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Research Article

MARINE ACTINOBACTERIA OF THE CORAL REEF ENVIRONMENT OF THE GULF OF MANNAR BIOSPHERE RESERVE, INDIA: A SEARCH FOR ANTIOXIDANT PROPERTY

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ABSTRACT

Actinobacteria isolated from the sediment samples of the coral reef environment of the Kurusadai Island of the Gulf of Mannar Biosphere Reserve, India registered a density of 0.2×10^2 CFU/g and they were screened for antioxidant property. *In vitro* antioxidant activity (Total Antioxidant Activity, Total Reducing Power, and Scavenging of Hydrogen peroxide and Nitric Oxide Radical Scavenging Activity of ethyl acetate extracts) of seven isolates (KRCR1 to KRCR7) was studied. Extract of the actinobacterium, KRCR1 showed maximum of Total Antioxidant Activity (0.599), Total Reducing Power (0.15), and Scavenging of Hydrogen peroxide (80.7) and Nitric Oxide Radical Scavenging Activity (88.5). This strain was identified as *Mycobacterium* sp. based on chemotaxanomical, micromorphological and molecular features.

Keywords: Coral reefs, Actinobacteria, Antioxidant activity, *Mycobacterium* sp.

INTRODUCTION

A free radical is a molecule with one or more unpaired electrons in the outer orbit. Many of these free radicals occur in the form of reactive oxygen and nitrogen species, due to the oxidative stress brought about by the imbalance of the bodily antioxidant defense system and free-radical formation. Reactive oxygen species such as superoxide radical, hydroxyl radical, peroxyl radical and nitric oxide radical attack biological molecules such as lipids, proteins, enzymes, DNA and RNA, leading to cell or tissue injury associated with aging, atherosclerosis carcinogenesis and may even lead to the development of chronic diseases in the cardio and cerebrovascular systems. Since oxidative stress caused by reactive oxygen species plays an important role in the development of various diseases, such as the Alzheimer and Parkinson diseases, screening of new microbial metabolites that have antioxidant activities has been the major area of focus for many researches who aim at developing new drugs (1,2).

Marine actinobacteria are potential providers of novel bioactive metabolites and have been currently emerging as an important source for natural products with unique chemical diversity. Members of the class actinobacteria especially *Streptomyces* sp. have long been recognized as prolific sources of useful bioactive metabolites, providing with more than 85% of naturally occurring antibiotics discovered to-date and continuing as a rich source of new bioactive metabolites (3). The actinobacteria play a very important role among the marine bacterial communities, because of their diversity and ability to produce novel chemical compounds of high commercial value (4, 5).

Recent studies are focusing attention on the antioxidant systems of bacteria, which is important in terms of biotechnology. The compounds isolated from marine *Streptomyces*, 2-allyoxyphenol and streptopyyrolidine have been reported to possess antioxidants and no cytotoxic activity (6, 7). To get insight into the role of the antioxidant response against hydrogen peroxide and paraquat stresses, superoxide dismutase and catalase activities and lipid peroxidation levels in relation to incubation time have been studied in *Streptomyces* sp. M3004, isolated from the Turkish soil (8). However studies on marine actinobacteria with respect to antioxidant and cytotoxic activities are very limited in the Indian sub-continent and most of the actinobacteria isolated are yet to be screened for antioxidant compounds. Hence, the present study to investigate the antioxidant properties of the marine actinobacteria isolated from the coral reef environment of the Kurusadai Island, Gulf of Manner Biosphere Reserve, India.

MATERIALS AND METHODS

Sample collection and pretreatment

Sediments from the coral reef environment of the Kurusadai Island of the Gulf of Mannar Biosphere Reserve, south east coast of India ($9^0$14'51.33N;$79^0$13'19.34"E) were collected with a corer. The collected samples were transferred to a sterile polythene bag and taken immediately to the laboratory. The sediment samples were aseptically air-dried in a laminar air flow and pretreated by incubating them at 55ºC in a hot air oven for ten minutes.

Isolation of actinobacteria

After the pretreatment, 10-fold serial dilution of the sediment sample was prepared, using filtered and sterilized 50% sea water. Serially diluted samples were plated in the Kuster's agar medium in triplicate. To minimize the bacterial and fungal contaminations, all the plates were supplemented with Nystatin (20 mg/ml) and Cycloheximide (100 mg/ml) respectively (9). The actinobacterial colonies were counted from 14th day onwards up to 28 days and the colonies were picked up and grown separately by streaking in Yeast extract, Malt extract Dextrose agar. Subcultures ensured for their axenicity and were maintained in slants.

Fermentation and extraction of secondary metabolites

Well grown slant culture of the strains was inoculated into 50 ml of nutrient broth in 250 ml Erlenmeyer flasks and incubated for 5 days in a rotary shaker (200 rpm) at 35°C. The inocula (10%) were transferred into 100ml of Yeast extract, Malt extract, and Dextrose broth in 250ml Erlenmeyer flasks. The inoculated culture broth was incubated for 5 days on a rotary shaker (200 rpm) at 35°C. After fermentation, the broth was centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was separated. The supernatant was extracted twice with equal volume of ethyl acetate and was concentrated under reduced pressure. The dried extract was dissolved in phosphate buffer (pH 6.6) and stored at 4 ºC until use.

Antioxidant Activity of Actinobacterial Extract

Total antioxidant activity

Total antioxidant activity of the actinobacterial crude extracts was determined according to the method of Prieto *et al.,* (10). Briefly, 0.3 ml of sample was mixed with 3.0 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Reaction mixture was incubated at 95ºC for 90 min in a water bath. Absorbance of all the sample mixtures was measured at 695 nm. Ascorbic acid (100 µg/ml) was used as positive control.

Total reducing power

Total reducing capacity of the actinobacterial extracts was determined according to the method of Oyaizu et al., (11). Actinobacterial extracts (100 µg/ml) in phosphate buffer (0.2 M, pH 6.6) were mixed with 1% potassium ferricyanide and the mixture was incubated at 50ºC for 20 min; 2.5 ml of 10% tri chloro acetic acid was added to the mixture and centrifuged at 5000 rpm 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% ferric chloride and the colour developed was measured at 700 nm. Ascorbic acid (100 µg/ml) was used as positive control.

Scavenging of hydrogen peroxide

Ability of the actinobacterial extracts to scavenge hydrogen peroxide was determined according to the method of Ruch et al., (12) with the slight modification of Green et al., (13): 40 mM hydrogen peroxide was prepared in phosphate buffer (7.4) and the hydrogen peroxide concentration was determined spectrophotometrically by measuring the absorption with the extinction co-efficient for hydrogen peroxide of $81M^{-1}cm^{-1}$. Extract (100 µg/ml, positive control) was added to 0.6 ml of 40 mM hydrogen peroxide solution and the absorbance was determined at 230 nm after 10 min incubation against a blank solution containing phosphate buffer without hydrogen peroxide. Percentage of scavenging of hydrogen peroxide was calculated as follows:

$$\text{Percentage of Inhibtion (I \%)} = (A_{\text{bland}} - A_{\text{sample}}) / A_{\text{blank}} \times 100$$

Nitiric Oxide radical scavenging activity

Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH interacts with oxygen to produce nitrite ions, which were measured by the Griess reaction (12). In this experiment, 3 ml of the reaction mixture containing 10 mM sodium nitroprusside and the actinobacterial extracts (100 µl /ml) in phosphate buffer were incubated at 25ºC 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 and the solution mixed. The mixture was allowed to stand for 30 min at 25ºC. A pink coloured chromophore developed was read at 540 nm against the corresponding blank solution. Ascorbic acid (100 µg/ml) was used as positive control. The Nitric oxide scavenging activity of the actinobacterial extracts is reported as % inhibition, and was calculated as in scavenging of hydrogen peroxide.

Identification of actinobacterial strain

The potential antioxidant strain was characterized chemotaxanomically (13) and morphologically. General morphology was determined on ISP2 agar and cultural characteristics were determined Cook and Meyers, (14). Spore morphology was determined by direct microscopic examination using the 15 day old cultures by observing under a light microscope and the acid fast stained culture was also observed under the light microscope. Whole cell sugar and cell wall amino acids were studied using thin layer chromatography.

16S rDNA sequence analysis

Genomic DNA was extracted from cultures grown on ISP 2 using the method of Sivakumar et al., (15).Each 50 µl of amplification reaction contained 1 µl template DNA (50–200 ng), 5 µl 10x PCR buffer with $MgCl_2$, 1 µl each PCR primer (20 mM) (27F, 1492R), 1 µl dNTP mix (10 mM), 2.5 U Taq DNA polymerase, 5 µl Dimethyl sulfoxide and 35 µl sterile MilliQ water. The reaction conditions were initial denaturation at 95 °C for 5min, followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 90 seconds. A final extension was performed at 72°C for 10min. Reaction products were electrophoresed on a 1% agarose gel and checked with ethidium bromide under UV light. The polymerase chain reaction products were purified and sequenced directly using a Taq Dye Deoxy Terminator Cycle Sequencing Kit and an ABI Prism 3730 automated DNA sequencer (Applied Biosystems). Both strands were sequenced as a cross-check by using forward and reverse primers Karuppiah et al., (16).

The 16S rDNA sequence of the test strain was aligned manually with available nucleotide sequences retrieved from the Ribosomal Database Project and European molecular biology laboratory/GenBank databases. An evolutionary tree was inferred by using tree-making algorithm, namely the neighbour joining algorithm. Evolutionary distance matrices were generated using the MEGA version 4.1 package. A bootstrap analysis of 1000 replicates was carried out. The root position of the tree based on the neighbour-joining method was estimated using B*acillus subtilis* as an out group (HM 105586).

RESULTS

Population density of actinobacteria

During the present investigation, the actinobacterial colonies were enumerated from the sediment samples of the Kurusadai island coral reef environment. The population density of the actinobacteria in Kuster's medium was 0.2 x 10^2 CFU/g and a total of 7 distinct morphological strains (KRCR1, KRCR2, KRCR3, KRCR4, KRCR5, KRCR6 and KRCR7) were obtained and used for antioxidant studies.

Total antioxidant activity

Fig.1 shows the total antioxidant activity of the actinobacterial extracts and the standard ascorbic acid (100 µg/ml) at 695 nm. Among the seven extracts, KRCR1 (100 µg/ml) showed grater activity (0.599) compared to the other strains, with the ascorbic acid equivalent between 100 µg/ml.

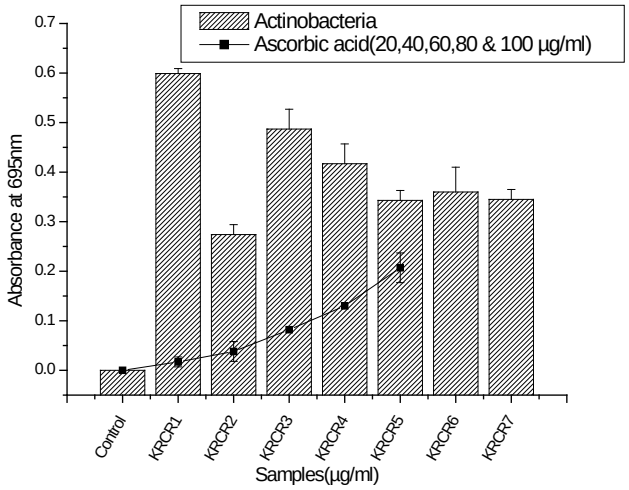

Fig. 1: Comparison of total antioxidant activity of actinobacterial extract (100µg/ml) with standard ascorbic acid (20-100µg/ml).

Total reducing power

Fig.2 shows the reducing capacity of the actinobacterial extracts and the standard ascorbic acid (100 µg/ml) at 700 nm. Among the seven extracts, KRCR1 (100 µg/ml) showed a strong activity of 0.151 absorbance, equivalent to the standard ascorbic acid, whereas others showed weak activity. Since the total reducing power indicates the potential antioxidant ability, KRCR1 can be considered as a potent source of antioxidant.

Scavenging of hydrogen peroxide

Fig. 3 indicates the percentage scavenging activity of the actinobacterial extracts (KRCR1, KRCR2, KRCR3, KRCR4, KRCR5, KRCR6 and KRCR7) with the standard ascorbic acid (100 µg/ml). Among them, KRCR1 (100 µg/ml) exhibited a maximum activity of 80.7% inhibition which was significantly higher than the standard L-ascorbic acid whose scavenging effect was only 61.3%.

Nitric Oxide Radical (NO) Scavenging Activity

Actinobacterial extracts decreased the amount of nitrite generated from the decomposition of sodium nitroprusside *in vitro* as depicted in Fig.4. The results showed that KRCR1 (100 µg/ml) had a scavenging activity of 88.5% of inhibition, which is significantly higher than that of the standard L-ascorbic acid (71.5%). Other extracts showed modest activity.

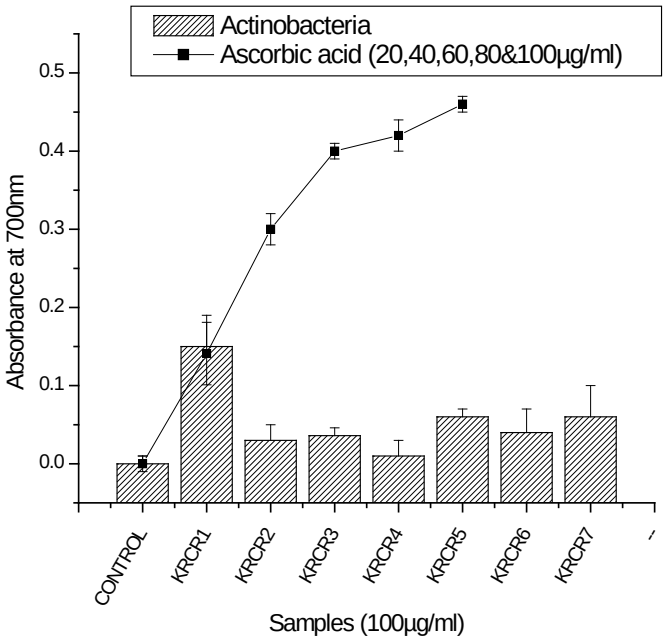

Fig. 2: Total reducing ability of actinobacterial extracts and standard ascorbic acid (20-100µg/ml).

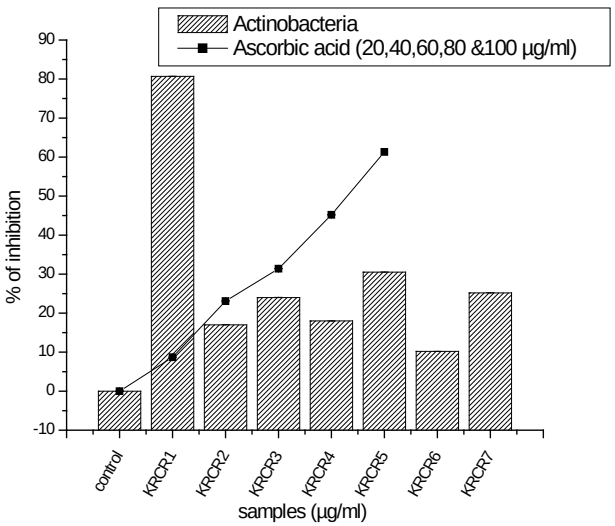

Fig. 3: Comparison of percentage scavenging of hydrogen peroxide radical by different actinobacterial extract (100µg/ml) with ascorbic acid (20-100µg/ml) as positive control.

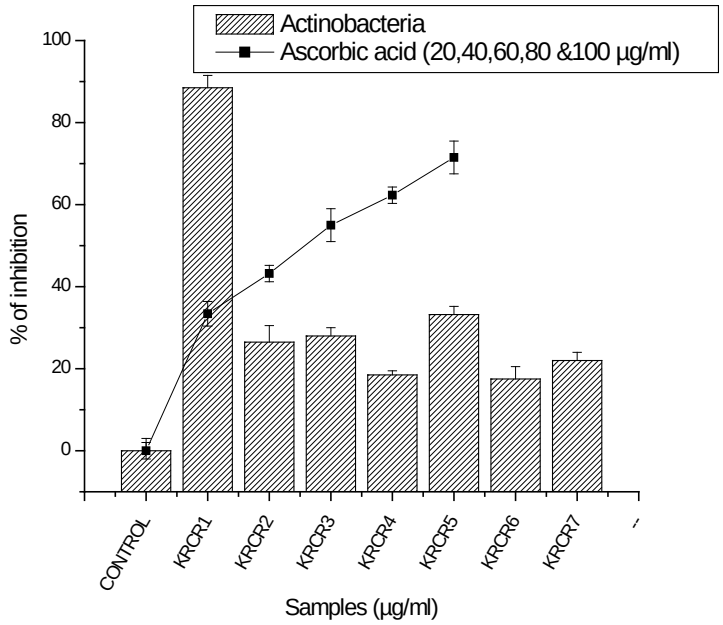

Fig. 4: Scavenging effect of actinobacterial extracts (100µg/ml) and standard ascorbic acid (20-100µg/ml) on Nitric Oxide.

Taxonomic investigation

As KRCR1 showed the potential antioxidant activity, it was identified based on the chemotaxonomical, micromorphological and molecular methods. Colony morphology of the KRCR1 was rough with a slight irregular margin and absence of aerial mycelium. The colony did not produce any pigments upon the prolonged incubation of two weeks in the ISP2, ISP7, nutrient and actinomycetes isolation agar. The cell wall of the strain possessed meso-DAP with galactose and arabinose as the whole cell aminoacids and sugars respectively, which indicates the cell wall type IV. The rod shaped cells observed under the light microscope, eliminated all the genera having the cell wall type IV with sugar pattern A, except *Mycobacterium.* The acid fast staining of the KRCR1 showed pink coloured cell aggregates with blue coloured background, suggesting that the KRCR1 belongs to the genus *Mycobacterium.*

16S rDNA sequence of the strain KRCR1 showed 1,452 bp (Gene Bank accession number HM105586). The phylogenetic tree was constructed for comparison with the isolate and 16S rDNA gene sequences of other 12 mycobacterial species with *Bacillus subtillis* as outside reference of operational taxanomic unit (Fig.5). Phylogenetic tree inferred that the strain KRCR1 belongs to the genus *Mycobacterium*. The level of similarity between the 16S rRNA gene of KRCR1 and *Mycobacterium microti* was 99.2 and its similarity level indicated that the KRCR1 is closely related to *M . microti.*

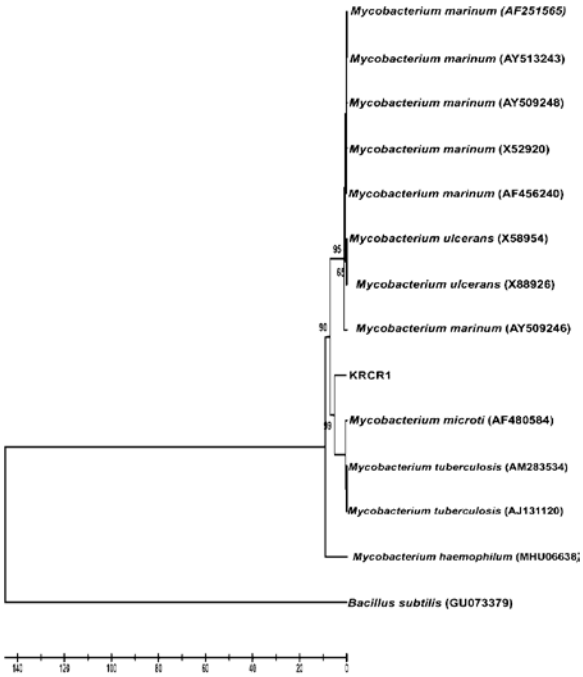

Fig. 5: Neighbour – joining tree based on 16Sr DNA sequences, showing relationship between the strain KRCR1 (1452 nucleotides) and 12 *Mycobacterium* species with *Bacillus subtilis* was incorporated as out group.

DISCUSSION

Isolation and enumeration of actinobacteria

Present study provides with the analysis of the actinobacterial communities in the sediments of the coral reef environment of the Kurusadai Island from the Gulf of Mannar Biosphere Reserve. The actinobacterial density was 0.2 x 10^2 CFU/g, which is less when compared to those of the mangroves (15), and seagrass environment Rajkumar (17). Though lesser in density, they can be pursued for their bioactive compounds, as opined by Ramesh and Mathivanan (18, 19).

Total antioxidant activity

Total antioxidant activity is based on the reduction of MO (VI) to MO (V) by the actinobacterial extracts and the subsequent formation of a green phosphate /MO (V) complex at acidic pH. Present investigation has shown that the extract of the KRCR1 showed higher activity (0.599) followed by KRCR3 and KRCR7 compared to others. KRCR4 showed poor activity. Similarly, total antioxidant activity of the leafy vegetables was investigated by (20, 21) who found different levels of antioxidant properties for the different systems. Kumar and Tamura (20) have reported total antioxidant activity in the range of 39.62 to 9.65 mg ascorbic acid equivalent for 1 g extract of seaweed. Considering this, activity of KRCR1 extract is better.

Total reducing power

Reducing ability of the actinobacterial extract depends on the presence of reductons in the extract which exhibit antioxidative potential by breaking the free radical chain by donating a hydrogen atom. Fig.2 illustrates that the strain KRCR1 has higher absorbance when compared to the control. This property is associated with the presence of reductons in the extract which are reported to be terminators of free radical chain reaction Duh, (22). Same trend has also been reported by (23) in the alcoholic extract of the seaweeds. The reducing capacity of KRCR1 is the significant indicator of its potential antioxidant activity.

Scavenging of hydrogen peroxide

Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells because it may give rise to hydroxyl radical in the cells Halliwell and Gutteridge, (25). Thus, removing hydrogen peroxide as well as O_2 is very important for protection of food systems. In the present study, actinobacterial extracts of KRCR1 (80.7), KRCR5 (30.05) and KRCR7 (25.2) showed higher scavenging activities as compared to that of the standard ascorbic acid.

Nitric oxide scavenging

Nitric oxide radicals play an important role in inducing inflammatory response and their toxicity multiplies only when they react with O_2 radicals to form peroxynitrite and damage biomolecules like proteins, lipids and nucleic acids Moncada et al., (26). Nitric oxide is generated when sodium nitroprusside reacts with oxygen to form nitrite. Actinobacterial extracts inhibit nitrite formation by competing with O_2 to react with nitric oxide directly Pandimadevi et al., (24). In the present study, phosphate buffer extract of the strain KRCR1 at 100 µg/ml exhibited 88.5% inhibition which is higher when compared to the standard ascorbic acid (100 µl). This suggests that KRCR1 could be a potent and novel source for obtaining therapeutic agents for scavenging nitric oxide.

Actinobacterial identification

The potential strain, KRCR1 which showed higher antioxidant activity compared to the other strains was selected for identification based on the chemotaxonomical, molecular and micromorphological methods. Results have shown that the cell wall of the strain possesses meso DAP with galactose and arabinose as the whole cell sugars, indicating that it belongs to the cell wall type IV Ruch et al., (12). Rod shaped cells with the free lying endospores have clearly indicated that it belongs to the genus *Mycobacterium* Tragg et al., (27). Phylogenetic analysis of the 16S rDNA sequence of KRCR1 also indicated that it belongs to the genus *Mycobacterium* by forming a separate clade within the *Mycobacterium* and showing closest similarly with *M.microti.*

Novel antioxidant gene noxR1 obtained from *Mycobacterium* by (28) indicated that it is having antioxidant activity. Similarly, in the present study, higher activity of scavenging of nitric oxide radicals and hydrogen peroxide and total reducing power were observed in the actinobacterial strain KRCR1 (*Mycobacterium* sp.), indicating that it can be pursued in greater details for large scale production of commercially important antioxidants.

CONCLUSION

The coral reef environment of the Gulf of Mannar Biosphere Reserve is a potential area for isolating novel actinobacteria. Among the seven actinobacterial strains, KRCR1 showed higher antioxidant activity, helpful in preventing or slowing down the progress of various oxidative stress related disorders and bacterial contaminations. The latter has many industrial uses such as preservatives in food and cosmetics and for preventing the degradation of rubber and gasoline. There are only few reports including the present me on the antioxidant activity of microbes. Hence, there is vast scope for further research is underway to analyze and isolate actinobacterial active compounds, having for antioxidant activity.

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REFERENCE

1. Adelman R, Saul RL, Ames BN. Oxidative damage to DNA: relation to species metabolic rate and life span. Proc Natl Acad Sci 1989; 85: 2706 – 2708.
2. Amstad P, Cerutti D. Genetic modulation of the cellular antioxidant defence capacity. Environ. Health Perspect.1990; 88: 77–82.
3. Berdy J. Bioactive microbial metabolites. A personal view. J Antibiot 2005; 58: 1–26.
4. Amador ML, Jimeno J, Ares LP, Funes HC, Hidalgo M. Progress in the development and acquisition of anticancer agents from marine sources. Ann Oncol 2003; 14: 1607–1615.
5. Hopwood DA. Therapeutic treasures from the deep. Nature Chem Biol 2007; 3: 457–458.
6. Shin HJ, Kim TS, Lee HS, Park JY, Choi IK, Kwon HJ. Streptopyrrolidine, an angiogenesis inhibitor from a marinederived *Streptomyces* spp. KORDI-3973. Phytochemi 2008; 69: 2363–2366.
7. Arumugam M, Mitra A, Jaisankar P, Dasgupta S, Sen T, Gachhui R, Mukhopadhyay UK, Mukherjee J. Isolation of an unusual metabolite 2-allyloxyphenol from a marine actinobacterium, its biological activities and applications. Appl Microbiol Biotechnol 2010; 86: 109–117.
8. Isik K, Ayar H, Kayali, Sahin N, Zturk EO, Gundogdu, Tarhan. Antioxidant response of a novel Streptomyces sp. M3004 isolated from legume rhizosphere to H2O2 and paraquat Process Biochemi 2007; 42 : 235–243.
9. Kathiresan K, Balagurunathan R, Masilamaniselvam M. Fungicidal activity of marine actinomycetes against phytopathogenic fungi. Indian J Biotechnol 2005; 4: 271–276.
10. Prieto P, Pineda M, Aguilar M. Spectrophotomeric quantitation of antioxidant capacity through the formation of a phosphomolybdemun complex: Specific application to the determination of vitamin E. Anal Biochem 1999; 268: 337-341.
11. Oyaizu M. Studies on product of browning reaction prepared from glucose amine. Jap J Nut 1986; 44: 307-315.
12. Ruch RJ, Cheng SJ, Klauing JE. Prevention of cytotoxicity and inhibition of intracellular communication by antioxidant catechins isolated from Chinesh green tea. Carcinogen 1986; 10: 1003-1008.
13. Green LC, Wanger DA, Glogowski J. Analysis of nitrate, nitrite and (15N) Nitrate in biological fluids. Anal Biochem 1982; 126: 371-382.

14. Cook AE, Meyers PR. Rapid identification of filamentous actinomycetes to genus level using genus-specific 16S rRNA gene restriction fragement patterns. Int J Syst Evol microbial 2003; 53: 1907-1915.
15. Sivakumar K, Sahu MK, Kathiresan K. Isolation of actinomycetes from the mangrove environment of the southeast coast of India. Eco Env Cons 2005; 11: 355-357.
16. Karuppiah V, Aarthi C, Sivakumar, K. Enhancementof PCR amplification of actinobacterial 16S rRNA gene using an adjuvant, dimethyl sulphoxide. Curr Sci 2011; 101, 22-23.
17. Rajkumar J. Purification and characterization of commercially important enzymes from marine actinomycetes isolated from the rhizophore of sea grass of the Gulf of mannar biospeare reserve, India. Ph.D. Thesis, Annamalai University, India. 2009; 150 pp.
18. Ramesh S, Mathivanan N. Screening of marine actinomycetes isolated from the Bay of Bengal, India for actinomicrobial activity and industrial enzymes. World J Microbiol Biotechol 2009; 25: 2103-2111.
19. Karthik L, Kumar G, Rao KVB. Diversity of marine actinomycetes from Nicobar marine sediments and its antifungal activity. Int J Pharm Pharmace Sci 2010; 2(1): 199-203.
20. Nabasree D, Bratati. Antioxidant activity of some leafy vegetables of India: A comparative study. Food chem 2007; 101: 471-474.
21. Ghaffari H, Ghassam BJ, Prakash HS. Evaluation of antioxidant and anti-inflammatory activity of stachys lavankulifolia. Int J Pharm Pharmaceu Sci 2012; 4(3): 691-696.
22. Kumar S, Tamura K. Molecular evolutionary genetics analysis software. Bioinformatics 2007; 17: 1244-1255.
23. Duh PD. Antioxidant activity of burdock (*Arctium lappa Linne*): it's scavenging effect on free radical and active oxygen. J Am Oil Chem Soc 1998; 75: 269-277.
24. Pandimadevi K, Suganthi N, Kesika P, Karuthapandian S. Bioprotective properties of seaweeds: In vitro evaluation of antioxidant activity and antimicrobial activity against food borne bacteria in relation to polyphenolic content. BMC Compl Alt Med 2008; 8: 1-11.
25. Halliwell B, Gutteridge JM. Free radicals in biology and medicine. Oxford: oxford university press 1999; 82-83 pp.
26. Moncada S, Palmer RM, Higgs EA. Nitric oxide physiology, pathophysiology and pharmacology. Pharmocol Rev 1991; 43: 109-142.
27. Tragg AB, Driks A, Stragier P, Bitter W, Broussard G, Hatfull G, Chu F, Adams KN, Ramakrishnan L, Losick R. Do mycobacteria produce endospores?. PNAS 2001; 107: 878-881.
28. Ehrt BS, Shiloh MU, Ruan J, Choi M, Gunzburg S, Nathan C, Xie Q, Riley LW. A Novel Antioxidant Gene from *Mycobacterium tuberculosis*. J Exo Med 1997; 186: 1885-1896.