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ORIGINAL ARTICLE

Isolation of Potential Anti-Microbial Compounds from Actinomycetes Found in Soil Surrounding Medicinal Plants of Saurashtra, Gujarat, India

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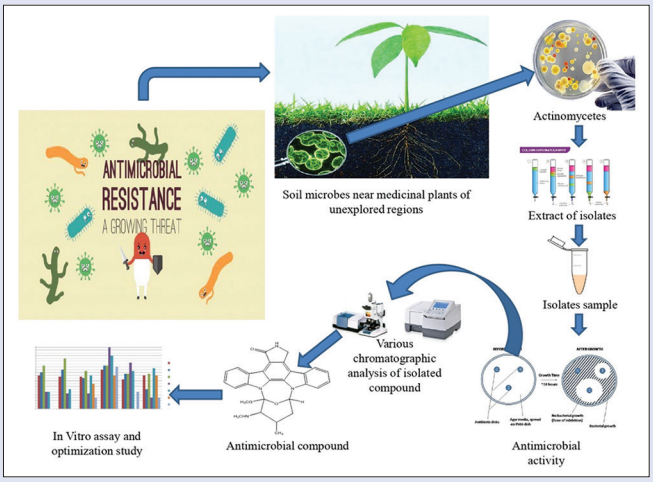
ABSTRACT

Objectives: Resistance developed by micro-organisms against antibiotics is now a global threat. There is a need to look at alternate sources from which substances useful against various diseases can be obtained. Soil surrounding the medicinal plants possessing anti-microbial activity was screened to find soil bacteria capable of producing anti-microbial compounds and to isolate such compounds therefrom. **Materials and Methods:** Eleven anti-microbial medicinal plants from three regions of Saurashtra, Gujarat, India, were selected, and soil surrounding those plants was collected. Sixty-six isolates of actinomycetes were obtained from the soil samples. Cross streak, agar well, and disk diffusion methods were used for primary, secondary, and final screening, respectively, of the anti-microbial isolates. **Results:** Among all, the isolate from the soil surrounding *Ocimum sanctum* in the Gir-Somnath region was found to be most potent and subjected to thin-layer chromatography, bio-autography, bio-chemical tests, enzymatic activity, 16S rDNA sequencing, and gel electrophoresis, which was confirmed to be *Streptomyces arenae*, followed by optimization of various conditions. Isolation and characterization of the compound were performed by using ultra-violet, Fourier transform infrared, nuclear magnetic resonance, and mass spectroscopy. A novel compound was isolated and characterized, which showed potent anti-microbial activity as compared to reference standards by *in vitro* anti-microbial assay. **Conclusion:** Soil surrounding medicinal plants which themselves have well-known anti-microbial activity may be a rich source of actinomycetes, which may produce compounds having anti-microbial properties. This can be an emerging strategy for researchers and clinicians to explore soil bacteria for the isolation of biologically active compounds for the management of various diseases.

Key words: Anti-microbial, bio-autography, characterization, resistance, *streptomyces arenae*

SUMMARY

- In the current work, a potential anti-microbial compound,2,3,10,11,12,13-hexahydro-9-methoxy-10-methyamino11-(methyl)-9,13-epoxy-1H,9H-diindolo[1,2,3-gh: 3′,2′,1′-lm]pyrrolo[3,4-j][1,7] benzodiazonin-3-one, was isolated from actinomycetes in the soil surrounding anti-microbial medicinal plants of the Gir-Somnath region of Saurashtra, Gujarat, India. The isolated compound had promising results having anti-microbial properties as compared to reference standards. There are vast opportunities to screen various ecological regions near many medicinal plants, and many bio-active molecules may be found from these sites.



Abbreviations Used: **TLC:** Thin-layer chromatography; **UV:** Ultra-violet; **FT-IR:** Fourier transform infrared spectroscopy; **NMR:** Nuclear magnetic resonance spectroscopy; **MS:** Mass spectrometry; **ISP:** International Streptomyces Project; **ZOI:** Zone of inhibition; **WHO:** World Health Organization.

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INTRODUCTION

Different micro-organisms, various plants, and animals are the main sources from which many natural products are obtained. For human use, these natural products have been exploited for thousands of years. Medicinal use of natural compounds from microbes is well known. Also, microbes play a critical role in the development of many useful medicines such as antibiotics. After the discovery of penicillin from *Penicillin notatum* fungus, scientists were encouraged to work on microbial applications in health.[1] Unique characteristics are also observed in the actinomycetes class of micro-organisms. Several molecules obtained from actinomycetes include antibiotics, various enzymes, secondary metabolites (bio-active), anti-tumor agents, immunosuppressive agents, anti-fungal agents, anti-cancer agents, anti-oxidants, anthelmintic agents, anti-malarial agents, anti-inflammatory agents, neurogenic agents, and so on.[2]

More than 7500 bio-active compounds have been produced so far from the *Streptomyces* genus of Actinomycetes. As a result, Actinomycetes

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attracts the attention biological scientists.[3] These groups of microbes are particularly very important in the pharmaceutical sector. In the past 500 years, 70% of antibiotics have been discovered from actinomycetes micro-organisms.[4] To isolate actinomycetes from different habitats in search of a newer anti-microbial compound has always been an area of interest among various researchers. Habitat differences are one of the crucial factors which play a significant role in the anti-microbial compound producing ability of actinomycetes.[5] Different types of chemical and physiological characteristics are observed in actinomycetes microbes because of a variety of biologically active enzymes. There are chances to develop newer bio-active molecules from actinomycetes found in the rhizosphere soil of medicinal plants.[6]

Antibiotic resistance is emerging as a critical health concern. Some microbes also develop resistance against multiple drugs, which has created major problems in the treatment of the diseases. Improper prescription by doctors, sub-standard drugs, and drug degradation because of poor storage indirectly lead to anti-microbial resistance.[7] There are many mechanisms for the development of resistance against anti-microbial compounds such as resistance gene transfer between bacteria, inactivation of the antibiotic, reduction in membrane permeability, target site modification, transport, or efflux of the antibiotic.[8-11] This invites a very interesting proposition: is it possible that actinomycetes surrounding anti-microbial medicinal plants can somehow produce anti-microbial compounds themselves?

The present research is focused on the isolation of actinomycetes from nearby soil of different anti-microbial medicinal plants, characterization of the most potent actinomycetes, and isolating a potent anti-microbial compound therefrom. Many ecological regions are still needed to be explored for screening purposes of useful actinomycetes. The Saurashtra area of Gujarat, India, is one of those regions.

MATERIALS AND METHODS

Chemicals and glassware

Chemicals required for research were obtained from different vendors such as Hi-media; Loba chemicals; Borosil Glass Limited, Ahmedabad, Gujarat, India; Duracell; Molichem; and Chemdyes.

Selection of area

Three districts (Rajkot, Gir Somnath, and Junagadh) of the region of Saurashtra, Gujarat, India, were selected randomly. These selected areas were not explored previously for this kind of study.

Selection of places

Places were selected randomly; the sample collection site details are Agriculture University Gardens, Junagadh (Latitude: 21.507063, Longitude: 70.447919), Girnar forest, Junagadh (Latitude: 21.125871, Longitude: 70.824014), the herbal garden of RK University, Rajkot (Latitude: 22.238540, Longitude: 70.901728), the herbal garden of the Noble Group of Institutions, Junagadh (Latitude: 21.589675, Longitude: 70.497716), a plant nursery in TalalaGir forest (Latitude: 21.064696, Longitude: 70.534494), and a plant nursery in Mangrol (Latitude: 21.134712, Longitude: 70.109603).

Selection of plants

Based on the literature review, 11 medicinal plants (*Aloe vera, Azadirachtaindica, Syzygiumcumini, Datura stramonium, Rosa indica, Pongamiapinnata, Ocimum sanctum, Allium sativum, Allium cepa, Trigonellafoenum-graecum, Psoraleacorylifolia*) were selected. According to the literature; parts of the plant such as seeds, leaves, roots, and so on may contain anti-microbial activity.

Selection of the soil part

Soil samples were collected from the soil microflora. This part of the soil contains a maximum number of micro-organisms. One foot near the medicinal plant soil was selected for sampling; first, up to 3 cm of the soil was separated from the surface of the site, and after that, soil samples were collected up to a 20 cm depth of the soil.[12] For the collection of the soil samples, previously sterile (autoclaved) articles were used. These samples were collected on sterile tubes and made airtight immediately after the collection of samples. Soil samples were air-dried at room temperature in laminar airflow for 1 day and stored at 2°C to 8°C until further process.

Sample treatment

One gram of dried soil was suspended in 99 mL sterile water in an Erlenmeyer flask, and the mixture was stirred in a rotary shaker for 1–2 hr. Afterward, 1 mL from the suspension was taken with the help of a sterile pipette and added into the sterile test tube along with 9 mL sterile water (Ranbaxy Laboratories Limited, Gurugram, India). The mixture was vortexed for 10 min, and 1 mL from the resultant solution was taken. The same method was applied, and diluted samples of 0.1 mL aliquot up to 10^6 were prepared. After sterilization of the media anti-fungal, Nystatin was added at a 50 $\frac{}{mL}$ concentration. Three types of media, Starch Casein Agar Media, Yeast Mannitol Agar Media, and Nutrient agar media, were used for the isolation of soil samples.[13] The colony of the actinomycetes was identified, and the media were selected based on their growth to prepare the pure colony of the actinomycetes by the streak plate method. 15% $\frac{w}{v}$ glycerol was added to the broths to maintain the viability of micro-organisms and was stored under -20°C until use.[14]

Characteristics of isolates

Actinomycetes colonies were identified and selected based on the morphology concerning the color of both aerial and vegetative mycelia. For morphological characters of isolates, the coverslip culture method was used.[15] Coverslip cultures of isolates were prepared in ISP media.

Microscopic examination

Microscopic observations were performed using coverslip culture based on the presence or absence of the substrate mycelium, fragmentation of the substrate mycelium, the presence of sclerotia or sporangium, sporulation on the substrate mycelium, and a spore chain morphology: rectiflexibilities, retinaculiaperti, or spirals. The identification was carried out according to Berger's manual of determinative bacteriology 9th edition.[16]

Primary screening for anti-microbial activity

For research in the field of novel molecules having anti-microbial properties, cross streak techniques are widely used.[17] The cross streak method was used in the initial stage of analysis with all actinomycetes isolates to evaluate any anti-microbial activity of actinomycetes isolates against various test pathogens. The modified cross streak method was applied by setting parameters such as the distance fixed at 1 cm among various test pathogens, the streak line length of test microbes fixed at 3 cm, the width of every streak fixed at 0.5 cm, the actinomycetes length for streak fixed at 7 cm, and the width of actinomycetes steak fixed at 0.5 cm. A control plate was maintained without inoculating actinomycetes to assess the normal growth of the bacteria.[18] The potential anti-microbial producer was selected by screening the actinomycetes isolates against a group of pathogens of microbial type

culture collection from National Chemical Laboratory Pune, India, as *Salmonella typhi* (NCIM2501), *Escherichia coli* (NCIM2685), *Bacillus subtilis* (NCIM2195), *Enterobacter aerogenes* (NCIM2340), *Proteus vulgaris* (NCIM2027), *Klebsiella aerogenes* (NCIM2283), *Staphylococcus aureus* (NCIM2602), *Pseudomonas aeruginosa* (NCIM2945), *Bacillus megaterium* (NCIM2054), and *Pseudomonas fluorenscens* (NCIM2639).

Secondary screening for anti-microbial activity

In the primary screening of 15 actinomycetes, isolates had shown good anti-microbial activity. These isolates were used in secondary screening. An Erlenmeyer flask (25 ml capacity, Borosil Glass Limited, Ahmedabad, Gujarat, India) was taken, and 5 mL of media was added for the inoculum (previously sterilize into a flask; autoclaving method). In the broth of media, 2.5% inoculum of actinomycetes was added under all aseptic conditions (under laminar flow). Afterward, the preparation was incubated under shaking conditions for 8 to 9 days at 37°C. The centrifugation process was performed at 4000 rpm for about 30 to 40 min, and the supernatant was collected. This was used for further process and used in the agar well diffusion method for secondary screening of the anti-microbial actinomycetes isolate.[19] For the evaluation of the anti-microbial properties of various plants and microbial extracts, the agar well diffusion technique was used. 20 to 100 µL of the sample was added into the agar well plate and incubated under specific conditions. The results were observed in terms of zone of inhibition (ZOI).

Anti-microbial efficacy of the ethyl acetate extract of the fermentation broth (Disk diffusion method)

Extraction of anti-microbial metabolites: Five actinomycetes isolates, OS-G-1, OS-J-1, OS-G-3, TF-G-1, and TF-J-1, were selected based on previous studies. A 500 mL capacity Erlenmeyer flask was taken, and the fermentation was added to it. The actinomycetes agar medium (AIA) was prepared, a 100 mL quantity of media was taken, and all five actinomycetes isolates were inoculated in these media under aseptic conditions with all necessary handling precautions. After that, the incubation process of media was performed under conditions such as 8 days of duration and a 30°C temperature. With centrifugation, the filtrate was separated. The solvent (ethyl acetate) was mixed with the filtrate in the proportion of 1:1 (*v/v*) under shaking conditions for 1 hr, which resulted in effective extraction. Purification of the residue was performed by the use of methanol. At the end of this process, a compound was obtained. The extracted compound was evaluated for anti-microbial activity by using disk diffusion.[20] Sterile paper discs (Whatman filter paper No. 1) were used to prepare anti-microbial paper discs for bioassays. 3 mg/mL of the crude extract was prepared in dimethyl sulfoxide (DMSO): methanol (1:1 *v/v*) (DM solvent), and using a microsyringe, 0.01 ml aliquots (0.03 mg) were adsorbed to each of paper discs, followed by the drying process at room temperature overnight.[21]

Thin-layer chromatography

Analysis of the ethyl acetate extract was carried out by using the thin-layer chromatography (TLC) technique. TLC of the crude ethyl acetate extract of the fermentation broth of actinomycetes isolates, OS-G-1, OS-J-1, OS-G-3, TF-G-1, and TF-J-1, was carried out. Anti-microbial spots were observed on the TLC slide. The crystals of the ethyl acetate extract were dissolved in a small volume of AR grade methanol, and the sample was used for TLC analysis. TLC analysis of the ethyl acetate extract was carried out on commercially available TLC silica gel 60 F_{254} plates using a mobile phase solvent system as acetic acid, N-butanol, and water in the proportion of 1:4:5. After that, a spot of the sample was seen under ultra-violet (UV) light, and iodine chamber development was performed.[22] A bio-autography study was also carried out for confirmation.

Taxonomic study of the most effective isolate

Based on experiments and results performed, the taxonomic study of the most effective anti-microbial producer was performed for the actinomycetes isolate. The electron microscopy method was used for the morphological study of actinomycetes isolates.[23] Various ISP media were prepared, and the growth of the most potent actinomycetes isolate was observed.

Bio-chemical characters of the most potent actinomycetes isolate

Gram staining, enzymatic activity, utilization of different sugars, Indol test, methyl red test, VP test, citrate test, and hydrogen sulfide production test were performed for detailed information of the actinomycetes isolates. A sensitivity study of the most potent actinomycetes isolates against various antibiotics was also carried out.[24]

16SrDNA sequence analysis

16S rDNA sequence analysis was performed for the identification of actinomycetes isolate OS-G-1 species by rDNA ribotyping and amplification of 16S rDNA with the sequencing of the amplicon.[25] An applied biosystem micro-sequence kit was used. The phylogenetic tree was constructed using the neighbor-joining method with a bootstrap test[26] in the MEGA 6 program.[27] The16S rDNA sequence was submitted to Genbank, NCBI, USA.

Optimization studies

The effect of various factors on anti-microbial compound production by *Streptomyces arenae* was studied to optimize the parameters. The major objective of this study was to optimize the parameters such as temperature; pH; suitable nutrient media; carbon and nitrogen sources; static and shaking conditions; and the concentration of NaCl, K_2HPO_4, humic acid, and sodium azide for anti-microbial production by isolate *Streptomyces arenae*.[28]

Different solvents for extraction of the anti-microbial

Various solvents were studied for the extraction process for the collection of an anti-microbial compound from samples. Various solvents were used, such as ethyl acetate, ethanol, butanol, methanol, propanol, carbon tetrachloride, hexane, benzene, acetone, diethyl ether, petroleum ether, chloroform, dichloromethane, dimethyl sulfide, and toluene; a proportion of 1:1 solvent was added to the supernatant of the broth, which was mixed well under agitation for 45 min duration using a homogenizer. A separating funnel was used for the solvent separation process; centrifugation was performed for trace removal at 5000 rpm speed and 15 min duration. The respective solvent was used as a control, and all extracts were used for anti-microbial activity by the agar well diffusion technique.[29]

Purification of the anti-microbial

The column chromatography method was used for partial purification of an anti-microbial compound; for this, 60–120 mesh (Merk) silica gel was used. Fractions in which anti-microbial activity was observed were purified with the same method of column chromatography; the off-white

yellow-colored compound obtained was stored in ampoule at 4°C. The fractions collected from column chromatography were subjected to TLC and tested for their anti-microbial activity.[30]

Analysis of the anti-microbial compound

The shape of the compound was observed under scanning electron microscopy (SEM); various parameters such as color, state, and melting point were studied. For detailed characterization of anti-microbial compounds, various chromatographic methods were used, such as UV, Fourier transform infrared (FT-IR) spectroscopy, nuclear magnetic resonance (NMR) analysis, mass spectroscopy, and an *in-vitro* microbial assay of anti-microbial compound checking through the cylinder plate method.[31]

Statistical analysis

Statistics was analyzed with SPSS version 14 (SPSS Inc., USA). Survival curves were plotted using the Kaplan–Meier method, and differences were estimated by the log-rank test. All experiments were repeated in triplicate. A *P* value of less than 0.05 was considered to be significant.

RESULTS

Colony morphology of actinomycetes isolates

As per colony appearance actinomycetes, colonies can be distinguished on the plate from those of fungi and true bacteria. They are compact and often leathery giving a conical appearance and have a dry surface. By using 11 plant soils and three regions of the Saurashtra area, a total of 66 actinomycetes isolates were found. It was found that the white color (57.58%) was dominant in the aerial mycelium of isolates and yellow (48.48%) was dominant in the case of vegetative mycelia of actinomycetes isolates.

Microscopic examination

Out of 66 actinomycetes isolates, 58 isolates were identified up to a generic level. It was found that out of 58 identified actinomycetes isolates, 30 isolates were belonging to *Streptomyces* (51.72%), 16 isolates were belonging to *Micromomospora* (27.59%), four isolates were belonging to *Intrasporangium* (6.9%), three isolates were belonging to *Saccharopolyspora* (5.17%), two isolates were belonging to *Streptosporangium* (3.45%), two isolates were belonging to *Rhodococcus* (3.45%), and one isolate was belonging to *Saccharomonospora* (1.72%). Diversity in actinomycetes genera was found from the study results.

Primary screening for anti-microbial activity

It was found that out of 66 actinomycetes isolates, 45 (68.2%) actinomycetes isolates were showing activity against test microbes and 21 (31.8%) actinomycetes isolates were not showing any activity against test microbes.

Secondary screening for anti-microbial activity

In the primary screening, 15 actinomycetes isolates, AI-G-1, RI-G-1, RI-G-2, RI-J-1, OS-G-1, OS-G-3, OS-R-1, OS-R-2, OS-J-1, AC-J-1, TF-G-1, TF-G-2, TF-J-1, PC-G-1, and PC-J-1, showed good anti-microbial activity. These isolates were used in the secondary screening of the Agar well diffusion method [Table 1]. Based on secondary screening, the most potent five actinomycetes isolates, OS-G-1, OS-J-1, OS-G-3, TF-G-1, and TF-J-1, were selected for further studies, for example, to check the anti-microbial study by the disk diffusion method, and this showed that OS-G-1 was the most potent actinomycetes isolate among others.

Microscopic examination of the actinomycetes isolate OS-G-1

The electron microscopy method was used for further study of the actinomycetes isolate OS-G-1. Various morphological parameters were studied, and both aerial and vegetative mycelia in the actinomycetes isolate OS-G-1 were observed; in electron microscopy images, spores were observed with characteristics such as long chains (spiral), circular spores were found, and the surface of spores was smooth; based on all these parameters, the OS-G-1actinomycetes isolate represents *Streptomyces* features and the genus was identified.

Cultural characteristics of the actinomycetes isolate OS-G-1

Cultural characteristics of the actinomycetes isolate OS-G-1 using different media were studied. Based on cultural characteristics, good growth of actinomycetes isolates was found with media such as ISP2 and ISP3 and actinomycetes agar media, reasonable growth of actinomycetes

Table 1: Anti-microbial activity of actinomycetes isolates by agar well diffusion (ZOI in mm)

Isolate code	Microbial species (ZOI in mm)							
	Ec	*Ka*	*Pv*	*Bs*	*Pa*	*Et*	*Sa*	*St*
OS-R-1	19.00*±1.00	15.33*±0.58	18.00*±1.00	18.67*±0.58	0.00±0.00	15.00*±1.00	17.00*±1.00	17.00*±1.00
RI-J-1	20.00*±1.00	19.33*±0.58	0.00±0.00	21.00*±1.00	17.00*±1.00	18.33*±0.58	21.67*±0.58	0.00±0.00
OS-R-2	17.67*±0.58	17.33*±0.58	15.00*±1.15	18.33*±0.58	0.00±0.00	16.67*±0.58	19.33*±0.58	15.67*±0.58
OS-J-1	18.33*±0.58	20.00*±1.00	18.67*±0.58	25.00*±1.00	0.00±0.00	21.00*±1.15	19.33*±0.58	20.00*±0.00
OS-G-1	20.00*±1.00	26.00*±1.00	24.67*±0.58	27.00*±1.00	0.00±0.00	18.33*±0.58	22.00*±1.00	19.00*±1.00
RI-G-2	20.00*±1.00	20.00*±1.00	0.00±0.00	21.67*±0.58	18.67*±0.58	15.33*±0.58	17.33*±0.58	0.00±0.00
TF-G-1	20.00*±1.00	20.67*±0.58	22.00*±2.00	18.67*±0.58	22.33*±1.15	0.00±0.00	20.33*±1.15	0.00±0.00
AI-G-1	20.00*±1.00	18.33*±0.58	0.00±0.00	19.67*±0.58	19.67*±0.58	0.00±0.00	22.67*±1.53	0.00±0.00
AC-J-1	18.33*±0.58	18.33*±0.58	19.00*±1.00	17.33*±0.58	0.00±0.00	16.33*±0.58	2.67*±0.58	15.00*±1.00
RI-G-1	18.00*±1.00	20.00*±1.00	0.00±0.00	19.33*±0.58	19.67*±0.58	16.67*±0.58	20.00*±1.00	0.00±0.00
OS-G-3	21.00*±1.15	18.67*±0.58	21.33*±0.58	20.00*±1.15	0.00±0.00	16.00*±0.00	20.33*±0.58	19.00*±1.00
TF-J-1	20.67*±1.15	21.33*±0.58	23.00*±1.15	20.67*±0.58	20.00*±1.00	0.00±0.00	22.67*±0.58	0.00±0.00
PC-J-1	19.33*±0.58	18.67*±0.58	0.00±0.00	18.33*±0.58	17.33*±0.58	0.00±0.00	17.67*±0.58	16.67*±0.58
PC-G-1	19.00*±1.00	17.67*±0.58	18.67*±1.15	18.33*±0.58	16.00*±1.15	0.00±0.00	19.33*±0.58	13.00*±1.15
TF-G-2	22.00*±1.00	20.67*±0.58	21.00*±1.00	18.67*±0.58	19.33*±0.58	0.00±0.00	19.33*±0.58	0.00±0.00

Values obtained from triplicate results,±SD: Standard deviation, *$P<0.05$: statistically significant, *PV: Proteus vulgaris, Bs: Bacillus subtilis, Et: Enterobacter aerogenes, Ka: Klebsiella aerogenes, Sa: Staphylococcus aureus, St: Salmonella typhi, Ec: Escherichia coli, Pa: Pseudomonas aeruginosa*

isolates was observed on ISP6 and ISP7 media, and poor growth of actinomycetes isolates was observed on ISP4 media.

Bio-chemical characters of the actinomycetes isolate OS-G-1

Gram staining

The actinomycetes isolate OS-G-1 was found to be Gram-positive and showed the violet color on the slide.

Enzymatic activity

The actinomycetes isolate OS-G-1 has been inoculated on a suitable medium by the streak and spot inoculation method to check different enzymatic degradative activities. It was found that the actinomycetes isolate OS-G-1 possesses positive results for protease, gelatinase, amylase, lecithinase, and cellulose and negative results for urease enzymatic activities. Thus, it was found that the actinomycetes isolate OS-G-1 was mostly enzymatically active.

Utilization of different sugars by the actinomycetes isolate OS-G-1 was tested

The utilization of different sugar molecules by the actinomycetes isolate OS-G-1 was studied with the use of various sugar sources as follows: A very good level of sugar utilization was observed with sugar sources such as maltose, D-mannitol, D-glucose, dextrose, and sucrose. D-mannitol can be utilized either moderately or fairly. Poor utilization was observed with various sugar sources such as L-arabinose, lactose, and L-rhamnose. Two sources, which are raffinose and D-xylose, of sugar were not utilized by isolates with observation and comparison to other used sugar sources. According to bio-chemical test results, Indol test, VogesProskauer test, and Citrate tests were found to be negative, whereas the methyl red test and hydrogen sulfide production test were found to be positive.

Sensitivity of the actinomycetes isolate OS-G-1 against antibiotics

The actinomycetes isolate OS-G-1 was showing sensitivity to lincomycin, ciprofloxacin, cefixime, erythromycin, amoxicillin, tetracycline, penicillin, streptomycin, kanamycin, and gentamycin, but it was showing resistance toward the antibiotics cefpodoxime and cephalexin. Thus, it was found that the actinomycetes isolate OS-G-1 was bio-chemically resourceful and it was possible to generate useful anti-microbials as well as many enzymes.

Separation of plasmids for anti-microbial production

The alkaline lysis method was used for plasmid separation in the *Streptomyces arenae*. There was no plasmid band observed on gel electrophoresis. The plasmid was absent in *Streptomyces areane*. Thus, it was found that the anti-microbial property was the property of the main chromosome and was not plasmid-oriented in the actinomycetes isolate *Streptomyces arenae*.

16Sr DNA sequencing and phylogenic analysis

The 16S rRNA gene sequence of OS-G-1 (GenBank accession number GOS1 MW504961) consisted of 771 bp [Figure 1]. BLAST analysis confirmed that the isolate belonged to the genus *Streptomyces*. The isolate OS-G-1 was deposited in the BIOTEC Culture Collection as *Streptomyces sp*. OS-G-1 (BCC74321). Phylogenetic analysis using the neighbor-joining method showed that strain OS-G-1 was most closely related to *Streptomyces arenae*.

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>GOS1
GGCGTCTTACACATGCAAGTCGTACCATGGTAACACGTAGGTGGGGATTAGTGGC
GAACGGGTGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGA
AACGGGGTCTAATACCGGATACTGACCACTGGGGGCATCTTTGATGGTCGAAAGC
TCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCT
CACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGG
CGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAAC
CTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCT
AACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCCGGAATTA
TTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGC
TTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCG
GAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGA
AGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC
AGGATTAGATACCCTGGTAGTCCACGCCGTAAACGGTGGGCACTATGTGTGGGCA
A
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Figure 1: 16S rDNA sequence of the actinomycetes isolate OS-G-1

Optimization studies

The effect of various factors on anti-microbial compound production by *Streptomyces arenae* was studied to optimize the parameters. A study was performed, and the ZOI was determined after the application of every single and derived result that was recorded as optimized parameters such as temperature (36°C); pH (8); suitable nutrient media (starch casein broth); carbon (maltose and starch) and nitrogen (casein and KNO_3) sources; shaking conditions; the concentration of NaCl (3 g/l), K_2HPO_4 (2.5 g/L), and humic acid (1g/L); and 8 days of incubation time, which were maximum for anti-microbial production by isolating *Streptomyces arenae*. All values were obtained from ± SD, n = 3.

Extraction and characterization of the anti-microbial produced by *Streptomyces arenae*

Extraction by using solvents: The maximum ZOI was found in the residue of anti-microbials obtained by using the ethyl acetate solvent [Table 2]. Thus, ethyl acetate was selected as a good solvent for extracting the filtrate obtained from the broth. From 1 L of the filtrate, 1.2 g of the crude compound was obtained from the ethyl acetate extract.

Purification of the anti-microbial

The column chromatography method was used for partial purification of an anti-microbial compound; for this, 60–120 mesh (Merck) silica gel was used. By the use of column chromatography, various fractions were collected and subjected to TLC and tested for their anti-microbial activity. TLC of active fractions was giving a single spot, and the active fraction had an Rf value of 0.58. The anti-microbial spot looks purple under UV light and yellow in the iodine chamber. Bio-autography of the anti-microbial separated spot was carried out against pathogens.

Analysis of anti-microbial compounds

Solid in the state, the off-white yellow color was observed through the necked eye; the round shape was observed by electron microscopy. It was found that the melting point of the anti-microbial compound was 179–182°C. Strong absorption at 330 nm was observed by UV spectroscopy, indicating the λ max value.

FT-IR spectroscopy

The spectrum was scanned using the KBr pellet technique. 1 mg of the sample was mixed with spectroscopically pure 100 mg of KBr pellets fixed in the sample holder, and analysis was carried out. The spectra were plotted against wavenumber, cm^{-1}, versus %Transmittance [Figure 2], showing 3700.20 cm^{-1}, 3778.8 cm^{-1}, 3830.50 cm^{-1} ($3800–3500$ cm^{-1} represents O-H stretching with the free hydroxyl group), 1219.5061 cm^{-1}, 1595.80 cm^{-1} ($1180–1360$ cm^{-1} C-N vibration, CO stretching, aromatic ring), 2375.5 cm^{-1}, 1720.60 cm^{-1}, ($1650–1780$ cm^{-1} C = O stretching),

2245.65 cm^{-1} (O-C-O stretching), 1029.70 cm^{-1} (1050–1150 cm^{-1} O-H bending alcohol), and 771.70 cm^{-1} (data observed in the range of 700–850 cm^{-1}, represents C-H bending with aromatic nature).

Mass and NMR spectroscopy

The mass spectrum of the anti-bacterial compound was taken using electron ionization mass spectrometry at 57 scan, and a graph was plotted as *m/z* versus % of base peak. An RTX-5 column was used by dimensions (30 mm length, 0.25 mm internal diameter, 0.25 µm film thickness). The injection temperature was 250°C. A 320°C temperature was maintained at the interface. With 1 ml/min flow rate, helium was used as a carrier gas. 1 µl sample was injected for analysis, and 1:25 split was given. With a 2 min hold time, ramping of temperature from 60°C to 310°C was given and a 10°C/min rise of temperature was set. A 13 min hold at 310°C was given. As per the spectrum result of the anti-microbial compound, the molecular weight was 466.2 gm/mol [Figure 3].

The NMR spectrum was taken using $CDCl_3$ as a solvent at 300 MHz. Chemical shift values were given in δ (ppm); the results indicate alcohol hydroxyl protons of 1.415 ppm (1.254 ppm, 1.115 ppm, 1.063 ppm, 1.014 ppm), within the range of 1–1.5 ppm, protons not attached to hetero-atoms, 0.880 ppm (0.856 ppm, 0.829 ppm, 0.698 ppm), within the range of 0.9–1.5 ppm, protons with hetero-atoms, 1.882 ppm and 2.379 ppm (1.760 ppm, 1.586 ppm, 2.353 ppm, 2.327 ppm, 2.260 ppm, 2.179 ppm, 2.000 ppm), within the range of 1.5–4.5 ppm, aromatic protons of 7.610 ppm (7.342 ppm, 7.264 ppm, 6.914 ppm), within the range of 6–8 ppm [Figure 4].

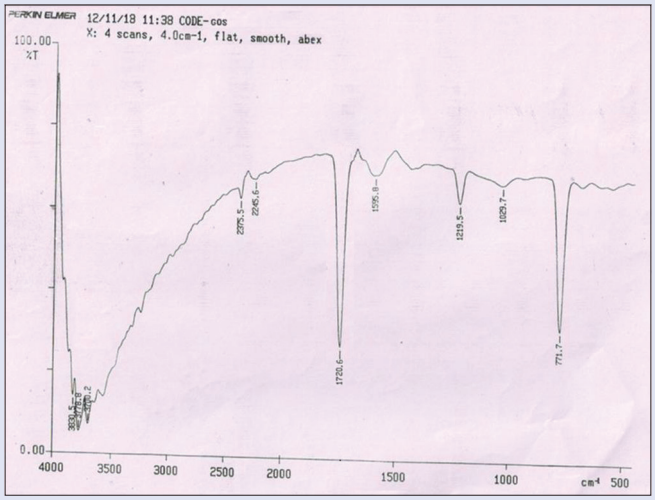

Figure 2: I.R. spectra of the isolated compound from the actinomycetes isolate OS-G-1

Based on spectral analysis of the anti-microbial compound structure, the following results were found [Figure 5]. The minimum inhibitory concentration of the crude antibiotic extract against seven test organisms was observed as against (A) Gram-positive bacteria such as *Bacillus megaterium* 1 mg/ml, *Bacillus subtilis* 0.5 mg/ml, and *Staphylococcus aureus* 0.5 mg/ml and against (B) Gram-negative bacteria such as *Pseudomonas fluorescens* 1.0 mg/ml, *Klebsiella pneumonia* 1.0 mg/ml, *Proteus vulgaris* 1.0 mg/ml, and *Escherichia coli* 0.5 mg/ml, followed by *in vitro* anti-microbial assay with standards as discussed in the below section.

DISCUSSION

Because of the misuse of antibiotics, superbugs are developing with a newer mechanism of resistance against available antibiotics; this is the keynote of the WHO 2020 report (https://www.who.int/en/news-room/fact-sheets/detail/antimicrobial-resistance). There is continuous research ongoing globally in the direction of novel molecules with anti-microbial properties; in most cases, research focused on structural modification of molecules, but unfortunately, success is rare. Therefore, there is a need to look at different sources from which researchers can develop a potential novel compound with anti-microbial properties. In the present study, the authors isolate a novel potential anti-microbial compound from unexplored sources. Out of 66 actinomycetes isolates, 45 (68.2%) actinomycetes isolates were showing activity against test microbes and 21 (31.8%) actinomycetes isolates were not showing any activity against test microbes. Soil actinomycetes used in the study have tremendous capability to produce potential anti-microbial molecules; in this aspect, many researchers work on soil actinomycetes, working on screening, identification, and isolation of actinomycetes for anti-microbial properties.[32] The scientist screened six soil samples; both types of screening, primary and secondary, were performed.

Table 2: Extraction of anti-microbial compounds by using different solvents with anti-microbial activity

Solvents	Microbial species (Zone of inhibition in mm)							
	Bs	*Sa*	*Pv*	*Ec*	*Ka*	*Pa*	*St*	*Et*
Ethyl acetate	29.67*±0.58	28.67*±0.58	29.00*±1.00	23.67*±0.58	25.00*±1.00	0.00±0.00	17.67*±0.58	19.00*±1.00
Ethanol	15.00*±0.00	14.00*±1.00	16.33*±0.58	14.33*±0.58	14.00*±1.00	0.00±0.00	9.67*±0.58	10.00*±1.00
Butenol	15.67*±0.58	16.00*±1.00	17.00*±0.00	12.00*±1.00	13.00*±0.00	0.00±0.00	10.67*±0.58	10.33*±0.58
Methanol	16.67*±0.58	15.33*±0.58	14.00*±0.00	13.33*±0.58	15.33*±1.15	0.00±0.00	8.00*±1.00	10.67*±0.58
Propenol	12.67*±0.58	13.00*±0.00	12.00*±1.00	10.67*±0.58	14.33*±0.58	0.00±0.00	7.00*±0.00	5.00*±1.00
Carbontetrachloride	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Hexane	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Benzene	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Acetone	17.33*±0.58	18.33*±0.58	15.33*±0.58	12.33*±0.58	12.00*±0.00	0.00±0.00	9.00*±1.00	8.00*±0.00
Diethylether	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Petroleum ether	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Chloroform	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Dichloromethane	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Dimethyl sulfide	15.00*±0.00	14.00*±1.00	13.33*±0.58	10.67*±0.58	11.67*±0.58	0.00±0.00	9.67*±0.58	10.67*±0.58
Toluene	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00

Values obtained from triplicate results,±SD: Standard deviation, *$P<0.05$: statistically significant, *PV: Proteus vulgaris, Bs: Bacillus subtilis, Et: Enterobacter aerogenes, Ka: Klebsiella aerogenes, Sa: Staphylococcus aureus, St: Salmonella typhi, Ec: Escherichia coli, Pa: Pseudomonas aeruginosa*

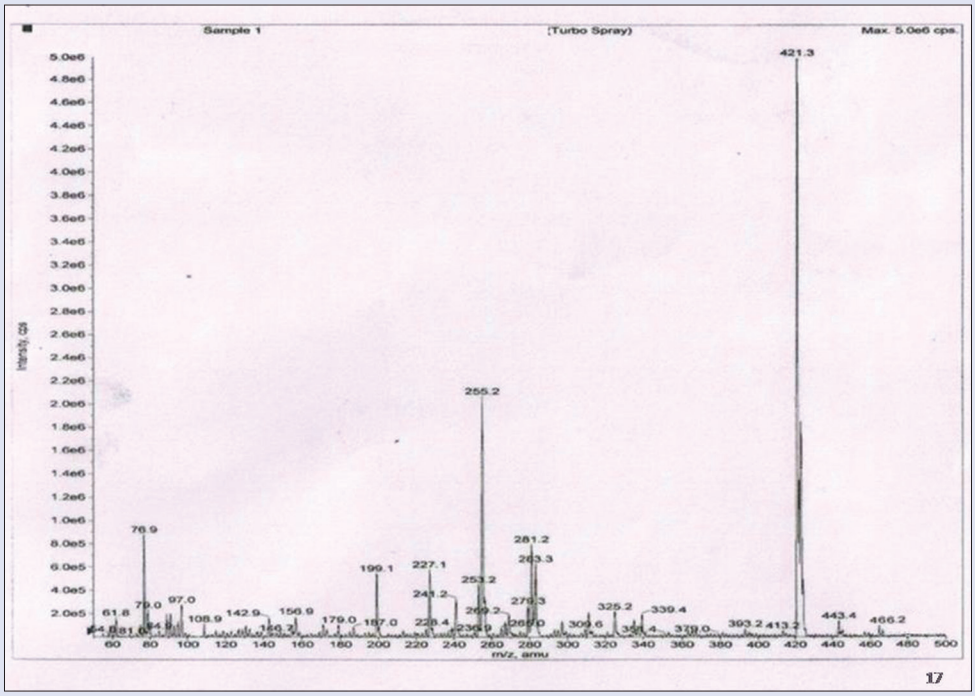

Figure 3: NMR of a compound from the actinomycetes isolate OS-G-1

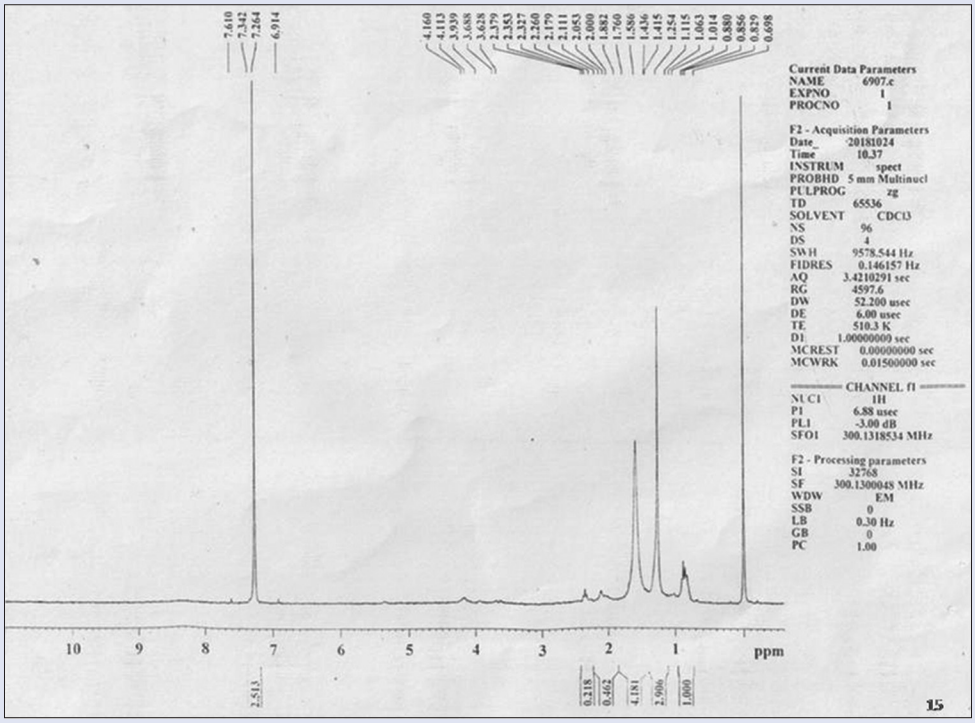

Figure 4: Mass spectra of a compound from the actinomycetes isolate OS-G-1

Partial characterization of the molecules by using various techniques was performed, and the possibility of biologically active molecules was reported in unexploited places. Singh and Bhoomi performed work on soil microflora from some national park region of Gujarat, studied soil microflora, and identified various interactions within microflora. In their study, many nitrifying bacterial and fungal species were recorded.[33,34]

In the present study, screening of isolates for anti-microbial properties was performed with various methods as discussed earlier and the most potent actinomycetes isolate was obtained based on ZOI. Some sites such as the Atlantic Sea and Pacific Sea were studied with regard to actinomycetesin search of secondary metabolites having bio-active properties. A study of 600 isolates were studied in this project, and a novel rare genus was identified, named *verrucosisspora*. This

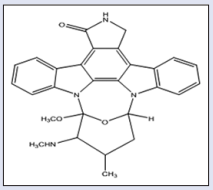

Figure 5: Structure of the isolated anti-microbial compound

work was performed by Fiedler and his associates in 2005.[35] Many researchers such as Subramani, Aalbersberg, Yarla, and Arvinda isolated potent antibiotic-producing actinomycetes from various habitats using different screening strategies and extracted new antibiotics.[36-38]

Isolate OS-G-1 was subjected for TLC and bioautography study for confirmation of bioactive properties as researchers were done their work on thin-layer chromatography and bioautography methods in respect to identification of the biological active area in slides of thin layer chromatography slide, also from results it was found that actinomycetes isolate OS-G-1 was mostly enzymatically active.[39] A study regarding the enzymatic activity of actinomycetes isolate was done by many researchers Stanford, Zhang and Zeng.[40]

Streptomyces OS-G-1 was confirmed as *Streptomyces arenae* as Mincer and Cook used 16s rDNA technology for identification of actinomycetes.[41,42] Various conditions or factors required for maximum production of bio-active anti-microbial compounds from the actinomycetes genus *Streptomyces* were studied by Wang.[43] They found that the starch casein medium was the best because the highest ZOI was observed with this medium in the study. The active fractions of antibiotics from column chromatography were collected. Various chromatography techniques were used to find the structure of the compound having anti-microbial properties. Similar methods were used by Augustine for structure elucidation; UV, FTIR, MS, and NMR were used for the structural study of antibiotics extracted from *Streptomyces albidoflavus* PU 23. Kim predicted rifamycin production in the marine isolate.[29,44]

In the present study, the antibiotic activity of the crude extract with standard antibiotics (streptomycin, tetracycline, and kanamycin) was studied, as shown in Figure 6, clearly indicating that the potency of the OS-G-1 actinomycetes isolate showed very promising results and confirming that the isolated compound 2,3,10,11,12,13-hexahydro-9-methoxy-10-methyamino11-(methyl)-9,13-epoxy-1H,9H-diindolo[1,2,3-gh: 3',2',1'-lm] pyrrolo[3,4-j][1,7] benzodiazonin-3-one had anti-microbial properties.

CONCLUSION

Currently, the global health care system is standing at a very crucial stage where some disease-producing microbes are developing complete resistance against available anti-microbials. There is an impending need to find alternate sources from where researchers can develop novel molecules having bio-active properties. This indicates that there may be a possibility of a natural exchange of constituents or properties between anti-microbial medicinal plants and their surrounding environment, including soil microbes. Also, there is a need to screen ecological regions previously not taken into consideration for the cultivation of microbes. This work also opens up possibilities to obtain bio-active molecules from microbes in the soil surrounding medicinal plants having different pharmacological activities.

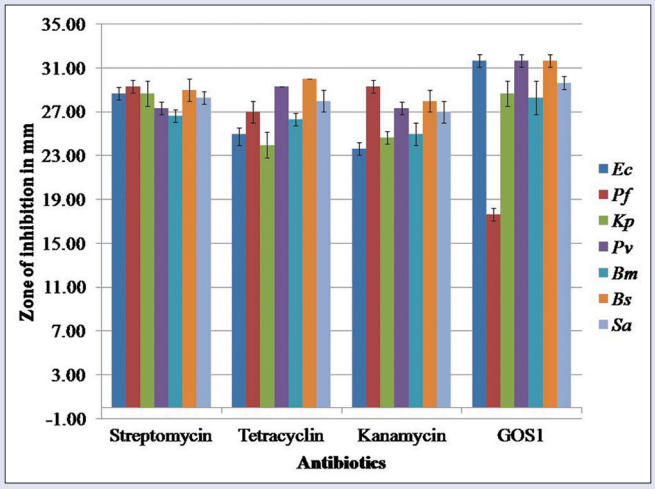

Figure 6: Comparison of anti-microbial activity against various test pathogens of streptomycin, tetracycline, and kanamycin with the compound produced from the actinomycetes isolate OS-G-1 (50 µl)

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Availability of data and materials

The datasets were used and analyzed during the current study available from the corresponding author on reasonable request.

Authors' contributions

Both authors read and approved the manuscript for publication. RD contributed to methodology, conceptualization, investigation, methodology, resources, literature review, and formal analysis of study data, draft, and edited the manuscript for intellectual content. PD was a project administrator, contributed to supervision, visualization, resources, literature review, and design of the study. Both authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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