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Isolation and screening of bioactive compound from actinomycetes isolated from salt pan of Marakanam district of the state Tamil Nadu, India

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ABSTRACT

Natural products either the source of the inspiration for a significant proportion of the new small molecule chemical entities introduced as drugs. Actinomycetes are the largest antibiotic producing genus in the microbial world discovered so far. The number of antimicrobial compounds reported from the different species of actinomycetes per year has increased almost exponentially for about two decades. As marine environment are different from terrestrial, it is concluded that marine actinomycetes might produce various types of novel bioactive compounds and enormous antibiotics. The objectives of this study is to isolate actinomycetes from salt pans of Marakanam to optimize and screen the production of secondary metabolites from the selected actinomycetes and to check the antimicrobial activity of these metabolites against pathogens. Out of 30 actinomycetes isolates Ros11 and Ros25 were screened and characterized based on antimicrobial properties. The secondary metabolites were extracted from Ros 11 and Ros 25 by chemical solvents, ethyl acetate and acetone. Using well diffusion method, it was observed that secondary metabolites of Ros 25 formed maximum inhibition zones, 10mm, 12mm and 15mm against *Staphylococcus aureus* and fungal pathogens *Aspergillus niger* and *Aspergillus fumigates*, respectively. The Ros 25 isolate could be the better candidate for the production of antibacterial and antifungal compounds.

Introduction

A large number of microorganisms, including certain bacteria, fungi and algae, produce secondary metabolites, which may have some degree of bioactivity, either against another microorganism or acting against certain physiological states of a diseased body. Actinomycetes, a group of free-living saprophytic gram positive bacteria belonging to the family Actinomycetaceae, is filamentous and sporulating in nature. It is widely distributed in nature like different soil, water and plant rhizosphere. Actinomycetes are the prime sources of novel bioactive compounds like antibiotics, enzymes and important other bioactive compounds. A large number of well known antibiotics were isolated from the microbial world so far. Two thirds of natural antibiotics, an alternative source of biologically active compound are obtained from actinomycetes (Behal, 2003). Since the discovery of streptomycin from *Streptomyces gresius* in 1942 by Waksman (1961), till now 22,500 bioactive compounds were isolated form different microbial sources, of them 45% from actinomycetes.

The emergence and dissemination of microbial resistance is raising a serious problem worldwide. As a result, development of new antibiotics is being urgently needed in present situation to combat against harmful pathogens. Rapid increasing bacterial resistance against commonly practiced bioactive substances, it is becoming a very essential condition to search of novel, safe and effective antimicrobials for replacement of invalidated antimicrobials.

Now days, marine derived antibiotics showed more competent in fighting microbial infections, because the terrestrial bacteria have not developed any resistance against them (Donia and Hamman, 2003). As marine environmental conditions are extremely different from terrestrial ones, therefore marine actinomycetes might produce different types of bioactive compounds are chemically diverse. Discovery of those new classes of therapeutics bioactive compounds could provide the new drugs needed to sustain us for the next 100 years in our battle against drug resistant infectious diseases (Williams, 1983). In this study we aimed to isolate actinomycetes from salt pan habitat as sources of bioactive secondary metabolites are antagonist against microbial species.

Materials and methods

Sample collection and isolation of actinomycetes

The soil samples were collected from five different marine salt pans (in different depth ranging from earth surfaces to 1/3 meter depth) at Marakanam district (12.2°N, 79.95°E coordinates), Tamil Nadu. From each location, 20-30 g of

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sample was collected from five different locations in pre-labeled sterile plastic bags and tightly sealed to avoid external contamination. The collected soil samples were air-dried at aseptic condition and stored at 4^0C until examined (Elliah and Ramana, 2004). The samples were pretreated with $CaCO_3$ (10:1 w/w) and incubated at 37^0C for 4 days and subsequently, samples were subjected to serial dilution up to 10^{-5} dilution by adding 1 g of soil sample in 9 ml of sterile double distilled water. Aliquots of (0.1 ml) of 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} diluted samples were spread on actinomycete isolation agar media (Himedia, Mumbai, India) supplemented with antibiotics, cycloheximide (25µg/ml) and nalidixic acid (25µg/ml) to minimize the fungal and bacterial growth (Lakshmanaperumalsamy *et al*. 1986). The plates were then incubated at 28^0C for 10 days and based on the morphology the actinomycetes cultures were selected and purified.

Morphological characterization

The morphology is observed after the incubation of 3-4 days of growth on medium. The morphology of actinomycete strains was examined using cover slip culture technique (Holt *et al*. 2000). The actinomycetes strains were stained by gram staining procedure (Cappuccino and Sherman, 1996).

Aerial mass colour and reverse side pigments

After appearing of powdery growth, colonies were subcultured on starch casein agar (SCA: @g/l- starch 10; casein 0.3; KNO_3 2; NaCl 2; K_2HPO_4 2; $MgSO_4$ 7 H_2O 0.05; $CaCO_3$ 0.02; $FeSO_4$ 7H_2O 0.01 Agar 18; pH-7.2), different ISP media (ISP 2, Yeast extract malt extract agar; ISP 3, Oat meal agar; ISP 4, Inorganic salt starch agar; ISP 5, Glycerol asparagine agar base; ISP6 and ISP 7, Tyrosine agar base) and Kuster Agar(Shirling and Gottlieb, 1966). The reverse side pigments of the isolated colony was further tested using Peptone yeast extract iron agar (ISP 6) and recorded as distinctive (+) or not distinctive (-). Production of melanoid pigments was tested on ISP 1(Das *et al*., 2008). The colour of mature sporulating aerial mycelium reverse side pigment colour was recorded (Table 1).

Source and maintenance of test pathogenic microorganism

Fungal strains, *Aspergillus niger*, *A. fumigates* and bacterial strain *Staphylococcus aureus* were used as test organisms in this study. The test organisms were isolated previously and maintained in laboratory, Department of Microbiology, Vellore Institute of Technology and University, Vellore, Tamil Nadu.

Antimicrobial activity of the actinomycetes

The antimicrobial activity of the isolated actinomycetes was determined by cross streak method (Oskay, 2009). Actinomycetes were inoculated singly by a single streak in the centre of plates and incubated at 28^0C for 10 days. The test microorganisms, *Aspergillus niger* and *A. fumigates*, and *Staphylococcus aureus*. were streaked at 90^0 angles to the actinomycetes isolates are the plates were then incubated at 37^0C for 24 hrs. Antagonism was determined by the growth inhibition of the test organisms.

Physiological and biochemical characterization

The physiological and biological characterization was carried out of the screened actinomycetes isolates (Ros 11 and Ros 25) which performed best antagonism against test microorganism (Gordon 1966; 1967). The carbon utilization ability of the isolates were studied using various carbon sources (like glucose, mannitol, fructose, xylose, sucrose, raffinose, inositol and arabinose. Carbon utilization agar (ISP 9) supplemented with 1% carbon sources (Nonomura, 1974) was used to test carbon source. For the optimizing nitrogen source, different nitrogen sources were tested for growth of the isolates.

Optimization of medium, pH, temperature and salt concentration for screened actinomycetes

To optimizing the best growth media for the isolates, Ros 11 and Ros 25, isolates were inoculated on different media such as SCA, Tryptone medium, ISP(No-1,2) and Kusters agar. and incubate at 28°C for 7 days (Pandey, 2005). To optimize pH, isolates were grown in medium with different pH ranged from pH-2 to 7, and incubate at 28°C for 7 days. Different concentrations of sodium chloride (3-8%) were added to the medium to test salt tolerant capacity of the isolates and the plates were inoculated at 28°C for 7 days. However, to optimize forwarded temperature for the growth of the isolates Ros11 and Ros25 were incubated in various temperatures, 20, 25, 30, 35 and 37°C. The growth of the isolates was measured using Colorimetric analysis at 630nm.

Extraction of crude secondary metabolite

The secondary metabolites were extracted from the isolates Ros 11 and Ros 25 was extracted. The isolates were inoculated in 50ml ISP 2 broth medium and incubated in shaker incubator (200 rpm) at room temperature for 4 days. The primary culture was then again inoculated in 200ml ISP2 broth media and incubated in shaker incubator (200rpm) for 7 days.

Extra cellular**:** After 7 days, cells of both isolates Ros 11 and Ros 25 were pelletized by centrifuging at 4000rpm for 10min. Extra-cellular secondary metabolite was extracted from supernatant of the total culture using equal volume (1:1 ratio; v/v) of ethyl acetate as a solvent and shaken vigorously for 2 hrs. The organic layer was collected and crude extract was then concentrated by evaporating solvent in rotary vacuum.

Intra cellular: The mycelia of Ros 11 and Ros 25 were air dried, grinded in sterile mortar and pestle in fine powder using liquid N_2. Acetone was added with powder as a solvent and kept in shaker (150 rpm) for one hour at room temperature. Intra cellular extract was then filtered through Whatman No.1 filter paper. The extracted intra-cellular crude secondary metabolites was then concentrated using rotary vacuum.

Assay of antimicrobial activity

The antibacterial activity of crude secondary metabolites extracted with different solvents was tested against selected pathogens by agar diffusion assay. Muller Hinton Agar and SDA/PDA plates were used for bacteria (*Staphylococcus aureus*) and fungul (*Aspergillus niger* and *A. fumigates*) test organism respectively. Media was bored and 100µl of crude extract was loaded into well(Bauer *et al*. 1966). The plates were incubated at temperature for 24 hours for bacteria and at room temperature for 3days for fungi. The presence of a zone of inhibition surrounding the well was observed and recorded. The diameter of the inhibition complete zone was measured to the nearest whole millimeter. Each test was repeated three times and the antibacterial activity was measured as the mean of diameter of the inhibition zones (mm) produced by the secondary metabolites. The inhibition zone were evaluated as percentage of inhibitoy >70 % (+++, strong inhibition), 50-70 % (++, moderate inhibition) and <50% mm (+/-, weak inhibition).

Results

Isolation and screening of actinomycetes

Actinomycetes were isolated from salt pans of five different locations of Marakanam. Based colony morphology, size, shape and colour, a total number of 30 actinomycetes strains, designated as Ros 1-Ros 30 were isolated from the soil samples cultured in different media size with different shape and colours colonies were small to medium. Two isolates from ISP-5 media, three from each SCA and ISP-3 media, four from each

media ISP-4 and ISP-6, five from ISP-2 media and finally seven from Kuster Agar isolated. All isolates were tested for their antimicrobial activity against selected bacterial and fungal pathogen. Of them 33% actinimycetes showed no antimicrobial activity; whereas 67% showed antagonism on tested microbes. About 57% of isolated actinomycetes were found to be inhibited the growth of both fungus and bacteria, whereas, 7% actinomycetes inhibited the growth only of bacteria, and 3% only of fungus. Among are isolated actinomyctes, 57% showed very weak interaction against tested fungal pathogens, whereas 22% showed moderate and only 6% showed high antagonism activity. Similarly actinomycetes showed antibacterial activities were categorized. Only 10% isolates showed high antagonism against tested bacteria. Among all the isolates, only two isolates Ros 11 and Ros 25 showed considerable antimicrobial activity against both selected fungal and bacterial pathogens (Fig. 1).

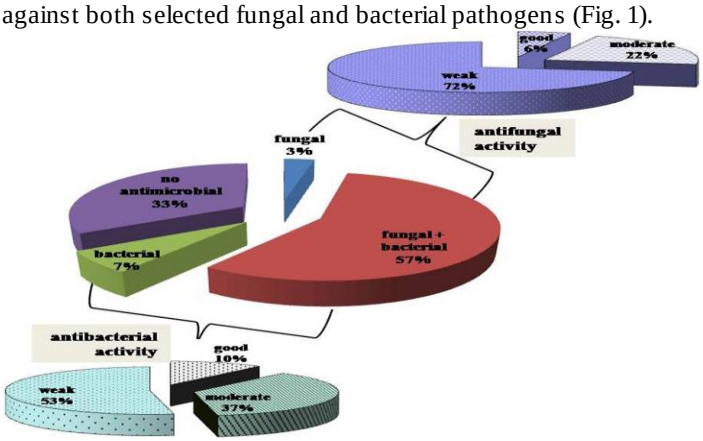

Fig. 1. Categorizations of the screened actinomycetes isolated from different salt pans of Marakanam district on the basis of antimicrobial activity against bacterial (*Staphylococcus aureus*) and fungal pathogens (*Aspergillus fumigates* and *Aspergillus niger*)

Phenotypic characterization

The isolates Ros 11 and Ros 25 were phenotypically characterized on the basis of spore morphology, mycelial colour, melanoid pigment and its solubility. Colour of aerial mycelia of both isolates was gray and melanoid pigment was present in both isolates. But only isolate Ros 25 was found to be produced soluble pigment. The spore surface of both isolates was examined. Smooth spore surface of Ros 11 and semi rough spore surface of Ros 25 was observed. Morphology of spore chains of both isolates were found to be rectiflexible in nature.

Utilization of carbon sources

Utilization of sole carbon sources was tested for isolates Ros 11 and Ros 25 using different carbon saccharides, arabinose, xylose, inositol, manitol, fructose, galactose, sucrose and raffinose. All carbon sources except manitol and fructose were found to be utilized by Ros 11, whereas Ros 25 utilized all carbon sources except arabinose (Table 2). Growth of isolates was examined on different media. Both isolates were showed excellent growth with profuse aerial mycelia on SCA, ISP-2 and Kusters agar. But growth inhibition of both the isolates was observed on Tryptone soya medium.

Biochemical tests

Different biochemical tests were carried out to characterize the biochemical properties of the isolates. The Gram staining test of isolates Ros 11 and Ros 25 revealed that both the isolates were Gram positive. To test of oxidase, nitrate reduction, indol, Simmon citrate and Voges Proskauer, (Holt.1989) for both isolates were found to be negative. But isolates were found to be positive for catalase, triple suger iron and citrate utilization test. To methyl red test, only Ros 25 was found to be positive (Table 3).

Optimization of medium, pH, temperature and salt concentration

The growing condition of Ros 11 and Ros 25 was optimized using SCA and Kusters agar media. The growth was studied in five different temperatures 20°C, 25°C, 30°C, 35°C and 37°C. Among the various temperatures, both the isolates showed maximum growth at 30°C(Fig. 2a).The pH of the media was also optimized by growing the isolates in media of different pH (pH 2-6). Among all the tested pH, pH-5 was optimized for the both isolates (Fig. 2b). The growth of the isolates evaluated in media with different salt concentration. 4% NaCl was optimized for maximum growth for the both isolates. The growth of the isolates was found to be declined either increase or decrease of the salt concentration of the media (Fig. 2c).

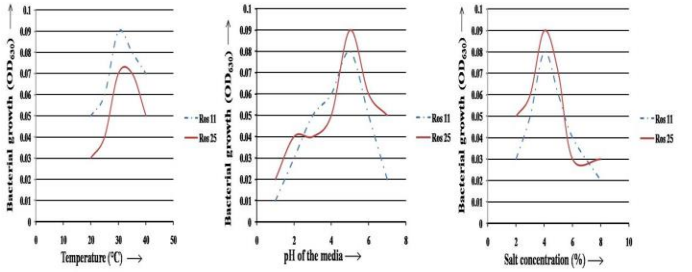

Fig. 2. Optimization of temperature, pH of media and salt concentration (NaCl) for growth of isolates, Ros 11 and Ros 25

Antimicrobial activity of extracted secondary metabolites

The antimicrobial property of secondary metabolites extracted from isolates Ros 11 and Ros 25 was tested by well diffusion method against bacterial pathogen, *S. aureus* and fungal pathogens, *A. fumigates* and *A. niger*. The isolates were grown at optimized growth condition at 30°C, pH 5 with 4% NaCl. After 7 days of growth in SCA media, secondary metabolite was extracted from the isolates. Extracted secondary metabolite was purified and antimicrobial activity was assessed *in vitro*. Extracted secondary metabolite from Ros 11 was found to have better antagonism than Ros 25 against *S. aureus* (Fig. 3a), formed 12 mm and 10 mm growth inhibition zone, respectively. But secondary metabolite of Ros 25 found to be most effective against fungal pathogen *A. fumigates* and *A. niger* (Fig. 3b), formed 15 mm and 12 mm (Fig. 3c) growth inhibition zone, respectively.

Discussions

Actinomycetes are an indispensable part of modern medical science. These are found in nearly all habitats. Actinomycete from marine environment was first discovered from the salt molds of St. Padenbur (Nadson, 1903). As actinomycetes are the major source of potential bioactive compounds, since early 1960s oceans micro flora is using as an unexplored source of novel bioactive compounds. Till now several species of actinomycetes have been identified from the marine environment and most of these are new genera (Dhanasekaran *et al*. 2005). As a result, over the past five decades more than 10,000 bioactive metabolites have been isolated and characterized from oceanic actinomycetes (Pickup,1993).

The rapid spreading of diseases with the emergence of antibiotic resistant pathogenic microbes is now growing concerns, challenging the commonly practiced antibiotics. Hence, for brawling against rising pathogens, novel families of antibiotics must be needed with new form in the market at regular intervals.

Table. 1. Coloured and colony texture of acitinomycetes isolated from different media and their antimicrobial activity

Isolates Name	Medium	Colour of colony	Colony texture	Antibacterial activity	Antifungal activity
Ros 1	SCA	Red	Powdery colony	-	-
Ros 2	SCA	Grey	Waxy colony	-	-
Ros 3	SCA	Grey-white	Waxy colony	+	+
Ros 4	ISP-2	Yellow	Powdery colony	+	++
Ros 5	ISP-2	Grey	Powdery colony	-	-
Ros 6	ISP-2	White	**Fine substrate mycelium**	++	+
Ros 7	ISP-2	Grey violet	Powdery colony	-	-
Ros 8	ISP-2	Grey	Powdery colony	+	++
Ros 9	ISP-3	Orange	Powdery colony	++	+
Ros 10	ISP-3	Red	Fine substrate mycelium	+	+
Ros 11	ISP-3	White	Powdery colony	+++	+++
Ros 12	ISP-4	Grey	Powdery colony	+	+
Ros 13	ISP-4	Grey-white	Powdery colony	-	-
Ros 14	ISP-4	White	Powdery colony	++	+
Ros 15	ISP-4	Yellow brown	Powdery colony	+	-
Ros 16	ISP-5	Reddish brown	Powdery colony	+	-
Ros 17	ISP-5	Orange	Powdery colony	-	-
Ros 18	ISP-6	Grey-white	Powdery colony	-	-
Ros 19	ISP-6	White	**Fine substrate mycelium**	-	-
Ros 20	ISP-6	Grey	Powdery colony	+	+
Ros 21	ISP-6	Grey-white	Powdery colony	+	+
Ros 22	ISP-7	Orange	Powdery colony	++	+
Ros 23	ISP-7	White	Powdery colony	++	++
Ros 24	Kuster Agar	Red	Powdery colony	-	+
Ros 25	Kuster Agar	White	Powdery colony	+++	++
Ros 26	Kuster Agar	Yellow	Powdery colony	+	+
Ros 27	Kuster Agar	Grey-white	Powdery colony	-	-
Ros 28	Kuster Agar	Grey	**Waxy colony**	-	-
Ros 29	Kuster Agar	Yellow	**Fine substrate mycelium**	++	+
Ros 30	Kuster Agar	Yellow	**Powdery colony**	++	+

+++: Good activity; ++: Moderate activity; +: Weak activity; -: No activity.

Table. 2. Comparative study of Ros 11 and Ros 25 with reference strain *S. aureofasciculus* using different parameters, morphological characters and utilization of sole carbon sources

Characteristics	*S. aureofasciculus**	Ros 11	Ros 25
Colour of aerial mycelium	Grey	Grey	Grey
Melanoid pigment	Present	Present	Present
Reverse side pigment	Present	Present	Present
Soluble pigment	Absent	Absent	Present
Spore chain morphology	Rectiflexibilis	Rectiflexibilis	Rectiflexibilis
Spore surface morphology	Smooth	Smooth	Semi Rough
Utilization of sole carbon sources			
Arabinose	+	+	-
Xylose	+	+	+
Inositol	+	+	+
Manitol	+	-	+
Fructose	+	-	+
Galactose	+	+	+
Sucrose	±	±	±
Raffinose	+	+	+

*References strain has been taken from Department of Microbiology Lab, VIT University for comparative study with screened isolates; (+), Positive response; (-) Negative response

Table. 3. Response of Ros 11 and Ros 25 in different biochemical tests

General characters	Results	
	Ros 11	Ros 25
Gram staining	(+)	(+)
Motility	(-)	(-)
Catalase test	(+)	(+)
Oxidase test	(-)	(-)
Nitrate reduction test	(-)	(-)
Indole test	(-)	(-)
Methyl red test	(-)	(+)
Simmon citrate test	(-)	(-)
Voges Proskauer test	(-)	(-)
Triple sugar iron test	K/K, H_2S +	K/K, H_2S +
Citrate utilization	(+)	(+)
Cell wall composition		
LL-DAP	+	+
Meso-DAP	-	-
Glysine	+	+
Whole cell Sugars	-	-
Wall type	I	I

(+), Positive response; (-), Negative response

Discovery of broad spectrum antibiotic can reduce the chances of emergence of drug resistant pathogenic microbes.

The aim in this study was isolation of actinomycete strains from salt pans of Marakanam district as source of novel bioactive compounds using the selective isolation media. Thirty different actinomycetes were isolated in different media, of which 10 isolates, nearly 33% actinomycetes showed no antagonistic activity on tested microbes. Among the remaining isolates only two actinomycetes Ros 11 and Ros 25 showed significant antimicrobial activity against selected bacterial (*Staphylococcus aureus*) and fungal pathogens (*Aspergillus fumigates* and *Aspergillus niger*).

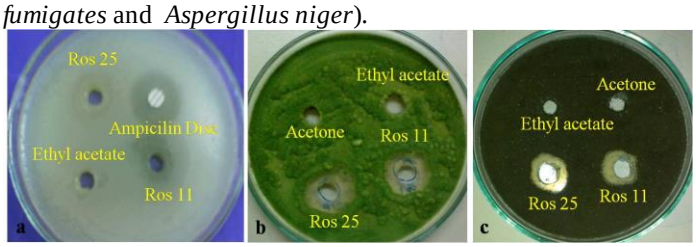

Fig. 3. Antimicrobial activity of crude isolates Ros 11 and Ros 25 (a) *Staphylococcus aureus*, (b) *Aspergillus fumigatus* and (c) *Aspergillus niger*

The cell wall type of Ros 11 and Ros 12 was characterized as wall type-I like *Streptomyces*, *Streptoverticillium*, *Chainia*, *Actinopycnidium*, *Actinosporangium*, *Elyptrosporangium*, *Microellobosporia*, *Sporichthya* and *Intrasporangium* (Lechevalier and Lechevalier, 1970). Both the isolates were gram positive and motile. The isolates were morphologically and biochemically characterized. The results for catalase test, citrate utilization, Indole test, Methyl red test, nitrate reduction test and Voges Proskauer test indicates that both the isolates belongs to the genus *Streptomycetes*.

Aspergillosis, the group of diseases caused by *Aspergillus* species infects people and animals and begins to grow inside them, especially in the lungs. *Aspergillus fumigatus* is more infectious among all *Aspergillus* species. It causes severe allergic reaction in humans. *A. niger* is less likely to cause human disease than some other *Aspergillus* species. But it causes otomycosis (fungal ear infections), which can cause pain, temporary hearing loss, and, in severe cases, damage to the ear canal and tympanic membrane. In this study, secondary metabolites were successfully extracted from the isolates Ros 11 and Ros 25 using solvent ethyl acetate. The metabolites showed antagonism against *Aspergilllus* spp. and *Staphylococcus aureus* in *in vitro* assay.

So many novel bioactive compounds were isolated characterized from marine *Streptomyces* sp. Presence of bioactive compounds, octalactins A and B (Tapiolas *et al*. 1991), five isoquinoline quinones, four new derivatives, Mansouramycin A-D, 3-methyl-7- (methylamino)-5,8-isoquinolinedione, dienoyl tetramic acids tirandamycin C and tirandamycin D (Carlson *et al*. 2009) were reported in *Streptomyces* sp. The antagonistic secondary metabolite produced by isolates Ros 11 and Ros 25 needs to be studied further to identify its chemical nature and characterization of its biological activity.

Since the need for antimicrobials is going up day by day due to emergence of new pathogens or due to drug resistance, so efforts are to be taken to discover newer and potent antimicrobials to combat these emerging diseases. Actinomycetes being the efficient producers could be exploited for the production of these drugs and we can diversify the range of antimicrobials only if we explore more and more undiscovered or unexploited species.

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