

Antimicrobial potential of actinomycetes isolated from soil samples of Punjab, India

Abstract

The present study was conducted to determine the antibacterial and antifungal potential of actinomycetes isolated from soil samples collected from two different locations of Punjab, India. The actinomycetes isolates showed various types of color pigments like pink, yellow, orange, red and brown which may have potential use in industries. Out of 23 actinomycetes isolates, 7 of them showed significant inhibition of different Gram positive and Gram negative bacteria and also some fungi. Actinomycetes isolate no. A4 inhibited *Klebsiella pneumonia* and *Salmonella enterica typhimurium* efficiently. Actinomycetes isolate no. A5 inhibited *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Salmonella enterica typhimurium* and the fungi *Alternaria alternata* and *Trichoderma viride*. The crude antimicrobial compound from the best isolates A4 and A5 were obtained by ethyl-acetate extraction and the MIC against two different bacteria *Klebsiella pneumonia* and *Salmonella enterica typhimurium* were 15 μ g/ml and 12 μ g/ml respectively. Determination of the nature of the antimicrobial compound by TLC analysis indicated that the compound may be alcohol, phenol or steroid in nature. The antimicrobial compound obtained from isolate A5 was stable even above 60°C and in the presence of proteinase K enzyme (5mg/ml). The results showed that isolate A5 which produced dark orange colour pigment has excellent antimicrobial characteristics with very low MIC value and stability at high temperatures and high concentration of proteinase K.

Keywords: antibacterial, antifungal, actinomycetes, inhibition, antimicrobial compound

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Introduction

Microorganisms are the main colonizers of the earth, bestowed with inherent physiological and functional diversity and have found applications in agriculture, medicine, industry and environment. Among the various industrially important microorganisms, actinomycetes are of prime importance and are primarily recognized as organisms of academic curiosity and producers of antibiotic compounds and biologically active secondary metabolites. Actinomycetes population has been identified as one of the major group of soil population, which may vary with soil type. Apart from soil, they are found in marine and terrestrial environments and also exist in symbiotic association with plants and other living organisms. The important genera of actinomycetes are *Streptomyces*, *Nocardia*, *Micromonospora*, *Thermomonospora*, *Actinoplanes*, *Microbispora*, *Streptosporangium*, *Actinomadura*, *Actinosynnema*, *Dactylosporangium*, *Rhodococcus*, *Actinosynnema*, *Kitasatospora*, *Gordona*, *Intrasporangium* and *Streptoalloteichus*. Actinomycetes from the genera *Actinoplane*, *Streptomyces* and *Actinopolyspora* have been reported to produce a number of broad-spectrum antibiotics. Apart from production of antibiotics, actinomycetes have been looked upon as potential sources of bioactive compounds and they are the richest sources of secondary metabolites. Actinomycetes synthesized about two-thirds of currently reported bioactive substances belonging to a great variety of chemical groups. Diversity and bioactivity of actinomycetes are well documented in various normal ecosystems all over the world. Actinomycetes also produce industrially important enzymes like cellulase, xylanase, pectinase, amylase, lipase and protease. Many actinomycetes also produce siderophores which have agricultural and clinical applications.¹ Actinomycetes producing bioactive compounds

with angiogenic or wound healing properties² and anti-tumorigenic properties have also been reported.³ New bioactive compounds of anthraquinone nature with potent antimicrobial, antitumor, anti-inflammatory and antiviral activities have also been reported from soil actinomycetes and its antitumour activity studied.⁴

Millions of strains have been isolated and screened over the decades in research and industrial laboratories, but there are still many explorations needed to discover untapped actinomycetes communities that might be associated with rhizospheres, plant endophytes, lichens, and endolithic bacteria, as well as marine sediments and invertebrates.⁵ Looking at the wide importance of actinomycetes in various fields, the present work reports the isolation of actinomycetes from different soil samples of Punjab and the study of the antimicrobial activity of the ethyl-acetate extracts of the isolates A4 and A5 to determine the antimicrobial compounds. The antimicrobial potential of extracellular metabolites produced by the soil-borne actinomycetes could be exploited for its future use in pharma industries.

Materials and methods

Collection of soil samples

The soil samples were collected from various regions of Punjab, India: agricultural soil of Chunni Kalan [30°65'17"N, 76°63'28"E], Fatehgarh Sahib district, barren land soil of Badali [30°44'15"N, 76°85'91"E], Mohali, and forest area of Chhatbir zoo [30°59'00"N, 76°78'00"E], SAS Nagar district. Soil is of alluvial type in Punjab. Soil samples were collected aseptically from a depth of 10-15 cm. Soil sample were collected using clean, dry and sterile polythene bags along with sterile spatula, marking pen and other accessories. The

samples were immediately taken to the Laboratory, where they were analyzed and if necessary, were stored at 4°C in the refrigerator.

Chemicals

The chemicals ethylacetate, chloroform, methanol, ethanol, hydrochloric acid, sulphuric acid, starch, casein, glucose, sucrose, arabinose, maltose, fructose, mannitol, urea, α -naphthol, potassium hydroxide, sodium chloride, p-dimethylaminobenzaldehyde, tetrazolium blue, sodium hydroxide, vanillin, naphthalene-1,3-diol and dimethyl sulfoxide, were obtained from Merck Limited, Mumbai, India. Nutrient agar, Mueller Hinton agar and Proteinase K were procured from Hi-media, Mumbai, India.

Sample processing and isolation of actinomycetes

20g of the soil was air-dried for 3days. The dried-soil was then ground into a fine powder in pestle (surface sterilized with ethanol). Later the soil samples were subjected to dry-heat treatment at 50°C for 1hr to depress the number of other bacteria and for preferential isolation of actinomycetes. One gram of soil was suspended in 100ml sterile distilled water and incubated in an orbital shaker at 30°C with shaking at 200rpm for 1hr. Mixtures were allowed to settle, and then serial dilutions of the spore suspensions were prepared up to 10-5. From each dilution, 0.1ml was taken and spread evenly over the surface of starch casein agar (SCA) with sterile L-shaped glass rod, then incubated at 30°C and were observed for the appearance of colonies. Actinomycete isolates were purified by streak-plate technique and the pure cultures were maintained on SCA slants at 4°C for further use.

Screening for antimicrobial activity

Primary screening of the actinomycetes isolates was done by perpendicular streak method on nutrient agar plates. The test organisms for primary screening used were the bacteria *E. coli*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Salmonella typhi*, *Klebsiella pneumonia* and the fungi *Fusarium oxysporum*, *Aspergillus niger*, *Trichoderma viride* and *Alternaria alternata*. Each of the isolates was streaked as a straight line on nutrient agar medium and incubated at 30°C for 7days (168hr). After the 7th day, different strains of micro-organisms were streaked at right angle, but not touching each other, and then incubated at 30-37°C for 24hrs in the case of bacteria and 27°C for 48hrs in the case of fungi. If the organism is susceptible to the antibiotic compound produced by the actinomycetes, then it will not grow near the actinomycetes.

The selected isolates were cultured in Starch Casein broth and incubated at 30°C for 7days. After 7days, actinomycetes cultures were filtered using 0.2 micrometer filter. Antimicrobial compound was recovered from the filtrate by solvent extraction with ethyl acetate. Ethyl acetate was added to the filtrate in the ratio of 1:1 (v/v) and incubated in shaker for 1hr for complete extraction. The ethyl acetate phase that contains an antibiotic agent was separated from the aqueous phase. It was evaporated to dryness in water bath at 80-90°C. The dried precipitate was dissolved in minimum amount of ethylacetate and was used to determine the antimicrobial activity.

Secondary screening was done by well-diffusion method on Muller Hinton Agar plates. Crude extract of antimicrobial compound which was produced by ethylacetate extraction was used. 100 μ l of the obtained compound was placed into the plates that were previously seeded with different pathogen bacteria. The plates were incubated at 30-37°C for 48hrs and examined for zones of inhibition.

Morphological and biochemical characterization

Only actinomycetes isolates that gave positive results in the screening for antimicrobial activity were characterized morphologically and physiologically. Phenotypic characteristics like aerial mass colour, reverse side pigments, morphology of spore chain, consistency, Gram's staining, growth on actinomycetes media, growth on *Streptomyces* media, etc. were done. The spore chain morphology was determined by direct microscopic examination using the 10days old cultures under a compound light microscope (Nikon, Japan) using 1000X magnification power. The observed structures were compared with Bergey's Manual of Determinative Bacteriology, ninth edition, and the organisms were identified. Bio-chemical characterization of actinomycetes was done by esculin hydrolysis, starch hydrolysis, casein hydrolysis, glucose utilization, sucrose utilization, citrate utilization, nitrate reduction, urea hydrolysis, catalase test and IMViC test, acid production from sugars, NaCl resistance and temperature tolerance.

Physiological characterization

These tests were performed as described by Gordon.^{6,7} Physiological tests included decomposition of Casein, Urea and Gelatin, and the ability to produce acid from various carbohydrates such as Glucose, Arabinose, Sucrose, Maltose, Fructose and Mannitol.

Preservation and maintenance of the actinomycetes isolates

Starch-casein agar slants were prepared and all the isolates that have been purified were streaked onto the slants. It was incubated at 30°C for 5days and after suitable growth the slants were sealed with paraffin and were maintained at 4°C.

Chemical screening of the antimicrobial compound

Silica gel plates, 10X20cm, 1mm thick, were prepared. These plates were prepared by spreading the slurry of silica gel evenly on the plates. They were activated at 150°C for half an hour. The antimicrobial compound was loaded on the plates with the help of capillary tube. Chloroform: methanol (30:70) was used as a solvent system. Spots were obtained after the solvent get eluted. After drying, the silica plates were sprayed with different types of reagents in order to detect the nature of the compound.⁸ One gram of dimethylaminobenzaldehyde was mixed with 25ml of 36% (v/v) HCl and 75ml of methanol. Then the stained sheets were heated to about 120°C for a few minutes till maximal colouration. This reagent was specific for primary amines. Reagent A consisted of 0.5gm of blue tetrazolium mixed with 100ml of methanol. Reagent B was prepared by mixing 24gm of sodium hydroxide in 50ml water with 50ml methanol. Reagent A and B were mixed 1:1 before use. The stained sheets were heated to about 120°C for few minutes till maximal colouration. Blue or violet colour zone formation or a light background will indicate the positive result. This reagent was specific for steroids and reducing compounds.⁸

One gram vanillin was mixed with 100ml concentrated sulphuric acid. The plates were heated to about 120°C for few minutes till maximal colouration. Coloured zones produced on a plate background were the indication of positive results. This reagent was specific for higher alcohols, phenols and steroids. Reagent 1 consisting of 0.2gm naphthalene-1,3-diol in 100ml ethanol was mixed 1:1 with reagent 2, which is 20% (v/v) Sulphuric acid, just before use. The stained plates were heated to about 120°C for few minutes till maximal colouration. Red, blue, green, violet, brown, orange or yellow coloured zone

formation were the indication of positive results. This reagent was relatively specific for sugars.⁸ Retention factor Rf of the compound was also determined after running thin layer chromatography.

Determination of the minimum inhibitory concentration (MIC)

MIC was determined by the serial dilution of the antimicrobial metabolite in Dimethyl Sulfoxide (DMSO). The concentrations of the antimicrobial compound for MIC determination ranged from 6mg/ml to 200mg/ml.

Stability of the antimicrobial compound

The stability of the antimicrobial compound was determined against temperature and Proteinase K enzyme. In case of proteinase K, 50µl of 10mg/ml proteinase K enzyme was mixed with 50µl of the crude extract of antimicrobial compound from the best isolate A5 and incubated in water bath for half an hour at 37°C. The mixture was then loaded onto the well of nutrient agar plate which was spreaded with the pathogenic bacteria to check the activity of the antimicrobial compound. In case of temperature stability, the antimicrobial compound from isolate A5 was incubated in water bath for 1hr at 60°C. Then after that antimicrobial activity was checked on nutrient agar plates against the pathogenic bacteria.

Results

Isolation of actinomycetes

Screening for antimicrobial activity: Five bacteria and four fungi were tested to screen the antibacterial effects of the secondary metabolites produced by the Streptomycete isolates. The results of primary screening indicated that 30% of the total isolates demonstrated antimicrobial activities. The aqueous extracts of the positive isolates were subjected to secondary screening using the same test pathogens. Isolates A4, A5 and A6 exhibited antimicrobial activity against all the bacteria used. A1 and A2 showed antimicrobial activity against *Klebsiella pneumonia* and *Salmonella typhi*, A3 showed antimicrobial activity against *Staphylococcus aureus*. Isolate A7 showed activity against *E. coli*, *Klebsiella pneumonia* and *Salmonella typhi*. Isolate A5 showed the highest antimicrobial activity (11-15mm) amongst all the isolates. It also showed antifungal activity against *Alternaria alternata* and *Trichoderma viride*. Therefore, this isolate has broad spectrum antimicrobial activity.

Morphological characterization of the isolates: After 10days of incubation, the colonies were observed with white, cream, grey, pale brown, yellow, orange, pink and red surfaces with hard and difficult to scrap textures which are the characteristics of actinomycetes. Some of them produced brownish and brackish pigmentations. Majority of the isolates showed appearance characteristics of *Streptomyces* as greyish-white on the aerial mycelia and pale yellow pigmentation on the reverse of the colonies. Gram's staining results showed filamentous structure with conidia typical of actinomycetes.

Biochemical characterization of the isolate A5: Isolate A5 showed the highest antimicrobial activity against both bacteria and fungi (Figure 1) (Figure 2), so, it was further characterized biochemically. The results of the biochemical tests are summarized in Table 1.

Minimum Inhibitory Concentration (MIC) determination: The crude antimicrobial compound from the best isolate A5 was obtained by ethyl-acetate extraction and the MIC against two different bacteria

Klebsiella pneumonia and *Salmonella typhi* was 0.012mg/ml. The inhibition zone diameters of different concentrations of the crude antimicrobial compound from A5 for the MIC determination are as shown in Table 2.

Table 1 Partial biochemical characterization of isolate A5

Biochemical tests	Results
Indole Production	-
Methyl Red	-
Vogesproskauer	-
Citrate Utilization	+
Starch Hydrolysis	+
Casein Hydrolysis	+
Gelatin Hydrolysis	+
Catalase Test	+
Oxidase Test	-
Nitrate Reduction	+
Growth in 1% NaCl	+
Growth in 3% NaCl	+
Growth in 5% NaCl	+
Growth in 7% NaCl	-
Growth at 4°C	-
Growth at 25°C	+
Growth at 40°C	+
Growth at 50°C	-
Glucose	+
L-Arabinose	+
Sucrose	-
Maltose	+
Fructose	+
Mannitol	+

Table 2 Inhibition Zone Diameter (IZD) shown by various concentrations of the antimicrobial compound from A5 against *S. typhi* and *K. pneumonia*

S. no.	Concentration (µg/ml)	IZD(mm) Against <i>S. typhi</i>	IZD(mm) Against <i>K. pneumoniae</i>
1	0.2	8	9
2	0.1	7	8
3	0.050	6	6
4	0.025	5	5
5	0.012	3	4
6	0.006	0	0

Chemical screening of the crude antimicrobial compound from A5: TLC analysis was done and sprayed with different reagents in order to determine the nature of the antimicrobial compound. For the antimicrobial compound extracted from A5, positive result was obtained by using vanillin as the spraying reagent. So, the compound

may be alcohol, phenol or steroid and the retention factor (Rf) using chloroform: methanol (30:70) as solvent system was 0.76 cm (Figure 3).

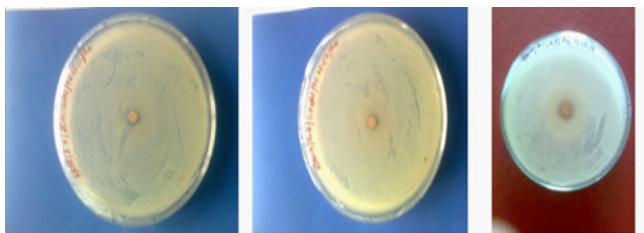


Figure 1 Antimicrobial activity of A5 against bacteria.

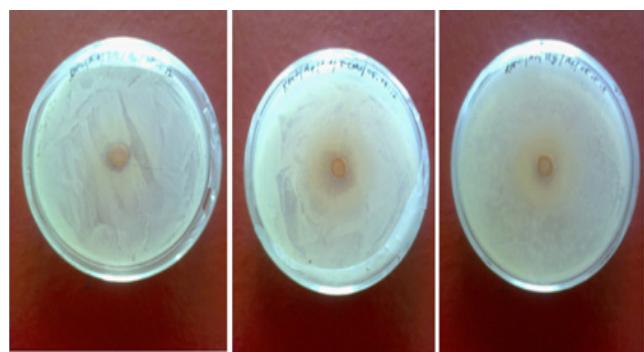


Figure 2 Antimicrobial activity of A5 against fungi.

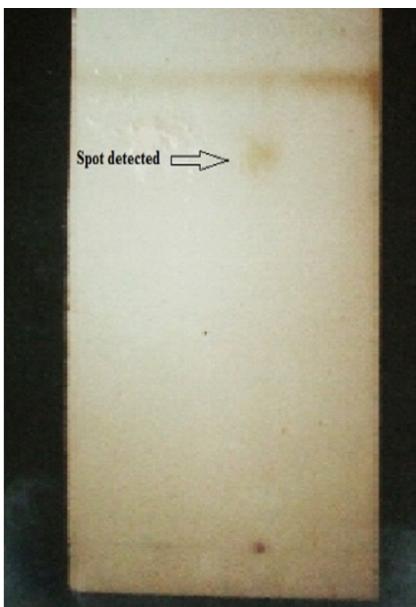


Figure 3 TLC analysis of the antimicrobial compound of A5.

Stability of the antimicrobial compound: It was found that the compound was stable even above 60°C and in the presence of proteinase K enzyme (5mg/ml). So it was proved that the actinomycetes isolate A5 is able to produce its antimicrobial effects in our body even in adverse conditions. Their resistance against temperature and proteinase K were shown by the retention of the zones of inhibition even after treatment with proteinase K for 1hr and incubation at 60°C for 30min.

Discussion

Three different soil samples were taken to isolate actinomycetes. They were agricultural, barren land and forest soil samples. It was observed that out of 23 isolates of actinomycetes, seven of them showed significant activity against bacteria and fungi. Primary screening results showed that isolate A5 inhibited *E. coli*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Salmonella enterica typhimurium* and the fungi *Alternaria alternata* and *Trichodermaviride*. Zone of inhibition ranged between 11 to 15mm. The morphological and biochemical characterization results show that isolate A5 is a isolate. For proper identification of genera and species of Actinomycetes, besides morphological and physiological properties, 16SrRNA sequencing needs to be determined. The identification of species and phylogenies has been made easier by molecular techniques in which primers that can target specifically the 16SrRNA sequence of the actinomycetes are used which makes identification fast and accurate.^{9,10} Actinomycetes isolates having broad spectrum of antimicrobial activity are of major importance for their study in the isolation of novel antimicrobial compounds. So far, quite a number of reports are available in which such novel compounds have been obtained and their study has been done. A strain of *Pseudonocardia* (VUK-10) isolated from the sediments of Mangrove ecosystems of Nizampatnam located in the south coastal region of Andhra Pradesh, India, was reported to show broad spectrum antimicrobial activity against bacteria like *Staphylococcus aureus* MTCC 3160, *Streptococcus mutans* MTCC 497, *Bacillus subtilis* ATCC 6633, *E. coli* ATCC 35218, *Enterococcus faecalis* MTCC 439, *Pseudomonas aeruginosa* ATCC 9027, the fungi *Fusarium oxysporum* MTCC 3075 and *Aspergillus niger* and the yeast *Candida albicans* ATCC 10231. The diameter of zone of inhibition ranged between 9 to 15mm.¹¹ In another report, three actinomycetes isolates K.6.3, K.14.2 and K.58.5 were found to show broad spectrum activity against Gram positive and Gram negative bacteria with zone of inhibition (up to 20mm). It was expected that the isolates might produce more than one antibacterial metabolites that made them effective inhibitor to both Gram positive and Gram negative bacteria.¹² In the present study, isolate A5 was obtained by ethyl-acetate extraction and the MIC against two different bacteria *Klebsiella pneumonia* and *Salmonella typhi* was 0.012mg/ml. This shows that the antimicrobial compound produced is a very effective compound. Low MIC value has been reported for isolate K.6.3 as 1mg/ml, and that of K.14.2 and K.58.5 as 2mg/ml against *S. aureus* ATCC 29213.¹² The MICs of the ethyl-acetate extract of *Streptomyces* (TR 007) ranged from 0.15 to 5mg/ml and that for *Actinopolyspora* (TR 008) varied between 0.039 and 5mg/ml when tested against different Gram positive and Gram negative bacteria.¹³ New antimicrobial compounds known as Napyradiomycins were isolated from a marine derived *Streptomyces* sp. SCSIO 10428 and they displayed antibacterial activities against the Gram-positive bacteria *Staphylococcus* and *Bacillus* strains with MIC values ranging from 0.25 to 32 μ g ml⁻¹.¹⁴ A novel alkaloid, xinghaiamine A, from a marine-derived actinomycetes *Streptomyces xinghaiensis* NRRL B24674T having sulfoxide containing compound was reported to exhibit broad-spectrum antibacterial activities to both Gram-negative persistent hospital pathogens (e.g. *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *E. coli*) and Gram-positive ones, which include *Staphylococcus aureus* and *Bacillus subtilis*. MIC values ranged between 0.69 to 11.04 μ M.³

The crude antimicrobial compound was stable even above 60°C when kept for 30min, and after treatment with proteinase K

enzyme (5mg/ml) for 1hr. Yucel and Yamac¹⁵ also reported a crude extract of an actinomycetes isolate identified as *Streptomyces* to be stable up to 60°C for half an hour.¹⁵ An actinomycete isolate of the genus *Actinomadura* isolated from the Saharan soil was reported to retain its broad spectrum antimicrobial activity at 60°C after treating for 1hr and at 80°C for 45min.¹⁶ Study of the stability of antibiotic metabolites from actinomycetes on subjection to different temperatures has also been reported in other studies.¹⁷⁻¹⁹ Uddin et al.²⁰ reported an antimicrobial compound from *Streptomyces albolorangus* isolated from chilli field which had stability at temperature ranges from 60 to 100°C and unstable after autoclaving.²⁰ Stability in the presence of proteinase K shows the non-proteinaceous nature of the antimicrobial compound.

Thin layer chromatography analysis of the crude antimicrobial compound extracted from A5 showed that the compound may be alcohol, phenol or steroid in nature as it gave positive result with spraying reagent vanillin-sulphuric acid.⁸ For further confirmation, bioautography analysis is required. The retention factor (Rf) using chloroform: methanol (30:70) as solvent system was 0.76cm. In one report, hexane extracted antimicrobial compounds isolated from *Bacillus* sp. strain B29 revealed presence of different antibiotics and antifungal compounds with different Rf values of 0.3 and 0.76 for antifungal compounds and of 0.12, 0.14, 0.19 and 0.3 for antibacterial ones.²¹ Bioactive compound obtained from *Streptomyces* sp. 2011 (JF751041) in one report showed that the ethyl acetate extract when subjected to High Performance Thin Layer Chromatography detected the presence of ester, quinone, macrolide and terpenoids. Rf values ranged between 0.01 to 0.95.²² In yet another report, *Streptomyces* isolates obtained from marine sponges produced antimicrobial compounds that showed Rf values ranging from 0.40 to 0.78 in TLC analysis. UV spectral analysis resulted in absorbance peaks ranging from 225 to 245 nm which confirmed the production of polyene substances.²³ For proper identification of the antimicrobial extracts it is necessary to obtain in pure form, which requires a series of purification process and different chemical analysis such as HPLC, Spectroscopy and other sophisticated techniques.

Conclusion

The present study reported the antimicrobial activities exhibited by different isolates of actinomycetes isolated from different biotopes of Punjab, India. Out of the many isolates, isolate A5 showed maximal antagonistic activity against the microorganisms used. It showed broad spectrum of antimicrobial activity as it inhibited both Gram positive and Gram negative bacteria and also some fungi. Therefore, this isolate proves to be a promising isolate which can be further studied for its applications in producing important pharmaceutical compounds and also as a biocontrol agent against plant pathogenic fungi. Further work on optimization of this isolate's antagonistic activity, purification of the important bioactive compound and study of its other properties like anti-tumorigenic and angiogenic activities are underway.

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None.

Conflict of interest

Author declares that there is no conflict of interest.

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