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Original Article

IN VITRO ASSESSSMENT OF ANTIOXIDANT AND ANTIMICROBIAL ACTIVITIES OF DIFFERENT SOLVENT EXTRACTS FROM LICHEN RAMALINA NERVULOSA

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ABSTRACT

Objective: The purpose of the existent study is to assess the *in vitro* antioxidant and antimicrobial activity of different solvent extracts of *Ramalina nervulosa.*

Methods: Folin ciocalteu's and Aluminium chloride method were used for total phenolic and flavonoid content estimation respectively. The antioxidant activity was evaluated through free radical scavenging activity by DPPH (2, 2-diphenyl-1-picrylhydrazyl), FRAP (ferric ions reducing antioxidant potential), cupric ion chelation ability. Agar diffusion method was used to assess the antimicrobial capacity. GC-MS analysis was carried out to identify the presence of compounds in the extracts.

Results: Ethanol extracts exhibited that the highest zone of inhibition with *Bacillus subtilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa, Klebsiella pneumoniae* and with two fungal strains such as *Fusarium oxyparum* and *Penicillium notatum.* The extract also has high inhibition capacity of 64.29% and 62.49% towards DPPH and Cu^{2+}ion respectively. But the aqueous extract showed higher amount of phenolic and flavonoid content of about 211.40 mg GAE/g dw and 50.72 mg QE/g dw respectively and Fe^{2+} ion reducing capacity of about 144.41 mol ascorbic acid equivalent/g db. Further analysis of GC-MS showed the presence of various bioactive molecules present in the lichen *Ramalina nervulosa*.

Conclusion: The results of FRAP assay were positively correlated with the total phenolic and flavonoid content. Ethanol extracts of *R. nervulosa* showed promising antibacterial, antifungal and antioxidant activities which can be considered as the potent source of antioxidant product and offer protection from oxidative stress under many pathophysiological conditions.

Keywords: DPPH, FRAP, Antioxidant, GC-MS, Free radical scavenging activity.

INTRODUCTION

Reactive Oxygen Species (ROS) or the free radical is a highly reactive atom capable of destructing all the biomolecules. An antioxidant is a molecule that hindering the oxidation of other molecules by protecting the key cellular components from the free radicals. During the unsympathetic condition, there is a dynamic imbalance between the amounts of free radicals and level of antioxidant to scavenge which lead to potential oxidative damage to DNA, proteins and several other biomolecules and fails to protect the body against ruinous effect [1, 2]. These free radicals may be either oxygen derived reactive oxygen species (ROS) such as hydroxyl (-OH), superoxide (O_2^-), peroxyl (ROO^-), alkoxyl (RO^-) as free radicals and hydrogen peroxide (H_2O_2) as non-radical or nitrogen derived (RNS, reactive nitrogen species) such as nitric oxide (NO^-), nitrogen dioxide (NO_2), dinitrogen trioxide (N_2O_3). The altered antioxidant balance during the increased production of oxidative species is called oxidative stress which is an important etiological factor concerned in several chronic disorders such as neurodegenerative diseases, cancer, diabetes mellitus, coronary heart ailments and aging process [3-5]. Many antioxidant compounds possess anti-cancerous, anti-inflammatory, anti-carcinogenic, anti-bacterial or anti-viral activities to a greater or lesser level [6-9].

Plants were producing a wide array of secondary metabolites such as phenolic compounds (phenolic acids, flavonoids, quinines and coumarins), nitrogen compounds (alkaloids and amines), vitamins, terpenoids and other secondary metabolites that have been proven as antimicrobial, antioxidant and antineoplastic agents [10]. These antioxidants have the wide range of biological effects that neutralize the oxidation of biological molecules by scavenging free radicals and chelating metals [11]. Today's challenges facing in any pharmaceutical industry lies in the discovery and development of new, pharmacological active molecules. Metabolites produced by microorganisms and fungi in particular, are a resource for which the therapeutic potential has been recognized but the one that remains largely unexplored and unexploited is approximately 20% of all the known fungal species which are obligate symbionts in lichens.

Lichens are nutritionally specialized fungi living together in symbiotic association with green algae and sometimes with cyanobacteria (or both). The growth condition occurs in diverse ecological locale where they subjugate at various substrates such as soil, barks of trees and rocks. Even though the lichens and lichen products have been used in traditional medicines for centuries and still hold considerable interest as alternative treatments in various parts of the world [12, 13]. The traditional usage of lichen in various medical applications remains conclusive and its properties were unexplored. In this regard, the present investigation was the first study report of lichen *R. nervulosa* in the Yercaud hills of Tamil Nadu. In fact lichen metabolites exert a wide variety of biological actions including antibacterial, anti-mycobacterial, antibiotic, anti-inflammatory, antiviral, anti-proliferative and cytotoxic effects. India has a rich diversity of lichen habitation which encompass of about 2450 (12.25%) species of which about 23% are endemic to the country. The prime need of the present study was to study the efficiency of various extracts of *R. nervulosa* to hinder the free radical and transition metal ion by *in vitro*. The objective of the present investigation was to determine the polyphenolic, flavonoid content and free radical scavenging activity determination via the reaction with the stable DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical and the ferric ions reducing capabilities were determined using the FRAP (ferric ion reducing antioxidant potential) assay and cupric ion concentration by CCA (cupric ion chelation assay). Further the proposed plan was to study the efficacy of antimicrobial activity of different extracts against the clinical pathogenic microorganisms. The results obtained from the present study will provide the basic data for understanding the antioxidant as well as antimicrobial properties of the different lichen extracts and allow the further researchers to involve in the development of pharmaceutical products.

MATERIALS AND METHODS

Plant material and collection

The lichen sample *Ramalina nervulosa* was obtained from Yercaud (Shevaroy hills) Tamil Nadu State, India according to the study from Sanjeeva *et al.* [14]. The samples were collected and stored in the KSRCT/BT lichen herbarium. The specimens were botanically identified as *Ramalina nervulosa* using the colour spot test such as *K test, C test, KC test, PD test* and confirm it through chromatography analysis using TLC.

Extraction

Samples were dried and grounded into powder using a mortar and pestle. The extract was obtained using Soxhalet with the help of three different successive solvent systems such as methanol, petroleum ether and water (aqueous).

Antimicrobial activity

Antimicrobial activity of different solvent extracts was evaluated by four bacterial strains such as *Bacillus subtilis* MTCC 441*, Proteus vulgaris* MTCC 1771*, Pseudomonas aeruginosa* MTCC 424*, Klebsiella pneumoniae* MTCC 3384 and five fungal strains as *Fusarium solani* MTCC 350, *Fusarium oxyparum* MTCC 1755, *Aspergillus Flavus* MTCC 277, *Aspergillus niger* MTCC 1344, *Penicillium notatum* MTCC 5108. Bacterial strains were cultured in nutrient agar plate and fungal species in potato dextrose agar (PDA). Both bacterial and fungal species were incubated at 37ºC for 24 h and 28 ºC for 72 h respectively. The antibacterial and antifungal activities of three solvent extracts of *Ramalina nervulosa* were determined by disc diffusion method [15]. Briefly 100 µl of suspension containing 10^8CFU/ml of bacteria cells and 10^4spores/ml of fungi were spread on Petri plates containing nutrient agar and PDA medium respectively. Tetracycline (10 µg/disc) and ampicillin (10 µg/disc) were used as a positive control for bacteria and fungi respectively. Discs without samples were used as a negative control. The inoculated plates were incubated for 24 h for pathogenic bacterial strains and 72 h for fungi isolates at 37 °C and 28 °C respectively. Antimicrobial activity was calculated by measuring the diameter of zone of inhibition in mm. The formation of an inhibition zone was made as triplicate values.

Total phenolic content determination

Total phenolic content of all the three extracts was determined using gallic acid as the standard solution (40-200 µg/ml) with slight modification from Slinkard and Singleton method [16]. 0.1 ml of the extracts were mixed with 1 ml of Folin ciocalteu's phenol reagent and vortexed vigorously and after 3 min mixed with 3.0 ml of 2% (w/v) Na_2CO_3. Reaction mixture was incubated for 2 hours at 37 °C and then the absorbance was measured at 760 nm. The total phenolic contents (TPC) of the extracts were expressed as mg gallic acid equivalents (GAE) per gram of extract on dry basis (db).

Total flavonoid content determination

Total flavonoid was determined by the method of Ordonez [17]. Briefly add 0.03 ml of 5% $NaNO_2$ with 0.1 ml of extracts along with 0.3 ml of distilled water. Incubated the reaction mixture for 5 minutes at 25 °C. To that add 0.03 ml of 10% aluminium chloride and add further of 0.2 ml of 1 mM NaOH after 5 min of incubation at room temperature. Diluted the whole mixture with 1 ml of distilled water and read the absorbance at 510 nm. Results were expressed as mM of flavonoid content per gram of dry extract powder as quercetin equivalent (QE).

DPPH free radical scavenging assay

The free radical scavenging activity was assayed by Genesys 10-S according to the method described by Leong and Shui [18] using DPPH as free radical. 0.1 mM solution of DPPH (2, 2-diphenyl-1-picrylhydrazyl) was prepared in methanol. An aliquot of 100 µl of different extracts was added to 3 ml of methanolic DPPH solution. The reaction mixture was shaken vigorously and incubated at 37ºC for 30 min in dark. The decrease in absorbance was measured at 515 nm using ascorbic acid as the standard. The scavenging activity of free radical (DPPH) in % was calculated by the following equation: $(A_0-A_1)/A_0 \times 100\%$ where A_o=Concentration of DPPH without extract and A_1= Absorbance of DPPH in the presence of sample.

Ferric reducing antioxidant potential (FRAP) assay

The ability to reduce ferric ions (Fe^+) of each extract was measured using the modified version of Benzie and strain method [19]. An aliquot of 100 µl of an extract was added to 1.5 ml of FRAP reagent (10 parts of 300 mM sodium acetate buffer at pH 3.6, 1 part of 10 mM.

TPTZ solution and 1 part of 20 mM $FeCl_3 \cdot 6H_2O$ in the ratio 10:1:1). The absorbance was read against the blank containing 1.5 ml of FRAP reagent and 0.1 ml of distilled water without the extract. The reaction mixture was incubated in a water bath at 37ºC for 10 min and the increase in the absorbance at 593 nm was measured. The antioxidant capacity based on the ability to reduce ferric ions of each extract was expressed as ferrous sulphate or ascorbic acid equivalent per gram of the extract.

Cupric reducing antioxidant capacity (CUPRAC) assay

The amount of cupric ion chelated was determined according to the Ruch method [20]. To 0.5 ml of extract, add 1 ml of $CuCl_2$. $2H_2O$ (0.01 M), 1 ml of ammonium acetate buffer of pH 7.0 and 1 ml of neocaproin solution (0.075M), The final volume of the reaction mix was made to 4 ml with distilled water and incubates the total mix at 37 °C for 1 h. The decrease in absorbance was measured at 450 nm using ascorbic acid as the standard.

Gas chromatography-mass spectrometry (GC-MS) analysis

The GC-MS analyses of the ethanolic extract were conducted with GC-MS-QP 2010 [Shimadzu, Japan]. Helium was used as the carrier gas with a flow rate of 1 µl per minute and Vf-5ms column was utilized. The injector and interface were operated at 250 and 380 °C, respectively. The oven temperature was raised from 70–290 °C at a heating rate of 5 °C min^{-1} and then held isothermally for 10 min. Helium at a flow rate of 1.0 ml min was used as the carrier gas. The sample of 1 µl of the ethanol extract (1:100) was injected in a pulsed split mode (the flow was 1.5 ml min^{-1} for the first 0.5 min and then set to 1.0 ml min^{-1} throughout the remainder of the analysis; split ratio 40:1). The MS conditions were as follows: ionization voltage of 70eV, acquisition mass range 35-500 and scan, time 0.32 s. The compounds were analysed using the chemical database.

Statistical analysis

For *in vitro* antioxidant activity of the extracts, the results were expressed as mean±standard error mean (SEM) (n = 3) and subjected to one-way analysis of variance (ANOVA) using Graph Pad Prism (Version 5.01, Graph Pad, Inc).

RESULTS

Antimicrobial activity

The results of *in vitro* testing of antimicrobial potential of three sequential extracts of *R. nervulosa* was tested against the different microorganisms in a job by the presence or absence of the zone of inhibition are shown in the table 1. The maximal inhibition zone for the tested microorganisms was in the range of 3.50-1.13 mm and 1.5-1.0 mm for bacteria and fungi respectively. The extracts showed the greater activity towards two bacterial species as *B. subtilis* and *Proteus vulgaris* but it had better effect towards most of the fungus. In particular, the analysis shows that all extracts had greater inhibition effect towards *Aspergillus niger, Aspergillus flavus* and PE extract towards *F. solani.* In antibacterial studies, all the extracts showed the greater potential towards two bacterial species as *B. subtilis* and *Proteus vulgaris* and there is no effect on the excluded bacterial species.

Total phenolic and flavonoid content

Phenolic compounds have the high level of antioxidants because they acts as the hydrogen donor and the single oxygen quenchers [21]. It has been demonstrated that antioxidant capacities of lichen extracts are dependent on their phenolic content [22]. The total

phenolic content of different solvent extracts of *R. nervulosa* was in the range of 211.40 to 43.67 mg GAE/g. The highest phenolic content was found in aqueous extract and lower in ethanol extract. The total flavonoid content was ranged from 20.16 to 50.72 mg QE/g and the higher content was found as same as phenolic content in aqueous extract.

Table 1: It shows zone of growth inhibition (mm) of *R. nervulosa* extracts against the clinical pathogens

Microorganisms		Diameter of Zone of Inhibition (mm)[a]			
		Extracts			Antibiotic
		PE	EE	AE	Control
Bacteria	*Bacillus subtilis*	2.17±0.12	2.57±0.12	1.67±0.15	1.20[1]
	Proteus vulgaris	2.20±0.15	3.50±0.17	2.37±0.18	1.77[1]
	Pseudomonas aeruginosa	1.13±0.15	1.27±0.12	0.00±0.00	2.53[1]
	Klebsilla pneumonia	1.43±0.18	1.30±0.12	1.97±0.09	2.53[1]
Fungus	*Fusarium solani*	1.47±0.12	1.00±0.12	1.23±0.15	1.33[2]
	Fusarium oxyparum	1.23±0.15	1.37±0.18	1.27±0.09	1.43[2]
	Aspergillus flavus	1.50±0.15	1.23±0.15	1.20±0.10	1.10[2]
	Aspergillus niger	1.23±0.15	1.07±0.09	1.13±0.13	1.13[2]
	Penicillium notatum	1.27±0.09	1.30±0.15	1.17±0.07	1.37[2]

The values were expressed in mean±SEM (n=3) and PE, EE, AE represents the petroleum ether extracts, ethanolic extracts, aqueous extracts respectively, [a]Inhibition zone diameter (mm) around the well by adding 20 µl of three different extracts, [1]Tetracycline as standard for bacteria and [2] Ampicillin as standard for fungus.

Table 2: It shows total phenolic and total flavonoids content of *R. nervulosa* extracts

Extracts	Total phenolic content (mg GAE/g dw)	Total flavonoids content (mg QE/g dw)
Ethanol extract (EE)	43.67±1.230	20.16±0.11
Petroleum ether extract (PE)	158.23±1.77	31.18±0.10
Aqueous extract (AE)	211.40±1.07	50.72±0.13

The values were expressed in mean±SEM (n=3) and GAE mg/g dw, QE mg/g dw, represent mg of Gallic acid equivalents, mg of Quercetin equivalents per g of dried extract respectively.

Ferric reducing power capacity

Results of ferric ion reducing power of *R. nervulosa* extracts were summarised in the fig. 1. The ability of an antioxidant to read the absorbance from the reduction of ferric-TPTZ (Fe(III)-TPTZ) complex into blue ferrous-TPTZ (Fe(II)-TPTZ) complex is based on the breaking of free radical chain by a single electron [23, 24]. The reducing ability of the ferric ion by the extract ranges from 44.840 to 144.410 of mol ascorbic acid equivalent per g db.

The results indicated that the ferric ion reducing power of the extract is due to the presence of high amount of phenolic content and the termination of free radical reaction occur while reacting with free radical and donated electron.

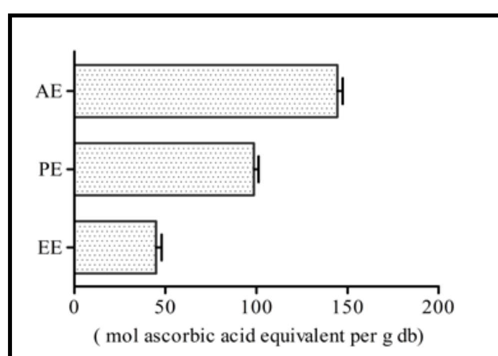

Fig. 1: Ferric reducing antioxidant power of different solvent extracts of *R. Nervulosa*

DPPH scavenging activity

Analyses from the fig. 2 concluded that all the extracts of *R. nervulosa* have higher scavenging effect with increase in the concentration of the extracts. The extracts of our study shows higher inhibition activity of free radicals about 64.29%, 15.67% and 55.55% even at low concentration of 100 µg/ml for ethanol, petroleum ether and aqueous extracts respectively.

The results revealed that the ethanol extract have higher DPPH free radical scavenging activity. The DPPH scavenging activity is also described as the IC_{50} value 0.023 for aqueous extract.

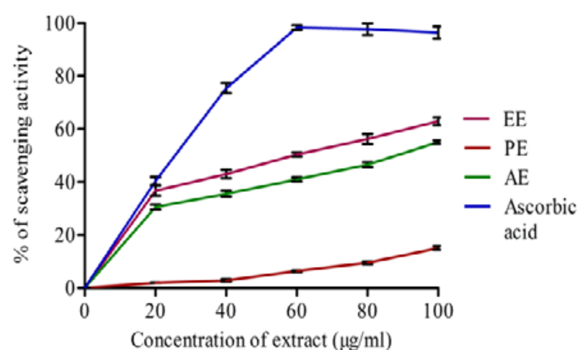

Fig. 2: DPPH radical scavenging abilities of different solvent extracts of *R. nervulosa* (n=3, mean±SD)

Cupric ion chelation activity

The chelation of cupric ion by the different extracts of *R. nervulosa* was shown in the fig. 3. The CUPRAC assay is based on the reduction of $Cu^{2+}$to Cu^{+} by antioxidants present in the extract. It was reported that the results obtained from the *in vitro* cupric ion (Cu^{2+}) reducing measurements obtained as 62.49%, 33.43% and 44.49% at a concentration of 100 µg/ml for ethanol, petroleum ether and aqueous extracts respectively. Similar to the result of DPPH, the

ethanol extracts also has high range of Cu^{2+}ion chelating activity. Thus the results showed that the extract has the ability of chelating agent by rendering the ions catalytically silent.

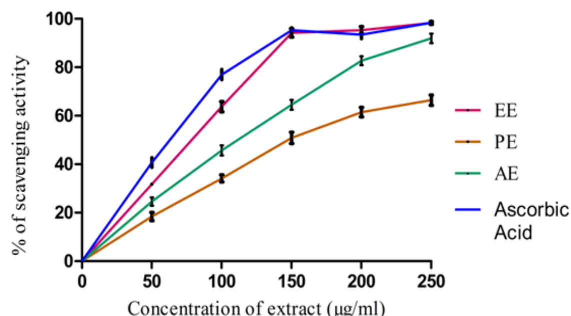

Fig. 3: The cupric ion chelating activities of various solvent extracts of *R. nervulosa* (n=3, mean±SD)

GC-MS analysis

Totally 25 compounds were identified in the ethanol extracts of *R. Nervulosa* and the identification is based on the peak area (%) and the retention time which was shown in the table 3.

The results of the above extract of R. *Nervulosa* shown the presence of three major compounds as azulene bicyclo decapentaene (8.367%), 9-octadecenoic acid, (e)-trans-. delta (16.14%) and stigmast-5-en-3-ol (41.88%).

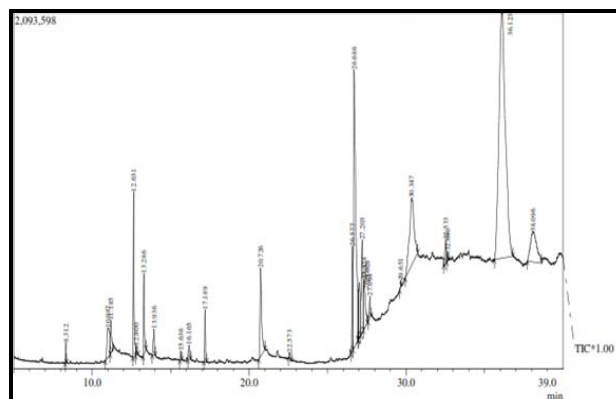

Fig. 4: GC-MS chromatogram with active ingredients of ethanolic extract of *R. Nervulosa.*

Table 3: It shows GC-MS analysis of ethanolic extract of *R. nervulosa*

Peak No	Retention time	Area (%)	Compound name
1	8.312	8.367	azulene bicyclo decapentaene
2	10.987	2.13	5-methyl-1,3-benzenediol
3	11.18	0.970	biphenyl 1,1'-biphenyl bibenzene
4	12.631	3.38	1-ethoxy-2-methoxy-4-methylben-
5	12.800	0.22	phenol, 3,5-bis(1,1-dimethylethyl)
6	13.286	1.67	5-sec-butylpyrogallol 5-sec-butyl-1,2,3-
7	13.936	0.7	1,2-benzenedicarboxylic acid
8	15.636	0.19	bicyclo[5.3.0]dec-1(7)-ene-2,5-dione
9	16.165	0.42	2-methoxy-4-amino-6-methylphen-
10	17.189	1.09	5,7-dihydroxy-4-methylcoumarin
11	20.726	3.71	n-hexadecanoic acid hexadecanoic acid
12	22.573	0.20	3,5-decadien-7-yne, 6-t-butyl-2,2,9,9-tetramet-
13	26.577	2.73	9,12-octadecadienoic acid
14	26.68	16.14	9-octadecenoic acid, (e)-trans-. delta-
15	26.950	0.44	2-amino-4-carbamoyl-6-piperidino-1,3,5-triaz-
16	27.028	2.55	9,12,15-octadecatrienoic acid, (z,z,z)-
17	27.203	3.77	2-aminoethanethiol hydrogen-
18	27.328	2.24	ethyl oleate 9-octadecenoic acid (z)
19	27.694	0.59	3-octadecyldihydro-2,5-furandio-
20	29.651	0.15	hexadecanoic acid, 1-(hydroxymethyl)-1,2-
21	30.347	8.32	stigmasterol stigmasta-5,22-dien-3-ol-
22	32.533	0.82	tridecanedial
23	32.640	0.25	butyl 6,9,12,15-octadecatetraenoate
24	36.123	41.88	stigmast-5-en-3-ol-
25	38.096	5.16	cholest-5-en-3-ol, 24-propylidene

DISCUSSION

Evidence in literature showed that the methanolic and aqueous extract of lichen *Peltigera* sp. showed the higher effect towards the bacteria *Pseudomonas aeruginosa, Aspergillus niger* and several *Fusarium* sp [25]. However, the results revealed that petroleum ether extracts shows maximum activity to almost all the microorganisms. In a study of Tatjana *et al.* [26] mentioned that the highest phenolic and flavonoid content was found in *Hypogymnia physodes* and *Parmelia sulcate* lichen respectively. Similar results of our study revealed that the extracts of *R. nervulosa* have very high phenolic content for the potential of antioxidant activity. By comparing the DPPH values with the previous study of Luo *et al.* [27] found that the inhibition percentage reached 55.8% at the concentration of 330 µg/ml in *Ramalina conduplicans* extract. But our results were also supportive that *R. nervulosa* have strong free radical scavenging activity and acts as greater constituents in food against the oxidative damage [28]. Olivetol (33.5%), atraric acid (17.2%) and olivetonide (15.7%) were the major components of the *H. Physodes* (parmeliaceae lichens) extract [29]. Gas chromatography study helped to analyse the chemical constituents present in the ethanol extracts. It will be used to find out the new drug molecule for the various properties like antimicrobial agents as well as antioxidant drug molecules.

CONCLUSION

The findings of the present study state the significant presence of antimicrobial and antioxidant activity in three different solvent extracts of *R. nervulosa.* The lichen possess rich amount of phenolic and flavonoid contents. Due to this presence, the lichen extracts have free radical scavenging activity and also having equivalent activity against bacteria and fungi. From this evidence, there is a good scope in developing natural medicine to decrease oxidative stress and microbial attack.

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CONFLICT OF INTERESTS

We do not have any conflict of interest

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