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Research Article

BIOLOGICAL CONTROL OF PHYTOPATHOGENIC FUNGI USING DIFFERENT EXTRACTS OF *CHAETOMIUM CUPREUM*

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ABSTRACT

Objective: This study evaluated the *Chaetomium cupreum* extracts as biocontrol agents against four plant pathogenic fungi (*Cladosporium cladosporioides, Fusarium oxysporum, Phomopsis azadirachtae, and Rhizoctonia solani*).

Method: The antifungal activity of n-butanol and ethyl acetate extracts of *C. cupreum* was evaluated against plant pathogenic fungi using food poison method.

Result: In n-butanol extract, the percentage inhibition of mycelial growth against *C. cladosporoides* was 88.3±0.1, *F. oxysporum* was 59.4±0.2, *R. solani* was 56.2±0.9, and *P. azadirachtae* was 52.0±0.1at 0.25 mg/ml, respectively. In ethyl acetate extract, the percentage inhibition of mycelial growth against *C. cladosporoides* was 86.0±0.5, *F. oxysporum* was 66.4±0.1, *P. azadirachtae* was 55.2±0.9, and *R. solani* was 52.0±0.1 at 0.25 mg/ml, respectively.

Conclusion: It was found that n-butanol extract is more effective than ethyl acetate extract of *C. cupreum*. Future studies will focus on the purification and characterization of compounds of *C. cupreum* and their biocontrol capacity with the mechanism for plant pathological applications.

Keywords: *Chaetomium cupreum*. Epipolythiodioxopiperazins. *Cladosporium* rot. Biopigments. Azaphilones.

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INTRODUCTION

The species of fungi such as *Phomopsis* sp., *Cladosporium* sp., *Rhizoctonia* sp., *Aspergillus* sp., *Penicillium* sp., and *Fusarium* sp. produce mycotoxins which cause heavy damage to fruits and vegetables during picking, transport and storage and thus affect their nutritional value and shelf life and make them unfit for human use [1]. Fungi also cause rot diseases in fruits and vegetables [2]. In India, neem tree is used for medicinal purposes. Ayurveda regards the tree as a "Sarva roga nivarini." The *Phomopsis azadirachtae* causes dieback disease in neem tree [3]. The fungus *P. azadirachtae* affects all parts of the neem tree. In the highly affected trees, it causes 100% loss of fruit productivity [4]. The field survey report indicates that all the trees in Mysore district in Karnataka were affected with *P. azadirachtae* [3]. The fungus *Rhizoctonia solani* causes leaf web blight to neem trees [4] and *Fusarium oxysporum* causes damping-off [5]. The fungus *R. solani* causes various diseases such as damping-off and wirestem in cabbage, cauliflower, and crucifer seedlings in the seedbed, bottom rot, head rot of older plants in the field, and root rot of rutabaga, horseradish, turnip, and radish. These diseases result in lower quality and product yield [6].

The fungus *F. oxysporum f. sp lycopersici* causes wilt disease in tomato crops worldwide [7]. Fusarium wilt is a banana disease is a caused in banana plants by *F. oxysporum* f. sp. cubense (Foc). During the harvesting stage, *C. cladosporioides* causes Blossom blight in strawberry in California, USA [8]. The *C. cladosporioides* also causes *Cladosporium rot* of red wine grapevines [9]. For controlling plant diseases, synthetic chemicals are used during transport and storage, but it is found that these synthetic chemicals have are toxic and non-biodegradable in nature and causes carcinogenicity, pathogenics to normal microorganisms, and pollutes the ground and soil environment [10]. Natural products, particularly microbial origin, are better alternatives and environmentally safe to control of plant diseases.

Chaetomium species are saprophytic ascomycetes, with *Chaetomiaceae* family. Since Kunze first established this genus in 1817, then 350 *Chaetomium* species have been described. The *Chaetomium cupreum* is a soil fungus exhibiting antagonism against various fungal phytopathogens [11]. In the present investigation, the ability of extracellular antifungal metabolites of *C. cupreum* was evaluated as biocontrol agents against plant pathogenic fungi (*Cladosporium cladosporioides, F. oxysporum, P. azadirachtae, and R. Solani*) were studied.

METHODS

Isolation and identification of fungus

The isolation of fungus *C. cupreum* was carried out from litter soil sample collected from the GKVK campus, Bengaluru, Karnataka, India, by Soumya *et al*., [12]. The strain with pigment production was selected and identified as *C. cupreum*-SS02 based on morphological and microscopic characteristics [13]. The morphological identity was confirmed by NFCCI, Agharkar Research Institute, Pune, India. To confirm the species, sequence analysis of the ITS region using universal primers (Forward primer, ITS 1 - TCCGTAGGTGAACCTGCGG, and Reverse primer, ITS 4 - TCCTCCGCTTATTGATATGC) was performed [14]. Nucleotide blast to the obtained sequence was performed in NCBI (www.ncbi.nlm.nih.gov/) using blast suite [15]. The *C. cupreum* culture was deposited in the National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India, with accession number NFCCI 3117. The sequence was deposited in NCBI Genbank with accession number KF668034. The stock culture was maintained on a potato dextrose agar (PDA) slant at 4°C.

Fermentation and extraction

In inoculum preparation, the fungus was grown on a PDA plate for 7–10 days and mycelial discs (5 mm) were transferred to 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth and incubated at 26±2°C on a rotary shaker at 120 rpm for 20 days to achieve the highest pigment production [16]. The culture of edible mushrooms produces high pigment production through submerged fermentation [17,18]. After 20 days of incubation, biomass was removed

by filtration through Whatman No. 1 filter paper and the broth containing the extracellular metabolites was obtained. The extraction of pigments was carried out according to the method described by Lathadevi *et al*.[19]. The broth obtained was used for the extraction of the different compounds by separating funnel using different organic solvents from non-polar to polar (chloroform, ethyl acetate, n-butanol, and methanol) in the ratio of 1:1. The solvent layer was collected, filtered, and removed by evaporation under vacuum using rotary evaporator at 45°C. The crude dried extract was obtained and stored at 4°C for future use.

Antifungal activity of *C. cupreum* extract by Antifungal activity

Fungal strains

Cladosporium cladosporoides, Fusarium oxysporum, Phomopsis azadirachtae and Rhizoctonia solani

Antifungal activity by food poison method

The antifungal activity of n-butanol and ethyl acetate of *C. cupreum* was evaluated against four plant pathogenic fungi using poisoned food method [20]. About 500 µl from each of different concentrations (0.25, 0.5, and 1.0 mg/ml) of n-butanol and ethyl acetate extract of *C. cupreum* was added to PDA medium before solidification. A 6 mm mycelium disc of test fungi was inoculated on the PDA petri plate containing fungal extract. Then, inoculated plates were incubated at 25±2°C for 7 days. Three independent replicates were maintained for each treatment. Carbendazim was used as standard compound, and dimethyl sulfoxide without fungal extract was used as negative control. The diameter of the growth of fungus in PDA Petri plates was used as a control for the calculation of percentage inhibition of test fungi. After 7 days of incubation, the colony diameter was measured in millimeters. The percentage of mycelial growth inhibition was calculated using the following formula [21].

Inhibition % $((D_0-D_1/D_0)\times 100$.

Where D_0 is the diameter of the mycelial mat in the control plate; D_1 is the diameter of the mycelial mat in the test plate.

Statistical analysis

All the measurements were taken in triplicate and expressed as mean value±standard deviation. The data were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test with GraphPad Prism 6 software (GraphPad Software, Inc., USA). The probability values $p<0.05$ were considered as statistically significant.

RESULTS

The antifungal activity of ethyl acetate and n-butanol extracts of *C. cupreum* was determined by employing food poison method. Among the four solvent extracts of *C. cupreum,* n-butanol and ethyl acetate extracts showed a concentration-dependent antifungal activity against phytopathogenic using food poison method.

Effect of n-butanol extract of *C. cupreum* on plant pathogenic fungi

The antifungal activity of the n-butanol extract of *C. cupreum* against plant pathogenic fungi such as *C. cladosporoides, F. oxysporum, P. azadirachtae, and R. solani* is presented in Fig. 1. Our results have shown that antifungal activity increases by inhibiting the growth of the fungi with an increase in concentration. In n-butanol extract of *C. cupreum*, the percentage inhibition of mycelial growth against *C. cladosporoides* was 88.3±0.1, *F. oxysporum* was 59.4±0.2, *R. solani* was 56.2±0.9, and *P. azadirachtae* was 52.0±0.1 at 0.25 mg/ml, respectively. Similarly, the percentage inhibition of mycelial growth against *C. cladosporoides* was 92.1±0.6, *F. oxysporum* was 72.0±0.1, *R. solani* was 70.8±0.5, and *P. azadirachtae* was 67.4±0.5 at 1.0 mg/ml concentration, respectively. The obtained results were compared with fungicide carbendazim (Fig. 1).

Effect of ethyl acetate of *C. cupreum* on plant pathogenic fungi

The antifungal activity of ethyl acetate extract of *C. cupreum* against plant pathogenic fungi such as *C. cladosporoides, F. oxysporum, P. azadirachtae, and R. solani* is presented in Fig. 2. In ethyl acetate extract of *C. cupreum*, the percentage inhibition of mycelial growth against *C. cladosporioides* was 86.0±0.5, *F. oxysporum* was 66.4±0.1, *P. azadirachtae* was 55.2±0.9, and *R. solani* was 52.0±0.1 at 0.25 mg/ml respectively. The obtained results were compared with fungicide carbendazim. Similarly, the percentage inhibition of mycelial growth against *C. cladosporioides* was 91.0±0.1, *F. oxysporum* was 80.0±0.2, *P. azadirachtae* was 70.8±0.5, and *R. solani* was 67.4±0.5 at 1.0 mg/ml, respectively. The obtained results were compared with fungicide carbendazim (Fig. 2 and Fig. 3).

DISCUSSION

The pure fungus culture was identified based on morphological and microscopic characteristics and named as *C. cupreum*. The organism *C. cupreum* was identified based on morphological and microscopic characteristics and molecular analysis and named as *C. cupreum*. The fungus *C. cupreum* fungus produces extracellular pigments in the fermented broth. The extraction of pigments was carried out by different solvents from non-polar to polar. The results of the present study have shown the significant antifungal activity of *C. cupreum* extracts against the phytopathogenic fungi. Among the four phytopathogenic fungi (*C. cladosporioides, F. oxysporum, P. azadirachtae, and R. solani*), *C. cladosporoides* was the most susceptible to both the n-butanol and ethyl acetate extract of *C. cupreum*. From the results, it was observed that growth of fungal mycelium decreases with the increase in the concentration of *C. cupreum* extracts.

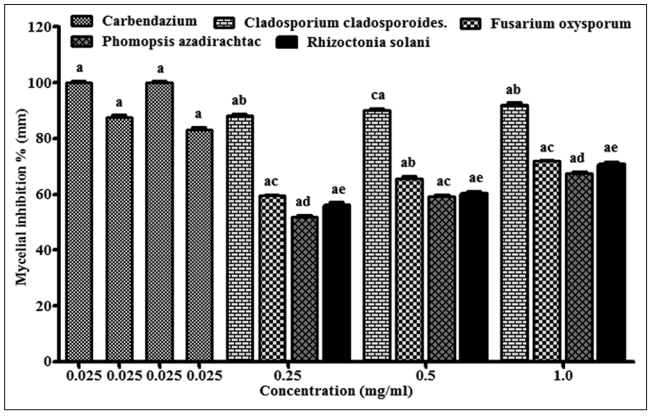

Fig. 1: Effect of antifungal activity of n-butanol extract of *Chaetomium cupreum* by food poison method. (Standard - carbendazim, Control - Dimethyl sulfoxide). Bars in each column with common letters indicate significant difference (p<0.05) according to Tukey's multiple comparison test. Experimental results are expressed as mean±SD (n=3)

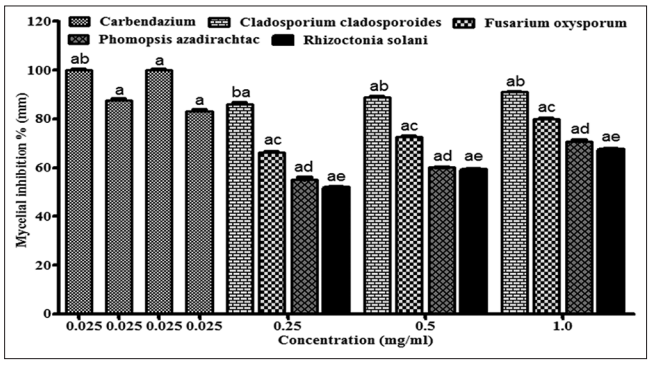

Fig. 2: Effect of antifungal activity of ethyl acetate extract of *Chaetomium cupreum* by food poison method. (Positive control - carbendazium, Negative control - Dimethyl sulfoxide). Bars in each column with common letters indicate significant difference (p<0.05) according to Dunnett's multiple comparison test. Experimental results are expressed as mean±SD (n=3)

In n-butanol extract (Fig. 1), the percentage of mycelium growth inhibition was high in *C. cladosporoides* (88.30%) followed by *F. oxysporum* (59.40%) and *R. Solani* (56.20%), whereas lowest in *P. azadirachtae* (52%) at 0.25 mg/ml. At higher concentration, the percentage of mycelium growth inhibition increases in *C. cladosporoides* (92.10%) followed by *F. oxysporum* (72%) and *R. Solani* (70.80%), whereas lowest in *P. azadirachtae* (67.40%) at 0.1 mg/ml. Whereas in reference standard carbendazim, the percentage of mycelium growth inhibition was highest in *C. cladosporoides* and *P. azadirachtae* (100%), followed by *F. oxysporum* (87.70%) and *R. Solani* (83%) at 0.025 mg/ml. The percentage of mycelium growth inhibition of n-butanol extract of *C. cupreum* and standard carbendazim decreased in the order of carbendazim > *C. cladosporioides* > *F. oxysporum* > *R. solani* > *P. azadirachtae*. The statistical significance using ANOVA followed by Tukey's multiple comparison test was performed, and probability values p<0.05 were considered as statistically significant. For statistical analysis, antifungal activity of n-butanol extract of *C. cupreum* was compared to the standard carbendazim, and it was observed that all phytopathogenic fungi except *C. cladosporoides* (carbendazim vs. *C. cladosporoides* p>0.05) showed significant difference (carbendazim vs.. *F. Oxysporum* p<0.05, carbendazim vs. *P. azadirachtac* p<0.05, and carbendazim vs. *R. solani* p<0.05) as shown in Table 1. In ethyl acetate extract (Fig. 2), the percentage of mycelium growth inhibition was highest in *C. cladosporoides* (86%), followed by *F. oxysporum* (66.40%) and *P. azadirachtae* (55.20%), whereas lowest in *R. Solani* (52%) at 0.25 mg/ml. At higher concentration, the percentage of mycelium growth inhibition increases in *C. cladosporoides* (91%) followed by *F. oxysporum* (80%) and *P. azadirachtae* (70.80%), whereas lowest in *R. Solani* (67.40%) at 0.1 mg/ml. Whereas in reference standard carbendazim, the percentage of mycelium growth inhibition was highest in *C. cladosporoides* and *P. azadirachtae* (100%) followed by *F. oxysporum* (87.70%) and *R. solani* (83%) at 0.025 mg/ml. The percentage of mycelium growth inhibition of ethyl acetate extract of *C. cupreum* and standard carbendazim decreased in the order of carbendazim > *C. cladosporoides* > *F. oxysporum* > *P. azadirachtae* > *R. solani.* The statistical significance using one-way ANOVA followed by Tukey's multiple comparison test was performed and probability values p<0.05 was considered as statistically significant. For statistical analysis, antifungal activity of ethyl acetate extract of *C. cupreum* was compared to the standard carbendazim, and it was observed that all phytopathogenic fungi except *C. cladosporoides* (carbendazim vs. *C.* cladosporoides p>0.05) showed significant difference (Carbendazim vs. *F. oxysporum* p<0.05, carbendazim vs. *P. azadirachtac* p<0.05, and carbendazim vs. *R. solani* p<0.05) as shown in Table 2.

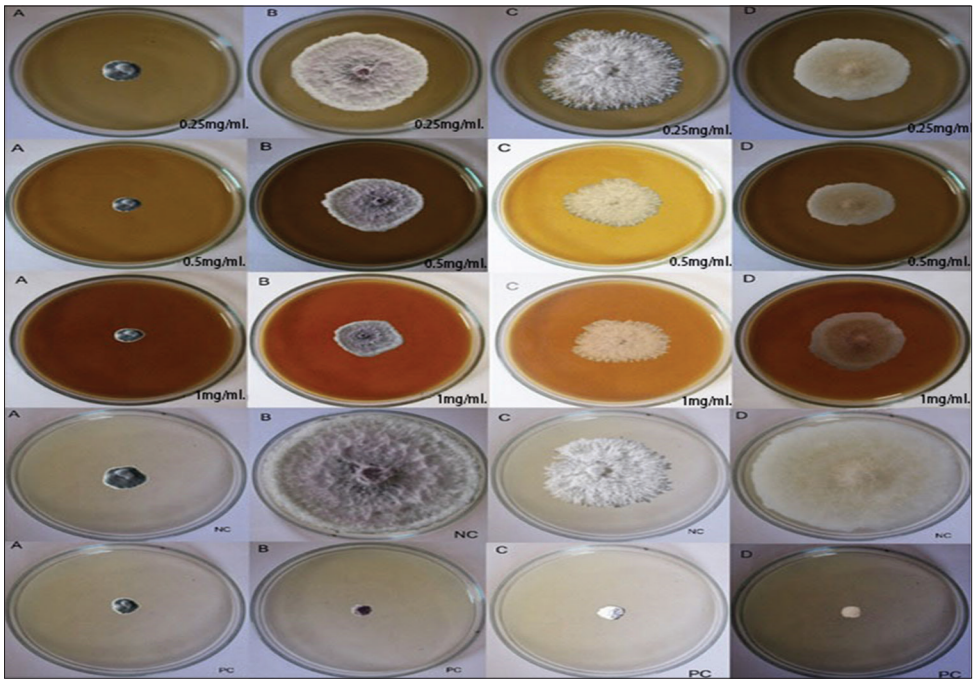

Fig. 3: Antifungal activity by of n-butanol extract of *C. cupreum*(A) *Cladosporium cladosporioides*, (B) *Fusarium oxysporum*, (C) *Phomopsis azadirachtae*, (D) *Rhizoctonia solani*

Table 1: Tukey's multiple comparison test for n-butanol extract of *C. cupreum* on phytopathogenic fungi and its comparison with carbendazim

Tukey's multiple comparison test	Mean difference	q	Significant (p<0.05)	Summary	95% CI of diff
Carbendazim versus *C. cladosporioides*	2.542	0.6683	No	ns	−14.85–19.94
Carbendazim versus *F. oxysporum*	27.04	7.111	Yes	**	9.647–44.44
Carbendazim versus *P. azadirachtae*	33.18	8.724	Yes	***	15.78–50.57
Carbendazim versus *R. solani*	30.31	7.970	Yes	**	12.91–47.70
C. cladosporioides versus *F. oxysporum*	24.50	6.026	Yes	**	5.905–43.10
C. cladosporioides versus *P. azadirachtae*	30.63	7.535	Yes	**	12.04–49.23
C. cladosporioides versus *R. solani*	27.77	6.830	Yes	**	9.171–46.36
F. oxysporum versus *P. azadirachtae*	6.133	1.509	No	ns	−12.46–24.73
F. oxysporum versus *R. solani*	3.267	0.8035	No	ns	−15.33–21.86
P. azadirachtae versus *R. solani*	−2.867	0.7051	No	ns	−21.46–15.73

The probability symbol * indicates significant difference (p<0.05). (*** p<0.001, ** p<0.01, * p<0.1, p<0.05 and NS: non-significant). The results are expressed as the mean of three measurements±standard deviation. The data were analyzed for statistical significance using ANOVA followed by Tukey's multiple comparison test.
C. cladosporioides: Cladosporium cladosporioides, F. oxysporum: Fusarium oxysporum, P. azadirachtae: Phomopsis azadirachtae, R. solani: Rhizoctonia solani

Table 2: Tukey's multiple comparison test for ethyl acetate extract of *C. cupreum* on phytopathogenic fungi and its comparison with carbendazim

Tukey's multiple comparison test	Mean difference	q	Significant (p<0.05)	Summary	95% CI of diff
Carbendazim versus *C. cladosporioides*	4.008	1.025	No	ns	−13.89–21.90
Carbendazim versus *F. oxysporum*	19.71	5.038	Yes	*	1.814–37.60
Carbendazim versus *P. azadirachtae*	30.64	7.832	Yes	**	12.75–48.54
Carbendazim versus *R. solani*	33.18	8.480	Yes	***	15.28–51.07
C. cladosporioides versus *F. oxysporum*	15.70	3.754	No	ns	−3.430–34.83
C. cladosporioides versus *P. azadirachtae*	26.63	6.368	Yes	**	7.503–45.76
C. cladosporioides versus *R. solani*	29.17	6.974	Yes	**	10.04–48.30
F. oxysporum versus *P. azadirachtae*	10.93	2.614	No	ns	−8.197–30.06
F. oxysporum versus *R. solani*	13.47	3.220	No	ns	−5.664–32.60
P. azadirachtae versus *R. solani*	2.533	0.6057	No	Ns	−16.60–21.66

The probability symbol* indicates significant difference (p<0.05). (***p<0.001, **p<0.01, *p<0.1, P<0.05 and NS: non-significant). The results are expressed as the mean of three measurements±standard deviation. The data were analyzed for statistical significance using ANOVA followed by Tukey's multiple comparison test. *C. cladosporioides: Cladosporium cladosporioides, F. oxysporum: Fusarium oxysporum, P. azadirachtae: Phomopsis azadirachtae, R. solani: Rhizoctonia solani.* CI: Confidence interval

It was observed that the *C. cupreum* extracts inhibited the growth of all phytopathogenic fungi. This antifungal activity may due to the different phytochemicals present in the *C. cupreum* extract. These results are of great importance, particularly against phytopathogenic fungi, which are responsible for heavy crop loss every year [22]. These results have shown that the ethyl acetate and n-butanol extracts of *C. cupreum* exhibit higher antifungal activity as compared to the crude extracts of *F. oxysporum* and *Fusarium poae,* isolated from *Ophiopogon japonicus*, investigated for their inhibition against several phytopathogens [23]. Furthermore, the crude extracts of *C. cupreum* exhibit higher antifungal activities as compared to the water and ethanol extracts from mushroom samples conducted against three plant pathogenic fungi [24]. The extracts of *C. cupreum* have shown higher antifungal activity as compared to the extracellular antifungal metabolites of actinomycetes against *Rhizopus stolonifer*, *Aspergillus flavus*, *F. oxysporum,* and *Alternaria* sp. [25]. The *C. cupreum* extracts exhibit higher antifungal activity as compared to methanolic extract of Abutilon theophrasti against Penicillium spp. [26]. The three new azaphilones such as rotiorinols A-C (1-3), with two new stereoisomers, (-)-rotiorin (4) and epiisochromophilone II (5), and a known compound, rubrorotiorin (6) were isolated from the fungus *C. cupreum* CC3003. The compounds 1, 3, 4, and 6 showed antifungal activity against *Candida albicans* with IC_{50} values of 10.5, 16.7, 24.3, and 0.6 µg/ml [27]. The commercially available broad-spectrum fungicide, Ketomium®, which is used in crop diseases control, was developed from *Chaetomium* species [28]. By observing these promising biological activities and diverse structural characters, azaphilones have opened a new subject for research. The present study revealed that *C. cupreum* extracts have significant potential for antifungal applications. This antifungal potential of *C. cupreum* is due to the presence of different types of phytochemicals.

CONCLUSION

The biological control with antagonistic microorganisms is a suitable and easy method for plant protection. The *C. cupreum* is producing extracellular metabolites which are easily extracted as compared to other fungi and mushrooms. Thus, it was found that n-butanol extract and ethyl acetate extract of *C. cupreum* showed significant antifungal activity due to the presence of phytochemicals. The flavonoids play an important role in biological activity [29]. The obtained results clearly confirmed that both the n-butanol and ethyl acetate extracts of the *C. cupreum* contain different phytochemicals particularly flavonoids that can be used in plant protection after their purification and characterization.

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DISCLOSURE STATEMENT

The author declares no conflict of interest.

AUTHOR'S CONTRIBUTIONS

NAW carried out all the assays, analysis, and interpretation of results and wrote the initial manuscript. ST was responsible for the idea of research and interpretation of the results and edited the manuscript.

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