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Original Article

ETHNOBOTANICAL SURVEY AND SCIENTIFIC VALIDATION OF MEDICINAL PLANTS USED IN THE TREATMENT OF FUNGAL INFECTIONS IN AGUMBE REGION OF WESTERN GHATS, INDIA

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ABSTRACT

Objective: Western Ghats is one of the plant biodiversity hot spot of India. Agumbe region of Western Ghats is known for rich plant diversity and traditional medicinal practices. The aim of the study was to document ethnomedicinal practices followed in this region to treat fungal infections and their scientific validation *in vitro*.

Methods: An ethnobotanical survey was conducted to identify the plants used to treat fungal infections in Agumbe region of Western Ghats, India. Aqueous extracts of the plants selected based on the ethnobotanical survey were subjected to *in vitro* antifungal activity assay. Significantly active plant extract was subjected to activity guided separation methods. The active fraction was subjected to phytochemical, TLC bioautographic and IR spectral analysis to identify the active principle.

Results: Ten plants were identified to be used to treat fungal infections. Scientific validation by *in vitro* antifungal activity assay revealed significant inhibitory activity by aqueous extract of *Combretum latifolium.* Phytochemical and Infra red spectral analysis of the active fraction revealed the presence of saponins, tannins and phenolic compounds.

Conclusion: The study presents the first report of antifungal potential of *Combretum latifolium* and *Humboldtia brunonis*. High polar tannin is identified as the antifungal active principle in the aqueous extract of *C. latifolium*.

Keywords: Antifungal, Western Ghats, Ethnobotanical, *Combretum*, Phytochemical, Tannin.

INTRODUCTION

The use of herbal medicine is evidenced as early as Neanderthal period. They are now in great demand, because of their better cultural acceptability, minimum side effects and better compatibility with the human body and not because they are less expensive [1]. Many of the rural populations are heavily dependent on traditional medicine systems. In the recent years, there is an exponential increase in the number of patients seeking an alternate medicine in place of conventional drugs [2, 3].

The complaints of athlete`s foot, dandruff, ringworm etc., caused by infectious fungal strains are common in tropical countries. Treatment of fungal infections is complicated by the emergence of resistant strains to the currently available antifungal agents. The antifungal agents are not only limited in number and costly, but are also associated with toxic side effects [4].

The aim of the study was to investigate the plant species used in traditional medicine to treat fungal infections in Agumbe region of Western Ghats, Karnataka, India and to determine the antifungal potentiality. Agumbe is the highest rain fall region of Karnataka with a mean annual rainfall of 7000-7500 mm, located at 12°55'N latitude and 74°53'E longitude at an elevation of 328.0 ft above the sea level, covered with an evergreen rain forest. The recent floristic survey carried out by the Foundation for Revitalization of Local Health Traditions has recorded 371 plant species of which 182 are medicinal [5]. This region was chosen for the study due to i) Access of local people to the rich plant diversity ii) Good knowledge of traditional medicinal practices iii) Least explored for ethnobotanical survey with respect to skin diseases exploiting wild phenerogams.

Agumbe region of Western Ghats has not been studied with reference to the use of plants to treat fungal infection/ diseases of skin. Hence the present investigation was undertaken to document the use of plants by herbalists and to justify their use by scientific validation.

MATERIALS AND METHODS

Ethnobotanical survey

An ethnobotanical survey on the medicinal uses of the test plants used against skin infections was conducted in the Agumbe region of Western Ghats. The villages covered were Agumbe, Someshwara and Hosagadde of Thirthahalli taluk, Shimoga, Karnataka, which spreads over an area of 1247 km2 with a population of 143 207 belonging to different communities with an unique ethnic groups such as Havyaka Brahmins and Sanketis [6-8].

The survey was carried out through a semi structured open ended interviews as suggested by Martin [9]. The subjects identified for the interview included Traditional healers, aged ladies as identified by the local people who have good knowledge of home remedies and forest department staff which included watchers and guards.

A simple questionnaire was developed for collecting the data with regard to the medicinal uses of the plants with special reference to fungal infections. One part of the questionnaire consisted of general questions regarding the plants generally used for skin infections and the data about the traditional healers. The second part of the questionnaire consisted of more specific questions regarding the specific plants used for dry skin infections, skin rashes coupled with itching and also the plants used for the treatment of dandruff.

The subjects were shown specific photographs of common fungal infections like Ring worm (Tinea corporis), Athlete's foot (Tinea pedis), Nail ringworm (Tinea unguium), Jock itch (Tinea cruris), dandruff etc., to make them aware of the symptoms with regard to the medicinal plants used for treating such symptoms.

The subjects were not pressurized to reveal their knowledge and were convinced that the information is exclusively for academic purpose. The survey was conducted twice in the same year choosing the same subjects to check the veracity of the information provided by them. Only such information which common between both the surveys has been included. The claims were compared with the

available reports emphasizing Indian floristic diversity, ethnobotany and medicinal plants [10-13] to find out the new claims of the plants used against skin infections.

Test plants

Based on the ethnobotanical survey following ten plants which are used in traditional medicine for skin infections were selected for scientific validation of traditional medicine. They are *Apama siliquosa, Barringtonia acutangula, Callicarpa lanata, Combretum latifolium, Dichapetalum gelonioides, Holigarna arnottiana, Humboldtia brunonis, Hydrocotyle javanica, Persea macrantha* and *Vateria indica*. The plant materials were collected in summer and authenticated specimens have been deposited at the herbarium of Department of Studies in Botany, Manasagangotri, University of Mysore, Mysore, Karnataka, India.

Preparation of aqueous extract

Apparently healthy leaf materials were collected washed thoroughly in running tap water. Ten gm of fresh leaf material was ground in 100 ml of distilled water, filtered with Whatman No.1 filter paper, sterilized and lyophilized. The extracts were then dissolved in sterile distilled water to a final concentration of 100mg/ml and stored at 4°C in a closed container until use [14].

Test fungi

Human pathogenic fungi *Candida albicans* (*C. a*) (MTCC 183), *Trichophyton rubrum* (*T. r*) (MTCC 296), *Microsporum gypseum* (*M. g*) (MTCC 2830) and *Microsporum canis* (*M. c*) (MTCC 2820) obtained from MTCC, Chandigarh and one clinical isolate each of *Aspergillus fumigatus* (*A. f*) and *Penicillium* sp. (*P.* sp) obtained from Mandya Institute of Medical Sciences, Mandya, Karnataka, India served as test fungi.

Antifungal activity assay

Preparation of inoculum

Cultures were grown on Sabouraud dextrose agar media for 48-72h at 37 °C. Colonies of similar stages of development were selected and inoculated into Sabouraud dextrose broth and inoculum turbidity was adjusted to 0.5 McFarland standards according to CLSI protocol [15].

Disc diffusion method

Disc diffusion method was performed according to CLSI M44-A document. 100 µl of the inoculum was seeded on the plates containing SDA medium. The plates were allowed to dry for 3-5 min. 100 µl of the extract was loaded to the sterile discs of 8 mm diameter and placed on the test plates. The plates were incubated at 37 °C for 48h for yeast and 72-96h for filamentous fungi. The diameter of the inhibition zones were measured in mm [15]. Discs loaded with respective solvents served as negative control and standard antifungal drugs Amphotericin-B (50 µg/disc), Fluconazole (25 µg/disc), Nystatin (50 µg/disc) and Miconazole (50 µg/disc) served as positive control. All tests were performed in triplicates.

Liquid-liquid partition and acid-base separation of the extract

The aqueous extract of *C. latifolium* which recorded significant antifungal activity was subjected to further studies for purification of the active principle by separation methods and each of these fractions was subjected to antifungal activity assay as described earlier.

Fifty ml of 1mg/ml concentration of aqueous extract was subjected to liquid-liquid separation with butanol and chloroform. Three fractions viz., butanol, chloroform and the remaining third aqueous fraction were collected. All the fractions were evaporated to dryness under reduced pressure using rotary evaporator and subjected to antifungal activity assay by disc diffusion method. Acid-base separation of the extract was carried out in a separating funnel with 5% Sodium bicarbonate, 5% Sodium hydroxide, 5% HCl and Diethyl ether [16].

Minimum Inhibitory Concentration (MIC)

MIC was determined by 96 well microtiter plate method according to Lee et al. [17] with some modifications. 100 µl of the inoculum was seeded in the wells of a 96-well microtiter plate containing 100 µl SDB media. 100 µl of extract was serially diluted to each well and the cell suspension was incubated for 24h at 37 °C. Ten µl of MTT [3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide] solution was added to each well and the plates were incubated further at 37 °C. 30 µl of 20% (w/v) SDS solution containing 0.02 mol HCl was then added and the plates were incubated at 37 °C for 16h to dissolve the formazan crystals that had formed [18, 19]. The turbidity of each well was measured at 630 nm using microtitre ELISA reader (Labtech LT-4000).

Phytochemical analysis

The third fraction of the aqueous extract which recorded significant antifungal activity was subjected to preliminary phytochemical analysis following the methods described by Harborne [20].

IR analysis of the active fraction

The aqueous fraction obtained from liquid-liquid separation which recorded significant antifungal activity was subjected to FT-IR spectral analysis using KBr discs on Jasco FT-IR 4100 infrared spectrometer.

TLC and bioautography

Aqueous active fraction 3 of liquid-liquid separation was subjected to Thin layer chromatography using three solvent systems chloroform: ethyl acetate: formic acid (CEF) (20:16:4), benzene: ethanol: ammonium hydroxide (BEA) (36:4:0.4) and ethyl acetate: methanol: water (EMW) (40:5.4:4), which were identified as the best solvent systems for the separation of phytochemicals in combretaceae members [21]. The TLC chromatograms were sprayed with potassium iodate solution and observed for the presence of tannins [22]. The developed chromatograms were subjected to agar overlay bioautography [23].

RESULTS AND DISCUSSION

The results presented in this paper are related to two parts of the investigation. The first part concerned with the ethnobotanical uses of the plant investigated. The second part concerned with the antifungal activity and phytochemical analysis of *C. latifolium*.

Ethnobotanical survey

Ethnobotanical survey has revealed that a notable/significant number of plant species were used by the local population to treat fungal infections. The present study documents the selected ten wild phenerogams (table 1) used exclusively against infectious diseases of both bacteria and fungi in general and skin infections, itching, wound dressing in particular. Significant attention has been drawn by these plants because most of the plants are exclusively confined to the study area, least availability of the earlier reports and were not scientifically validated with respect to the antifungal activity.

Among these ten plants, first report on the ethnomedicinal use of *C. latifolium* and *H. brunonis* were claimed from our laboratory [24] wherein *in vitro* antibacterial activity against both human and plant pathogenic bacteria were demonstrated. Although ethnobotanical survey with respect to plants used in treatment of wounds and skin diseases was carried out in Uttara Kannada district (nearby district of study area), the reports have not cited the use of these plants [8, 25].

Antifungal activity assay

Aqueous extract of *C. latifolium* significantly inhibited the growth of all the test fungi except *C. albicans* and the aqueous extracts of remaining plants did not show activity (table 2). The zone of inhibition was concentration dependent and it increased with increase in the concentration of the extract. Maximum inhibition of 31.33 mm was observed against of *M. canis* at 100 µl followed by *T. rubrum, M. gypseum, Penicillium* sp. and *A. fumigatus* (table 3). Significant inhibition in the range of 10-19 mm was observed against all the test fungi except *A. fumigatus* and *C. albicans* at the lowest test concentration of 10 µl/disc.

Among the standard antifungal discs tested for their inhibitory efficacy against the test fungi revealed significant inhibition of all test fungi by Nystatin and Miconazole. Amphotericin-B did not

inhibit the growth of *M. canis, M. gypseum* and *T. rubrum* while Fluconazole did not inhibit the growth of *A. fumigatus, M. canis, M. gypseum* and *Penicillium* sp. at the respective recommended test concentrations. The comparative efficacy of different concentrations of the aqueous extract with that of the standard antifungal agents at the respective doses revealed that 25 µl concentration of the crude extract produced the inhibition zone of test fungi equal to that of Nystatin (50 µg) and Miconazole (50 µg).

Table 1: Ethnobotanical results concerning ethnomedicinal uses of the plants species documented during the survey

Name of the Plant	Family	Part used	Reported literature on ethnomedicinal uses	Ethnomedicinal uses as reported during expedition
Apama siliquosa Lamk.	Aristolochiaceae	Leaves, Root, Seeds	Healing ulcers, burns, cholera, diarrhea, dysentery, leprosy, skin diseases	Stomach ache, poison bites, burns, diabetes, dysentery, skin itching
Barringtonia acutangula (L.) Gaertn.	Lecythidaceae	Bark, Fruit	Skin diseases, diarrhea, dysentery, malaria	To treat cold in chest and fever in children, Skin diseases, joint pain in pregnancy
Callicarpa lanata L.	Verbenaceae	Leaves	Anthelmintic, wound dressing, asthma, cough	As a betel leaf substitute, as a mouth freshener
Combretum latifolium Bl.	Combretaceae	Roots, Leaves	Leaves having insecticidal property	Skin diseases, Inflammation, tumor in breast and thigh
Dichapetalum gelonioides Roxb.	Dichapetalaceae	Fruit, Leaves	To treat amenorrhea, mouth ulcers	Swelling, Pit viper biting, polydipsea, treating wounds
Holigarna arnottiana Hook.	Anacardiaceae	Leaves	To increase body power, arthritis, skin diseases, cancer	Skin diseases, obesity, inflammation
Humboldtia brunonis Wall.	Fabaceae	Bark, Leaves	In menstrual problems	Over bleeding during menstruation, treating wounds
Hydrocotyle javanica Thunb.	Apiaceae	Leaves	To treat cutaneus diseases, dysentery, blood purifier, nervousness	Dysentery, toothache, skin diseases, antidote against dog bite
Persea macrantha (Nees) kesterm	Lauraceae	Leaves, bark	To treat asthma, rheumatism, ulcer	Treating wounds
Vateria indica L.	Dipterocarpaceae	Leaves, Bark, oil	To heal hemorrhoids, inflammations and hasten healing, skin eruptions, wounds, ulcer	Cough, asthma, leprosy, skin eruptions, wounds, dysentery, diarrhoea, bleedings

Table 2: Antifungal activity of the aqueous extracts of test plants by disc diffusion method

Test plants	Test fungi					
	A. f	***C. a***	***M. c***	***M. g***	***P.* sp**	***T. r***
A. siliquosa	–	–	–	–	–	–
B. acutangula	–	–	–	–	–	–
C. lanata	–	–	–	–	–	–
C. latifolium	+	–	+	+	+	+
D. gelonioides	–	–	–	–	–	–
H. arnottiana	–	–	–	–	–	–
H. brunonis	–	–	–	–	–	–
H. javanica	–	–	–	–	–	–
P. macrantha	–	–	–	–	–	–
V. indica	–	–	–	–	–	–

Key: +→ Activity present, -→ Activity absent, Foot note: *A. f*: *Aspergillus fumigatus*, *C. a*: *Candida albicans*, *M. c*: *Microsporum canis*, *M. g*: *Microsporum gypseum*, *P.* sp: *Penicillium* sp., *T. r*: *Trichophyton rubrum*

Table 3: Inhibition zone (in mm) of the aqueous extract of *C. latifolium* by disc diffusion method against test fungi

Test fungi	Aqueous extract					Standard antifungal discs (in µg /disc)			
	1.0 mg	**2.5 mg**	**5.0 mg**	**7.5 mg**	**10 mg**	**AB (50)**	**FLU (25)**	**NYS (50)**	**MIC (50)**
A. f	0.00±0.0	11.33±0.2	14.33±0.2	18.66±0.2	22.66±0.2	12.00±0.0	0.00±0.0	26.00±0.0	11.66±0.2
C. a	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0	10.33±0.5	24.00±1.0	19.66±0.5	14.33±0.5
M. c	16.33±0.2	24.00±0.0	27.33±0.2	29.33±0.2	31.33±0.2	0.00±0.0	0.00±0.0	20.00±0.0	21.66±0.2
M. g	13.66±0.2	20.33±0.2	25.00±0.0	27.00±0.0	29.33±0.2	0.00±0.0	0.00±0.0	11.33±0.2	18.00±0.0
P. sp	10.33±0.2	13.00±0.0	15.33±0.2	20.33±0.2	23.66±0.2	18.00±0.0	0.00±0.0	23.66±0.2	14.33±0.2
T. r	19.33±0.5	22.66±0.2	25.00±0.0	28.66±0.5	30.33±0.2	0.00±0.0	30.66±1.0	14.66±1.0	19.66±0.5

Values are given as mean of three replicates±standard error , Key: AB→ Amphotericin-B, FLU→ Fluconazole, NYS→ Nystatin, MIC→ Miconazole

Liquid-liquid partition and acid-base separation of the extract

The aqueous extract (1.3 g) when subjected to liquid-liquid separation, the extract yield with butanol was 90 mg, with chloroform 170 mg and the 3rd fraction of aqueous extract was 980 mg. Similarly, the aqueous extract (1.23 g) when subjected to acid-base separation yielded 70 mg of neutral fraction, 140 mg of acidic fraction, 85 mg of basic fraction and 760 mg of phenolic fraction. Liquid-liquid partition and acid base separation of the aqueous extract of *C. latifolium* revealed inhibitory activity against all the test fungi except *C. albicans* in fraction 3 only. Butanol and chloroform fractions of liquid-liquid partition and all the 4 fractions of acid base separation did not show any inhibitory activity against all the test fungi. Aqueous fraction 3 of liquid-liquid partition recorded a marginal increase in the inhibitory activity over aqueous crude extract (table 4). This increase in zone of inhibition could be due to

the reduction of other impurities and residing of active principle in the aqueous extract after liquid-liquid partition. The antifungal activity of the polar extracts indicates that tannins or polyphenolic compounds could be responsible for the activity [26].

Table 4: Antifungal activity (zone of inhibition) of different fractions of crude extract of *C. latifolium*

Test fungi	Aqueous crude extract	Liquid-liquid partition			Acid-base separation			
		Butanol fraction (F 1)	Chloroform fraction (F 2)	Aqueous fraction (F 3)	Neutral fraction (F 4)	Acidic fraction (F 5)	Basic fraction (F 6)	Phenolic fraction (F 7)
A. f	22.66±0.2	0.00±0.0	0.00±0.0	28.00±0.1	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0
C. a	-	0.00±0.0	0.00±0.0	-	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0
M. c	31.33±0.2	0.00±0.0	0.00±0.0	39.66±0.1	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0
M. g	29.33±0.2	0.00±0.0	0.00±0.0	35.00±0.1	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0
P. sp	23.66±0.2	0.00±0.0	0.00±0.0	27.66±0.2	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0
T. r	30.33±0.2	0.00±0.0	0.00±0.0	41.33±0.2	0.00±0.0	0.00±0.0	0.00±0.0	0.00±0.0

Values are given as mean of three replicates±standard error.

Table 5: MIC (mg/ml) of the crude aqueous extract and aqueous fraction 3

Test fungi	Crude aqueous extract	Aqueous extract fraction 3
A. fumigatus	1.25±0.0	0.62±0.0
C. albicans	-	-
M. canis	0.31±0.0	0.15±0.0
M. gypseum	0.31±0.0	0.15±0.0
Penicillium sp.	1.25±0.0	0.62±0.0
T. rubrum	0.31±0.0	0.07±0.0

Minimum inhibitory concentration (MIC)

MIC of the crude aqueous extract and 3rd fraction of the aqueous extract are tabulated in table 5. MIC was observed between a range of 0.31 mg and 1.25 mg against all the test fungi in case of crude aqueous extract, where as MIC has reduced to half the concentration of the MIC of the aqueous extract in case of 3rd fraction of the aqueous extract. Against *T. rubrum*, the MIC has reduced to quarter of the concentration (0.07 mg) of the aqueous extract.

Phytochemical and IR spectral analysis

Phytochemical analysis of the active fraction 3 of liquid-liquid partition tested positive for phenols, saponins, tannins, monosaccharides and reducing sugar while it tested negative for alkaloids, steroids, terpenoids, flavonoids and cardiac glycosides.

The FT-IR spectral analysis of the active fraction 3 of liquid-liquid separation revealed the existence of various characteristic functional groups of the phytochemicals (fig. 1). It records major stretching vibrations at 1643.05 cm^{-1}, 2927.41 cm^{-1}, 3096.15 cm^{-1}, 3407.6 cm^{-1} and 3618.77 cm^{-1}, which support the presence of tannins, Saponins and phenolic compounds. The signals detected at 3618.77 indicate the presence of free –OH group, signal detected at 3407.6 indicate the presence of aromatic primary amines, which is supported by a strong asymmetrical stretch at 1643.05 for nitro compounds (Primary amines). Broad stretch at 2927.41 indicates the presence of $–CH_2$ and the medium stretch at 3096.15 indicate the aromatic alkenes.

Literature survey documents the antifungal potential of a variety of phenols, saponins and tannins isolated from different plant sources. *Combretum* species have been found to contain flavonoids, coumarins, anthracene glycosides, Saponins, steroids, triterpenoids and tannins [27-31]. Many of which could be potentially active against microbes. Tannins are known to possess rather weak antimicrobial activity, but their effects are dependent on the overall composition and concentration in the herbal medicine. The antifungal potential of tannins from different species of *Combretum* has reported by Kolodziej [32].

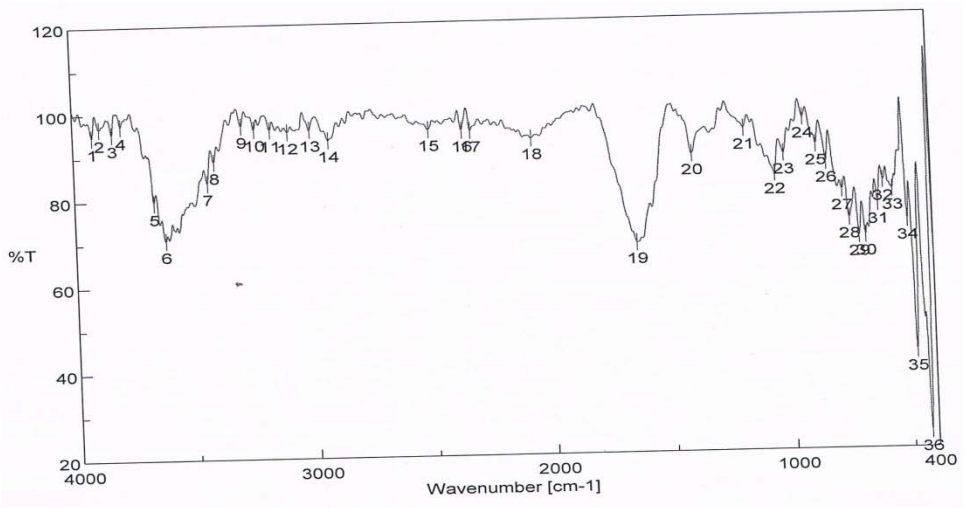

Fig. 1: FT-IR Spectra of the aqueous fraction 3

TLC and bioautography

TLC bioautography helps in direst visualization of active spots on the chromatogram. Among three solvent systems used to develop the chromatogram, EMW system was found to capable of eluting the active principle up to 0.2 R_f. Bioautography has exhibited the inhibition zone at 0.2 R_f in EMW solvent mixture, where as the inhibition zones were detected at the extract application spot in the other two solvent mixtures. The result indicated that the solvent mixture EMW alone was able to elute the active principle while the remaining two solvent systems BEA and CEF were not able to elute the active principle. Subsequent increase in the polarity of the solvent systems and subjecting to bioautography recorded the same result described earlier, thus indicating the high polarity of the active principle. The TLC chromatogram sprayed with Potassium iodate solution appeared as a pinkish red spot, confirmed tannin as the active principle and the appearance of the pinkish red spot matched with the spot of antifungal activity observed in bioautography.

CONCLUSION

The present investigation is the first documentation of antifungal activity of *C. latifolium* and *H. brunonis*. High polar tannin is the antifungal active principle in *C. latifolium*. The results suggest that *C. latifolium* is an important candidate plant for further work of isolation and characterization of the antifungal active principle for commercial exploitation.

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CONFLICT OF INTERESTS

Declared None

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