Research Article

Study on cultivation of cyanobacteria and extraction of antimicrobial compounds against pathogenic bacteria

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Abstract

Objective: Focusing on bioproducts, ongoing patterns in drug research have demonstrated that microalgae are promising organisms to outfit novel and more secure naturally vital compounds. Cyanobacteria are considered as potential source of antibiotic production. **Materials and Methods:** Twenty cyanobacterial strains from different habitats belonging to various genera were isolated cultured and tested for their antimicrobial activity. Among the isolates, percentage of incidence and frequency of *Microcystis* was more in Badrakali freshwater lake and Adilabad mavala lake. The biomass of cyanobacterial sp were lyophilized and extracted with ethyl acetate and crude extracts were subjected to TLC purification twice and tested for antibacterial activity against two Gram+ve bacteria (*Staphylococcus aureus, Micrococcus luteus*) and four Gram-ve bacteria (*Escherichia coli, Klebsiella pneumonia, Salmonella typhi, Salmonella paratyphi*) by Agar well diffusion method. **Results:** Ten strains showed activity against at least two of the test organisms. Bioactivity was more by extract of ethyl acetate. Extracts of *Oscillatoria* strains (MBKG02) were shown more antibacterial activity on *E. coli* (22mm) and *S. paratyphi* (19mm) where as *Microcystis* sp (MBKG16, MBKG08) shown more activity against *S. paratyphi* (20mm). *Microcystis* (MBKG 07) strain has shown more inhibitory effect on *S. paratyphi* (10mm). **Conclusion:** The results obtained in the present study clearly suggest that the production of antibacterial products from cyanobacterial sp could represent a viable and eco-friendly to reduce the use of synthetic drugs. The presence of antibacterial activity was independent of the strain origin.

***Keywords*:** Cyanobacteria, thin layer chromatography, antibacterial activity

Introduction

Since ancient time nature has provided man with broad and diverse amount of pharmacologically active compounds which were isolated and utilized as highly effective drug to combat deadly diseases or manufacture synthetic drugs similar to that found in nature. Till date the antibiotics mostly used are derived from microorganisms of terrestrial soils and aquatic habitats (Bhattacharyya et al., 2013). Pharmaceutical drug discoveries since years depended heavily on the process of empirically screening of large number of pure compounds to provide new leads. Historically, actinomycetes have been the most prolific producers of new bioactive metabolites. But the cyanobacteria (blue green algae) are among the oldest phototropic organisms. Their cultivation without organic substrate can be an economical advantage over the microorganisms (Kaushik and Chauhan, 2008). The search for cyanobacteria with antimicrobial activity has gained importance in recent years due to growing worldwide concern about alarming increase in the rate of infection by antibiotic resistance microorganisms. Biologically active substance was proved to be extracted by cyanobacteria. Different strains of cyanobacteria are known to create intracellular and extracellular metabolites which have a huge fascination as normal well spring of bioactive atoms with an expansive scope of organic exercies such as antibiotics, antiviral, anti tumoural , anti oxidant and anti inflammatory (TÜney İ Cadirci et al., 2006; Cardozo et al., 2007; Patra et al., 2008; Mohammed et al., 2015). The bioactive molecules are produced in an attempt for biological adaptations and

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tolerance to environmental stress and to confer advantages for their survival (Bhattacharyya et al., 2013).

Algae and cyanobacteria are swiftly proving to be an extremely important source of biologically active secondary metabolites (Gademann and Portmann, 2018). Report on antibiotic activity of cyanobacteria was first reported by (Patterson et al., 1994). More than 150,000 algae are found in all over freshwater on the earth. The screening of extracts from various regular sources is a typical method to find organically unique metabolites. In such research exercise on microalgae like cyanobacteria were observed to be a rich well spring of different product of commercial, pharmaceutical and toxicological premium: primary metabolites, for example proteins, fatty acids, vitamins, pigments and various secondary metabolites with different bioactivities (antifungal, antiviral, antibiotic and other) or cyanotoxins like hepatotoxins and saxitoxins were isolated from cyanobacteria (Volk et al., 2006). Cyanobacteria can be considered as one of the important taxa to be explored for their efficiency (Bhattacharyya et al., 2013). Algae are well springs of amino acids, terpenoids, phlorotannins, steroids, phenolic compounds, halogenated ketones, alkanes and cyclic polysulphides (Taskin et al., 2007).

The aim of study reported here was to isolate the cyanobacteria in Warangal and Adilabad districts of Telangana and study their occurrence in fresh water lakes by calculating their incidence and frequency and to examine the antibacterial activity of various concentrates of cyanobacterial isolates by agar diffusion method. In this investigation we have attempted to assess the antibacterial activity of different organic extracts of cyanobacteria on *E. coli, S. aureus, K. pneumonia, M. luteus, S. typhi, S. paratyphi* as test organisms.

Materials and methods

Isolation and culturing of cyanobacteria

The cyanobacterial samples were collected from the freshwater lakes and canals of Warangal and Adilabad districts. The samples were collected from pegadapalli lake, stagnant water pool from Reddypalem village, Reddypalem yellamma lake, Badrakali lake, Waddepalli freshwater lake, Adilabad mavala lake at mid winter season in month of August to December by Capillary method. Cyanobacterial strains were isolated and identified by standard microbiological methods (Desikachary. 1959; Rippka et al., 1979). Water samples were inoculated on Blue green agar media (Yadav et al., 2012; El-Aty et al., 2014). Media components in g/lit [$NaNO_3$ 1.500, K_2HPO_4 0.0314 , $MgSO_4$ 0.036 , $CaCl_2.2H_2O$ 0.0367, Na_2CO_3 0.020, Na_2 Mg EDTA 0.001 , citric acid 0.0056 , ferric ammonium citrate 0.006 , final pH after sterilization(at 25°C) 7.1] and Allen Arnon agar media [$K_2HPO_4.3H_2O$ 28.0 g/500ml, $MgSO_4$ 20g/500ml, $CaCl_2.2H_2O$ 6g/500ml, NaCl 20g/500ml, A&A Fe-EDTA solution 160ml, $MnCl_2.4H_2O$ 360mg/l, MoO_3 85% purity 36.0 mg/l or $Na_2MO_4.2H_2O$ 99% purity 61.1mg/l, $ZnSO_4.7H_2O$ 44.0mg/l, $CuSO_4.5H_2O$ 15.8mg/l, H_3BO_3 572mg/l, NH_4VO_3 4.6mg/l, $CoCl_2.6H_2O$ 8.0mg/l] by pour plate method and simultaneously cultured in broth of same media. The plates and flasks were incubated for 20days under continuous emission of white cool fluorescent light maintained for 16x 8 L/D cycles with 30µ mol photon $m^{-2}s^{-1}$ referred as 30°C. The obtained cyanobacterial colonies were sub cultured several times to obtain pure cultures. Identification was done using morphological variation studies and toxonomical approaches according to Desikachary (1959). The percentage of incidence and frequency were calculated by employing the following formula:

$$\% \text{ of Incidence} = \frac{\text{No. of colonies of species in all plates}}{\text{Total No. of colonies of the all the species in all plates}} X100$$

$$\% \text{ of Frequency} = \frac{\text{No of observation in which species appeared}}{\text{Total no of observations}} X100$$

Preparation of algal extracts

The axenic cyanobacterial cultures *Oscillatoria sp, Microcystis sp, Dictyospaerium sp, Nostoc sp, Chroococcus sp, Anabeana sp* were selected and screened for their antimicrobial activity against *Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Salmonella typhi, Salmonella paratyphi, Klebsiella pneumonia.* For extraction a volume of 3L of 25th day culture was reduced to 200ml by rotary evaporator (SENCO technology Co., Ltd. China) at 40°C. 300ml of ethyl acetate was added and the mixture was shaken for 24hours. The ethyl acetate layer was separated from the aqueous layer. The aqueous layer was shaken again with 300ml of fresh ethyl acetate. The ethyl acetate layers were combined and dried with Na_2SO_4 in rotary evaporator at 40°C to remove the organic solvent. The remaining residues of ethyl acetate were removed by air dry at 32-38°C. The aqueous layer was discarded. The extracts were weighed and stored at -20°C until use (Bui, 2014).

Chromatographic analysis and purification of cyanobacterial extracts

Thin Layer Chromatography technique can be used to detect compounds by the phenomenon of capillary action, injecting a thin layer adsorbent material into solvent system. The solvents travels due to the different attraction of the analysis to the stationary phase.

The retention factor for each spot can be determined by the equation below:

$$Rf = \frac{\text{Distance travelled by the spot(cm)}}{\text{Solvent front (cm)}}$$

The analysis of TLC moderately depends on the adsorbent material, temperature and pressure in the solvents as well as the spotting distance. The value of the retention factor is not constant in practice. The dried cyanobacterial ethyl acetate extracts (100mg) were dissolved in 3ml of methanol (25%) for purification using TLC plates (10x10cm; 0.25mm layer thickness). The aliquots were directly spotted two centimeters from the edges of a silica gel coated TLC plate. Separations of crude extracts were done using ethyl acetate: methanol: water (7:2:1) as mobile phase (Bui, 2014). Spots developed on such TLC plates were observed under UV illumination. The illuminated orange spots were eluted separately and dissolved in 3ml methanol (25%), each elute again subjected to TLC purification using Ethyl acetate: methanol: water (7:2:1). Now all spots obtained at second stage were bio assayed for antibacterial activity by agar well diffusion method (Maheep et al., 2014).

Determination of relative percentage of inhibition

The relative percentage of inhibition with respect to positive control was calculated by using the formula:

$$\text{Relative percentage of inhibition of test extract} = \frac{100 \times (a - b)}{c - b}$$

Where,

a, Inhibition zone of test extract

b, Inhibition zone of the solvent

c, Inhibition zone of the standard drug

Results and discussion

Isolation and culturing of cyanobacteria in different growth media

A total of 20 strains of cyanobacteria belongs to genera *Anabeana, Spirulina, Chlorella, Microcystis, Oscillatoria, Nostoc, Chroococcus, Gleocapsa, Nostoc carneum, Lyngbye, Gleotricha, Microchaete, Cylindrospermum, Aphanotheca, phormidium, Dictyospaerium, Merismopedia, Coelospaerium, Rivularia* and *Tolypothrix* were isolated from freshwater lakes of Warangal and Adilabad districts and purified in BG11 and Allen media. Among the isolates, percentage of incidence of *Nostoc* (43.5) and *Gleotrichia* (33.3) was more in Waddepalli lake of Warangal, where *Spirulina* sp and *Microcystis* sp have shown high P.I (23.7) in Pegadapalli lake and Khanapur lake of Adilabad (27.2) respectively. In all the cyanobacterial genera *Gleocapsa* sp, *Nostoc* sp and *Microchaete* sp shown highest percentage of frequency (66.6) in Reddy pallem pond, stagnant water of Warangal and Khanapur lake of Adilabad. In case of *Spirulina* highest P.F (80.0) was observed in Waddepalli lake as shown in (table 1). There is

Table 1. Percentage of Incidence & frequency of cyanobacterial sp collected from different water sources cultured in BG11 medium

Organisms	Pegadapalli lake		Reddypallum pond		Waddepalli lake		Reddypallum lake		Canal water (Pegadapalli)		Reddypallum village lake		Badrakali lake		Adilabad mavala lake		Adilabad khanapur lake		Kovali freshwater lake	
	PI	PF	PI	PF	PI	PF	PI	PF	PI	PF	PI	PF	PI	PF	PI	PF	PI	PF	PI	PF
Anabeana sp	20.3	46.6	-	-	-	-	-	-	29	60	-	-	-	-	7.3	20	-	-	-	
Spirullina sp	23.7	53.3	-	-	23.07	33	-	-	-	-	-	-	-	-	2.94	13.3	-	-	-	-
Microcystis sp	16.94	20	13.6	33.3			9.3	53.3			23	66.6	24.1	66.6	14.7	46.6	27.2	66.6	-	-
Oscillatoria sp	15.25	20	16.6	60	-	-	-	-	22.5	33.3	5.76	20	24.1	66.6	-	-	-	-	-	-
Nostoc sp	11.86	26	-	-	43.5	80	-	-	32.2	66.6	-	-	-	-	13.2	40	-	-	-	-
Chroococcus sp	-	-	12.1	26	-	-	11.6	33.3	-	-	19.2	33.3	-	-	-	-	-	-	12.9	33.3
Gloeocapsa sp	-	-	19.6	66.6	-	-	-	-	-	-	7.69	13.3	-	-	11.7	40	6.8	13.3	-	
Nostoc carneum	-	-	25.7	53.3	-	-	-	-	16.1	33.3	-	-	-	-	5.8	20	-	-	-	-
Lyngbye sp	-	-	12.1	33.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gloeotrichia sp	-	-	-	-	33.3	60	-	-	-	-	-	-	-	-	-	-	-	-	9.25	26.6
Microchaete sp	-	-	-	-	-	-	17.4	66.6	-	-	-	-	-	-	16.17	66.6	22.7	66.6	-	-
Cylindrosperm um sp	-	-	-	-	-		10.4	11.9	-	-	17.3	46.6	-	-	-	-	13.6	26.6	-	-
Aphanotheca sp	-	-	-	-	-	-	12.7	40	-	-	-	-	14.5	60	8.82	40	18.1	40	25.9	66.6
Phormidium sp	-	-	-	-	-		16.2	46	-	-	13.4	33.3	-	-	-	-	-	-	-	-
Dictyoshaeriu m sp	-	-	-	-	-	-	8.1	33.3	-	-	-	-	12.9	40	-	-	-	-	-	-
Merismopedia sp	-	-	-	-	-	-	-	-	-	-	3.84	20	-	-	19.11	66.6	-	-	9.25	20
Coelosphaeriu m sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	20.37	60
Rivularia sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	16.6	53.3
Tolyprhrix sp	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	5055	13.3

P.I : Percentage of Incidence P.F : Percentage of Frequency

no particular correlation between P.I & P.F of isolated strains, because of variability in requirements of cyanobacteria such as vitamins, organic and inorganic constituents from species to species. According to Enevoldsen, (2003) cyanobacteria sp also need different growth conditions like concentration of pH, salinity, temperature, light aeration etc.

Table 2. Bioassay of TLC bands against *S. aureus, E. coli, K. pneumonia, S. typhi, S. paratyphi, M. leteus* with Streptomycin as control. MBKG: Microbiology Kuraganti Guna

Cyanobacterial sp	TLC Bands with Rf values	Zone of inhibition (mm) against the test organism, streptomycin as control						
		S. aureus	*E. coli*	*K. pneumonai*	*S. typhi*	*S. paratyphi*	*M. luteus*	Streptomycin
Microcystis sp	A - 0.47	--	--	--	--	--	--	17
(MBKG16)	B - 0.79	--	--	--	--	--	--	
	C - 0.84	12	8	8	--	20	10	
Microcystis sp	A - 0.277	--	--	--	--	--	--	17
(MBKG08)	B - 0.6	--	--	--	--	--	--	
	C - 0.766	13	8.2	--	--	17	10	
Microcystis sp	A - 0.22	--	--	--	--	--	--	17
(MBKG32)	B - 0.458	--	--	--	--	--	--	
	C - 0.550	8	9.4	--	--	15	11	
	D – 0.623	--	--	--	--	--	--	
Dictyospaerium sp	A - 0.27	--	--	--	--	--	--	17
(MBKG35)	B - 0.57	--	--	--	--	--	--	
	C - 0.66	--	--	--	--	--	--	
	D – 0.81	10	8	12	--	20	13	
	E – 0.88	4	--	--	--	7	2	
Oscillatoria sp	A - 0.252	--	--	--	--	--	--	17
	B – 0.368	--	--	--	--	--	--	
(MBKG02)	C - 0.578	--	--	--	--	--	--	
	D – 0.821	--	--	--	--	--	--	
	E – 0.884	6	22	--	--	19	10	
	F- 0.968	--	16	--	--	8	--	
Nostoc sp	A - 0.27	--	--	--	--	--	--	17
	B - 0.35	--	--	--	--	--	--	
(MBKG07)	C - 0.56	--	4	--	--	--	--	
	D – 0.06	--	--	--	--	--	--	
	E – 0.917	8	10	7	--	8	10	
Microcystis sp	A - 0.28	--	--	--	--	--	--	17
(MBKG07)	B - 0.44	4	--	--	10	16	12	
Oscillatoria sp	A - 0.91	--	6	10	--	14	10	17
(MBKG03	B - 0.78	--	--	--	--	--	--	
Chroococcus sp	A - 0.27	--	--	--	--	--	--	17
	B – 0.59	--	2	6	--	--	--	
(MBKG24)	C - 0.69	--	--	--	--	--	--	
	D – 0.76	8	10	10	--	14	10	
	E – 0.78	--	--	--	--	--	--	
	F- 0.85	--	--	--	--	--	--	
Anabeana sp	A - 0.32	--	--	--	--	--	--	17
(MBKG29)	B – 0.41	--	--	--	--	--	--	
	C - 0.51	--	--	--	--	--	--	
	D – 0.65	--	--	--	--	--	--	
	E – 0.76	--	--	--	--	--	--	
	F- 0.88	--	--	--	--	--	--	
	G – 0.97	5	8	12	--	16	12	

TLC spots and antibacterial activity

All the ten cyanobacterial strains under study showed antibacterial activity against one or other bacteria (Table 2). Ethyl acetate extracts of 10 cyanobacterial strains showed significant antibacterial activity against Gram positive or Gram negative bacteria. Among the isolated strains extracts of *Microcytis* sp, *Oscillatoria* sp, *Nostoc* sp, *Anabeana* sp, *Chroococcus* sp, *Dictyospaerium* sp were shown remarkable antibacterial activity. *Microcystis* MBKG16, MBKG08, MBKG32, MBKG07 exhibited widespread spectrum of antimicrobial activity against both Gram positive and Gram negative bacterial strains (Table 2). The C band of *Microcystis* MBKG16 Rf value is 0.84 has shown inhibition zones of 12mm, 8mm, 8mm, 20mm, 10mm on *S. aureus*. *E. coli, K. pneumonia, S. paratyphi, M. luteus* respectively. *Microcystis* MBKG08, MBKG32 have shown remarkable inhibitory effect on *S. paratyphi* and *M. luteus* (17mm, 10mm). D and E spots of *Dictyospaerium* shown good antibacterial activity on *E. coli, K. pneumonia, S. paratyphi* and *M. leteus*. The spots E and F (Figure 1) of *Oscillatoria* MBKG02 inhibited the growth of *E. coli* with maximum inhibitory zone of 22mm, *S. paratyphi* with 19mm (Figure 2).

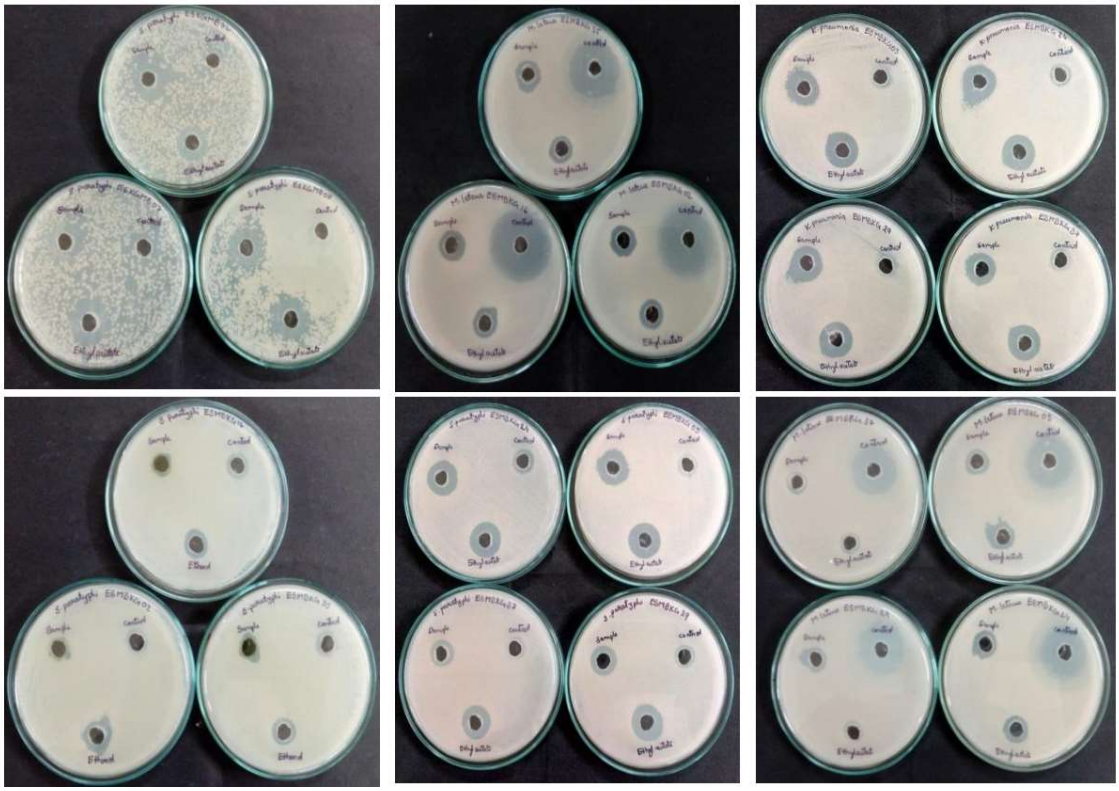

Figure 1. Zone of Inhibition of cyanobacterial extracts against *S. aureus, E. coli, S. typhi, S. paratyphi, M. luteus, K. pneumonia*

Figure 2. TLC developed in solvent system (ethyl acetate: methanol:water (7:2:1 v/v) MBKG: Microbiology Kuraganti Guna

Ethyl acetate extracts of *Chroococcus sp* MBKG24, *Anabeana sp* MBKG29 showed antibacterial activity against *S. aureus. E. coli, K. pneumonia* and *S. paratyphi*. The results showed that broad spectrum antibacterial activity was predominantly observed by all the cyanobacterial strains, due to secondary metabolites secreted by them, which may have various functional groups like carbonyl, nitro methane, alcoholic and phenolic groups (Maheep et al., 2014). Many researchers reported that *Nostoc* produced bioactive compounds with antibiotic activity against *S. aureus, Bacillus cereus* (Mian et al., 2003). Skulberg (2000) reported that antibiotics produced by *Nostoc sp* inhibits the growth of bacteria, notably multidrug resistance *S. aureus* and biocide resistance *P. aeruginosa*. Those results are in harmony with the report of our cyanobacterial strains which have biocidal against Gram +ve, Gram –ve pathogenic bacteria. The ability to produce antimicrobial compounds may be significant not only as a defensive mechanism for the cyanobacterial strains but also a good source of the new bioactive compounds from medical point of view. In this work crude ethyl acetate extract of the *Dictyospaerium*, *Microcystis* were found to be active when compared with other extracts. Cyanobacteria make collection of bioactive blends including 405 lipopeptides, 5.6% amino acids, 4.2% fatty acids, 4.2% macrolides, 9% amides (Singh et al., 2005). Many of these compounds have been reported to posses antibiotic, pharmacological effects such as antibacterial, antifungal, antiviral, anticancer activities (Mian et al., 2003; Ghazala and Shameel, 2005; Soltani et al., 2005). The use of organic solvents provides more efficiency in extracting compounds which have antimicrobial activity (Cardozo et al., 2007; Mohammed et al., 2015). These cyanobacterial strains exhibit antibacterial activity which makes them significant species for screening of natural products. Therefore it is important to pay more attention to develop antibiotics which could fight against many dreadful diseases.

Conclusion

The study reveals that all the cyanobacterial TLC bands of extracted compounds showed considerable antibacterial activity with ethyl acetate extracts of *Oscillatoria* strains (MBKG02, 03) against *E. coli* (22mm,16mm,6mm), *S. paratyphi* (19mm,10mm,8mm). *Microcystis* strains (MBKG 16,08,32,07) showed activity with zone diameter of 20mm,17mm,15mm,16mm against *S. paratyphi* whereas 12mm,13mm,8mm,4mm against *S. aureus*.. The study emphasizes the importance of exploring the bioactive metabolites from cyanobacteria and their antimicrobial activity against pathogenic microorganisms for future biotechnological applications. However whether such extracts will act as effective therapeutic agents remains to be investigated.

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Conflicts of interest

There are no conflicts of interest among the authors.

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