 500 µg/mL.

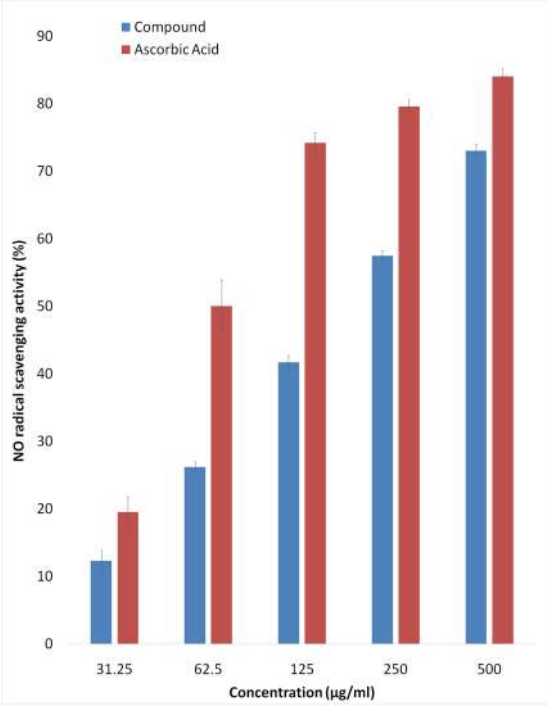


Fig. (3). Nitric Oxide radical scavenging activity of the diketopiperazine compound extracted from *Streptomyces* sp. VITMK1.

3.3. DPPH Free Radical Scavenging Assay

After the assay, colour change from purple to yellow was observed. The DPPH free radical has absorption maxima at 517 nm. The decrease in the absorbance corresponds to the percentage of DPPH free radical scavenging capacity. As can be seen from Fig. (**4**) the compound at increasing concentration showed higher DPPH free radical scavenging activity as follows: 10.40±2.79% at 31.25 µg/mL, 16.76±2.75% at 62.5 µg/mL, 37.85±1.28% at 125 µg/mL. 62.27±1.58% at 250 µg/mL and 72.48±0.32% at 500 µg/mL.

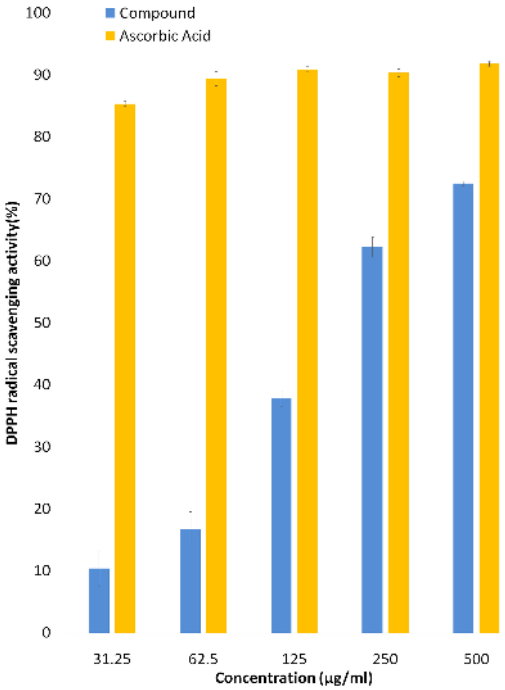


Fig. (4). DPPH radical scavenging activity of the diketopiperazine compound extracted from *Streptomyces* sp. VITMK1.

3.4. MTT Cell Viability Assay

It was observed that at 31.25 µg/mL, the compound did not affect the cells and cell viability of 94.31±0.03% and 91.95±0.06% were registered at concentration of 62.5 µg/mL and at 125 µg/mL. The compound exhibited concentration dependent cell viability on RAW macrophages 264.7 cell line.

4. DISCUSSION

It is a widely known fact that the free radicals have a critical role in various diseases these days [19, 20]. ROS and reactive nitrogen species (RNS) have been reported to cause DNA damage [21]. It necessitated the investigation of potential antioxidant compounds from natural sources. A wide spectrum of sources is available for natural antioxidants, among which actinomycetes occupy a key position. There have been earlier reports of antioxidant activity reported from actinomycetes. Kim reported the isolation and identification of actinomycetes from marine source and the antioxidative effects of its culture supernatant. Initially the DPPH scavenging activity of the isolate was 60%. When the conditions were optimized the DPPH radical scavenging activity increased to 88% [22]. This suggests that the optimization of conditions to obtain optimal cell growth and antioxidant activity would improve the antioxidant activity of the actinomycete isolate and the compounds that they produce. Intracellular and extracellular extracts of *Streptomyces* species VITTK3 were assessed for antioxidant activity and it was found that the extracellular extract showed 96% and the intracellular extract showed 22% at a concentration of 5 mg/mL [23]. The DPPH radical scavenging activity of our study was 72.48±0.32% at 500 µg/mL. The concentration with which we worked was ten times less that the concentration used by Thenmozhi *et al*. Diketopiperazines isolated from other sources have been evaluated for their antioxidant potential. A diketopiperazine isolated from the bacterium *Pseudoalteromonas haloplanktis* TAC125 was evaluated for its antioxidant activity by DPPH free-radical scavenging assay. It showed 72% activity at 10 mmol and 51% activity at 1 mmol when compared to hydroquinone which had 100% activity [24]. The potential antioxidant activity of three diketopiperazine compounds cyclo(prolyl-valyl), cyclo(prolyl-leucyl) and cyclo (prolyl-ioslleucyl)

isolated from the mushroom *Sarcodon aspratus* showed DPPH free radical scavenging activity. The EC50 of the three compounds were reported to be 0.15, 0.17, 0.18 mM respectively [25]. The reducing capacity of a compound serves as a significant indicator of its potential antioxidant activity. In order to study the reducing ability of the compound, we studied the transformation of Fe^{3+} to Fe^{2+} in the presence of the extracted compound. The ferrous ferric complex formed was read at 700 nm for absorption using a UV Spectrophotometer. The results observed from the reducing power assay showed that the diketopiperazine compound is a strong antioxidant. It is essential to make use of a free radical scavenging assay to evaluate the antioxidant activity of the compound. The DPPH assay is one of the most common and relatively quick methods used for testing radical scavenging activity of bioactive metabolites [26]. Griess assay used to determine the level of over produced cellular nitric oxide induced by cytokines and endotoxin was the choice to evaluate the nitric oxide radical scavenging assay [27]. The compound was observed to exhibit strong DPPH free radical scavenging activity and NO radical scavenging activity. In all the assays ascorbic acid was used as the standard and it can be seen that the compound exhibits similar antioxidant activity at higher concentrations. The MTT assay results on the RAW 264.7 macrophage cell line suggest that the compound pyrrolo [1, 2-A] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)- was nontoxic to RAW macrophages 264.7 cell line.

CONCLUSION

The diketopiperazine compound pyrrolo [1, 2-A] pyrazine-1, 4-dione, hexahydro-3-(2-methylpropyl)– extracted from *Streptomyces* sp. VITMK1 is a nontoxic potential antioxidant agent capable of scavenging free radicals. The study also emphasizes that actinomycetes are an inexhaustible source of secondary metabolites with varied biological activity.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

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