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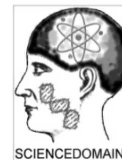

In-vitro Antifungal Activities of *Cola nitida* Schott & Endl. (Sterculiaceae) against Five *Candida* species and Four Dermatophytes

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Authors' contributions

This work was carried out in collaboration between all authors. Author BAA initiated and designed the study, wrote the protocol, supervised the work with statistical analysis, corrected the drafts of the manuscript and managed literature searches. Author TOL co-supervised of the work, managed the analyses of the study, corrected the drafts of manuscript while authors OOM and KEN collected data, managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: Various medicinal and pharmacological values have been observed in species of *Cola* including their use to treat mouth infections, whooping cough and ringworms. Some studies have been done on their antibacterial and anti-mycobacterial but little or none on antifungal potential. This study was designed to study the antifungal activities of crude extract of *Cola nitida* against five *Candida* species and four dermatophytes implicated in various oral and skin infections *in vitro.*
Place and Duration of Study: Department of Pharmaceutical Microbiology, University of Ibadan, Ibadan, Nigeria, during March 2007 to July 2011.
Methodology: Preliminary phytochemical screening was done using standard conventional methods. Antifungal activity was carried out using the agar cup diffusion technique at 2.0 mg/mL while the minimum inhibitory concentration (MIC) was carried out using the agar dilution method. The kill kinetics study was done using viable counting technique.

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Results: The methanol extracts of *Cola nitida* leaf and *Cola nitida* seed exhibited significant inhibitory actions against all the 9 pathogens with the formal more effective than the later. Both the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of the plant extract ranged from 0.0625 to 1.00 mg/mL. The antifungal activity of *Cola nitida* compares favorably well with the control ketoconazole. Phytochemical analyses reveal the presence of tannin, saponin and alkaloids, although at varying degrees. Kill kinetics reveals a concentration dependent decline in the microbial load of the tested organisms with time. The rate of decline increased with increase in concentration.
Conclusion: Current study supports the ethno-medicinal uses of the plants as potential antifungal agents.

Keywords: Cola nitida; MIC/MFC; antifungal; kill kinetics.

1. INTRODUCTION

Since ancient times, mankind has used plants to treat common diseases and some of these traditional medicines are still included as part of the habitual treatments of various maladies. Folk medicine mainly based on plant enjoys a respectable position today, especially in the developing countries where the availability of modern health facilities and services are limited.

Cola Schott & Endl. (*Sterculiaceae*) is a genus of about 125 species of trees indigenous to the tropical rain-forest African region [1]. The species of *Cola* are found mostly in the relatively dry part of the rain forest, although, *Cola milleni* and *Cola gigantea* are widely distributed in wet and dry forest environments [2,3]. Various medicinal values have been observed in species of *Cola*. *Cola* are used to treat whooping cough and Asthma [4,5]. Odugbemi [6] also reported that the leaves of *Cola milleni* are used in the treatment of ringworm, scabies, gonorrhoea, dysentery and ophthalmic.

Extracts of *C. nitida* bark have been reported to have antibacterial activity [7] while the methanol extract of root bark of *C. nitida* and *C. millenii* have been reported to be active against *Mycobacterium* species [8]. There is little or no information about the antifungal activity particularly anti-dermatophytic activity of the plants. Hence this study is designed at justifying the ethno-pharmacological claims of the investigated plant and its antifungal activities in relation to oral and skin infections.

2. MATERIALS AND METHODS

2.1 Plant Materials

Cola nitida leaves and seed were collected from various locations in Ibadan, identified and authenticated at the Botany Department of the University of Ibadan where voucher specimen with No UIH-22487 was deposited. The samples were air dried and pulverized before use for the study.

2.2 Preparation of Plant Extracts

Two kilograms (2 kg) of each pulverized plant sample were subjected to soxhlet extraction with methanol as extraction solvent. Extracts were filtered, evaporated to dryness *in-vacuo*, weighed and stored at -4°C until needed.

2.3 Phytochemical Screening

The pulverized samples of the leaves and seeds of *Cola nitida* was examined for the presence of anthraquinones, tannins, saponins, alkaloids and cardenolides. The procedure used was as previously described by [9,10].

2.4 Microorganism

The five strains of *Candida* spp. and four dermatophytes used in this study are *Candida valida, Candida glabrata, Candida tropicalis, Candida albicans, Candida krusei, Trichophyton interdigitalis, Trichophyton tonsurans, Epidermytophyton rubrum Trichophyton rubrum.*

2.5 Media

The media used were Saboraud dextrose agar (SDA) and Tryptone soy broth (TSB) which are products of BIOTECH.

2.6 Determination of Antimicrobial Activity

This was carried out using the agar cup diffusion technique described by [9]. Saboraud dextrose agar plates were seeded with each fungus. Using sterile Pasteur's pipette, 0.2 ml of the standardized inoculums ($1x10^7$ CFU/mL) of the organism in tryptone soy broth was placed on the

surface of each SDA plate. This was uniformly spread with the aid of a sterile glass spreader. The seeded plates were allowed to dry in the incubator at 37°C for 20 minutes. A standard cork borer of the 8mm diameter was used to cut equidistant wells on the surface of the agar into which was added 0.2 ml solution of each extract. Ketoconazole was used as chemotherapeutic control. The plates were incubated at room temperature for 72 h after which, diameters of zones of inhibition were measured. Results are average of triplicate experiments.

2.7 Determination of Minimum Inhibitory Concentration (MIC)

This was carried out using the agar dilution method [11]. Different concentrations of the extracts were prepared to final concentration in the range of 2.00 mg/mL to 0.0078 mg/ml. A 2 ml of the extract was mixed with 18 ml of molten SDA and poured into sterile petri dish allowing the agar to set. The surface of the set agar was allowed to dry before streaking with overnight broth cultures of fungal. Plates were incubated at 25°C for 48-72 hr and examined for the presence or absence of growth. The lowest concentration preventing growth was taken as the MIC of the extract.

2.8 Determination of Minimum Fungicidal Concentration (MFC)

This was determined by a modification of the method of [8]. To a 0.5 ml at different concentrations as used in the MIC assay that shows no visible growth on the agar plate was added 0.5 ml of test organisms in tubes. These were incubated at 25°C for 24-48 hrs. Samples were streaked out from the tubes onto Saboraud dextrose agar to determine the minimum concentration of the extract required to kill the organism. These concentrations were indicated by failure of the organism to grow on transfer to these media plates. The lowest concentration that prevented fungal growth after days of incubation was recorded as the minimum fungicidal concentration (MFC). All tests were performed in duplicates to ensure accuracy. Agar plates without extract and another agar plate without organism were also incubated to serve as positive and negative control.

2.9 Time Kill Kinetics Assay

This was determined using the viable counting technique. A 0.5 ml. of each culture was subculture into a warm (37°C) 4.5 ml. Tryptone Soy Broth and incubated for 90 minutes using a Gallenkamp orbital incubator to give a logarithmic phase culture. A 0.1 ml of the logarithmic phase culture was then inoculated into a 4.9 ml. of tryptone soy broth containing the MIC/MFC concentrations of the tested extract to give 1 in 50 dilution of the culture (equivalent to approximately 1x 10^7 colony forming units) and 4.9 ml TSB inoculated with only the test organism to serve as the positive control. A 1 ml of the test sample (extract culture mixture and the control) were withdrawn immediately, diluted out in normal saline and two drops of each dilution plated into an oven dried Sabourand dextrose agar to give culture count at 0 minutes. Samples were taken at an interval of 30, 60, 120, 180, 240 and 360 minutes respectively. The procedure was carried out in duplicates to ensure reproducibility. Plates were incubated at 25°C for 48-72 h before counting the colonies. The positive control plates were also incubated. The number of colony forming unit were counted after the period of incubation. The numbers of surviving bacterial cells per ml were calculated by taking into consideration the dilution factor and the volume of the inoculums. All the procedure was repeated for MFC, 2 x MFC and 4 x MFC respectively. The percentage survival of the organism was calculated for each of the time intervals at various concentrations and the control. A graph of percentage survivors of the organisms against contact time in minutes was plotted on a semi-logarithm graph.

3. RESULTS

The result of the phytochemical screening of the seed and leaf samples for secondary metabolites is presented in Table 1 and reveals the presence of alkaloids, tannins, flavonoids and saponin while anthraquinone and cardenolides were absent (Table 1).

The results of the antibiogram assay and antifungal screening of the crude methanol extracts of *Cola nitida* are presented in Table 2 and Table 3 respectively. The antifungal screening shows that the methanol extracts of *Cola nitida* leaf and seed had activity on all the tested fungal though the formal was more effective.

The results of the minimum inhibitory concentration (MIC) and the minimum fungicidal concentration (MFC) of both the crude methanol extract is presented in Table 4. The MIC of the

crude *Cola* extracts ranged between 0.0625 and 1 mg/mL and the order of potency is *Cola nitida* leaf > *Cola nitida* seed while the minimum fungicidal concentration (MFC) also ranged between 0.0625 and 1 mg/mL with most of them having the same MIC and MFC (Table 4).

4. DISCUSSION

The choice of plants used in this study was due to their reported activities in the treatment of various diseases and this study further elucidates on their antimicrobial activities. The phytochemical screening reveals the presence of alkaloids, tannins, flavonoids and saponin and the absence of anthraquinone and cardenolides.

[12,13] reported that methanol extraction leads to higher yield of flavonoids and in turn leads to a higher antimicrobial activities of the extract in which they are present. This was reflected in the varying zones of inhibition of the individual extract on the organism. The zone of inhibition produced by the plant extract is an indication of the susceptibility of tested microorganisms to the plants. Also, the diameters of zones of inhibition were observed to vary from one organism to another and from one plant to another. These differences in the zones of inhibition observed could be explained according to Prescott [14] that the effect of an antimicrobial agent varies with the target species.

In this study, the degree of antifungal activity may be accounted for by the flavonoids synthesised by plants in response to fungal infection [15], and the active components in the crude extract may be acting synergistically to produce good antimicrobial effects [16].

Table 1. Phytochemical composition of the crude extracts of medicinal plants

Sample	Alkaloids	Saponin	Anthraquinones	Flavonoids	Terpenoids	Cardenolides	Tannin
CNS	+	+	-	+	ND	-	+
CNL	+	+	-	+	ND	-	+

CNS: – Cola nitida (seed); CNL: –Cola nitida (leaf); ND: Not determined; +: Positive; -: Negative

Table 2. Antibiogram test of organisms

Organisms	Antibiograms
Candida valida	Flu^s Clo^s Ket^s Gri^r Nys^s
Candida glabrata	Flu^s Clo^s Ket^s Gri^s Nys^s
Candida tropicalis	Flu^s Clo^s Ket^s Gri^s Nys^s
Candida albicans	Flu^r Clo^s Ket^s Gri^r Nys^s
Candida krusei	Flu^r Clo^s Ket^s Gri^r Nys^s
Trichophyton interdigitalis	Flu^s Clo^s Ket^s
Trichophyton tonsuran	Flu^s Clo^s Ket^s
Epidermophyton rubrum	Flu^S Clo^S Ket^S
Trichophyton rubrum	Flu^S Clo^S Ket^S

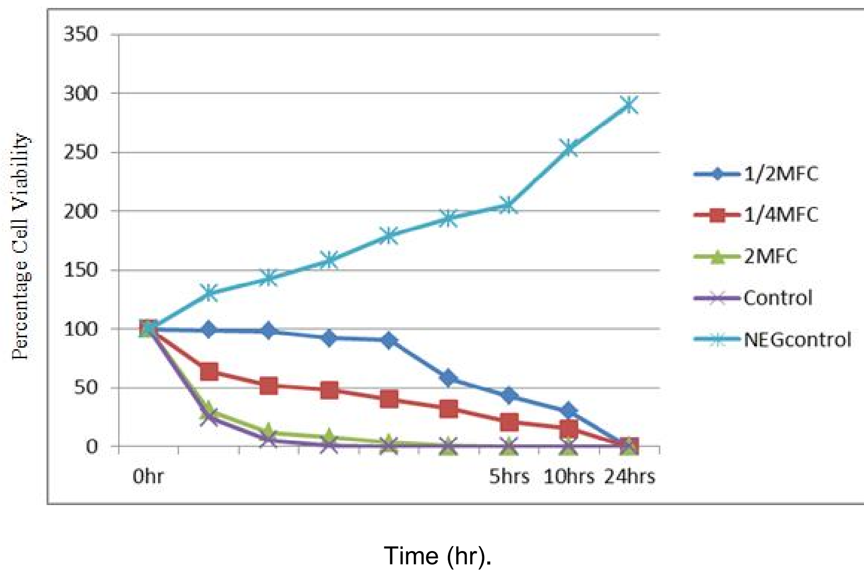
r= Resistant; Flu=fluconazole, Clo=clotrimazole, Ket= ketoconazole, Gri=griseofulvin,
s= Susceptible; Nys=nystatin

Table 3. Antimicrobial activities of 2 mg/ml of crude methanolic extract

Microorganisms	Diameter zone of inhibition(mm)*		
	CNL	CNS	Ketoconazole
Candida valida	20±0.2	14±0.1	22
Candida tropicalis	19±0.1	15±0.1	21
Candida glabrata	20±0.2	13±0.1	22
Candida albican	30±0.2	13±0.1	28
Candida krusei	15±0.2	10±0.1	16
Trichophyton interdigitalis	21±0.2	15±0.1	20
Trichophyton tonsurans	18±0.2	15±0.2	19
Trichophyton rubrum	22±0.2	18±0.2	18
Epidermophyton rubrum	12±0.1	11±0.1	22

*CNS: – Cola nitida (seed); CNL: –Cola nitida (leaf), * Average of triplicate experiment*

The almost competitive action of the extract with the pure compound contained in the standard ketoconazole may be due to the mixtures of bioactive compounds present in the extract [17]. The result of the kinetic study showed that the extract exhibited great kill rate on the tested organisms. As the time of exposure to the extract increases, the rate of kill also increases. At 4h of exposure to the extract, the percentage survival of organism has reduced and at 8h time of exposure, the reduction is significant. The Time-kill kinetics study has been reported to form the basis of *in vitro* investigations for pharmacodynamic drug interaction. In this study the extract exhibited fungistatic and fungicidal effects against the bacterium used. The result obtained showed that the extract exhibited significant ($p<0.05$) fungicidal activity at 4hours post treatment and complete clearance of microbes at 24 hours post treatment. This same accelerated rate of kill was observed in Figs. 1-5.



Time (hr).

Fig. 1. Kill kinetics of Cola nitida leaf on *T. rubrum*

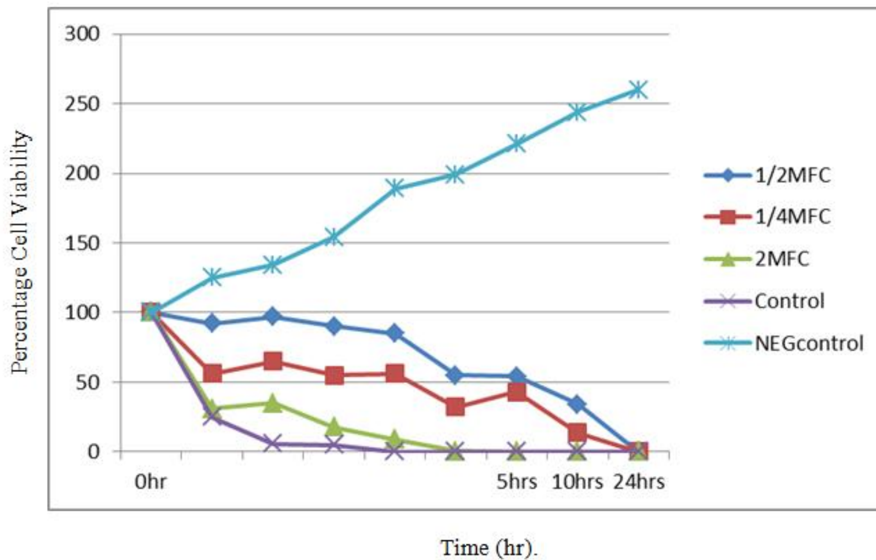


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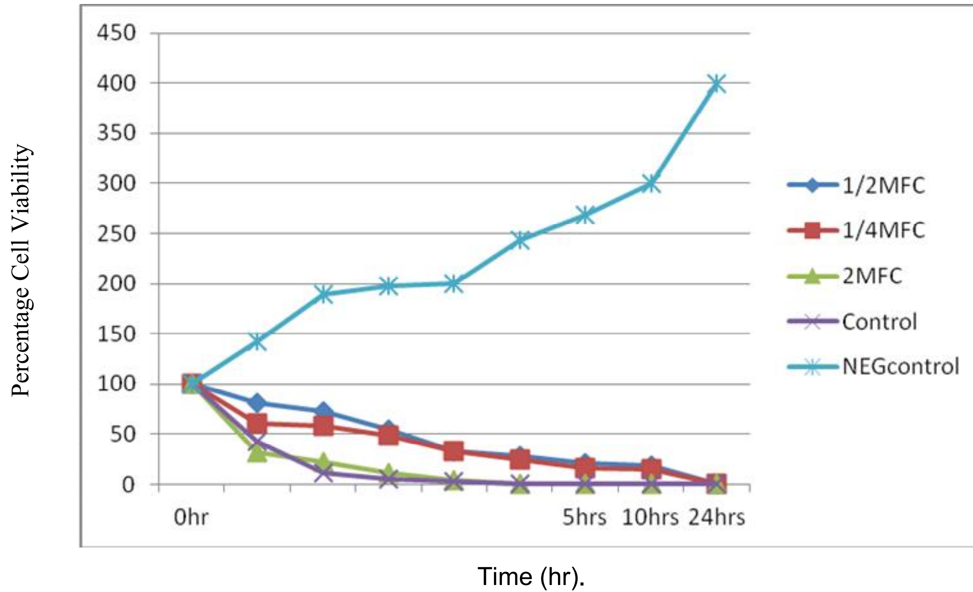
Fig. 2. Kill kinetics of *Cola nitida* leaf on *Candida valida*

Table 4. The MIC/MFC of crude plant extract on the name organisms

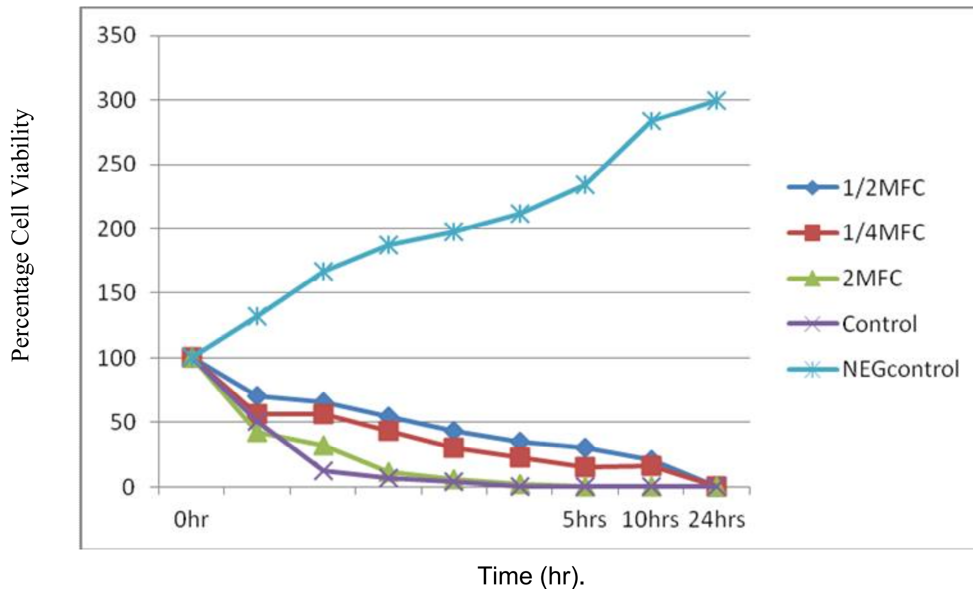
Plant	mg/mL	Cv	Ct	Cg	Ca	Ck	Ti	Tt	Tr	Er
CNS	MIC	0.125	0.500	0.125	0.250	0.500	0.250	0.500	0.50	0.50
	MFC	0.250	1.000	0.250	0.500	1.000	0.500	1.00	1.00	1.00
CNL	MIC	0.0625	0.500	0.125	0.125	0.500	0.0625	0.500	0.50	0.50
	MFC	0.125	1.000	0.250	0.125	1.000	0.0625	1.00	1.00	1.00

CNS: – *Cola nitida* (seed); CNL: –*Cola nitida* (leaf); Cv: –*Candida valida;* Ct: – *Candida tropicalis;* Cg: – C*andida glabrata*; Ca: – *Candida albicans;* Ck: – *Candida Krusei;* Ti: – *Trichophyton interdigitalis;* Tt: – *Trichophyton tonsurans;* Tr: – *Trichophyton rubrum;* Er: – *Epidermophyton rubrum*



Time (hr).

Fig. 3. Kill kinetics of *Cola nitida* leaf on *Candida tropicalis*



Time (hr).

Fig. 4. Kill kinetics of *Cola nitida* seed on *Candida albicans*

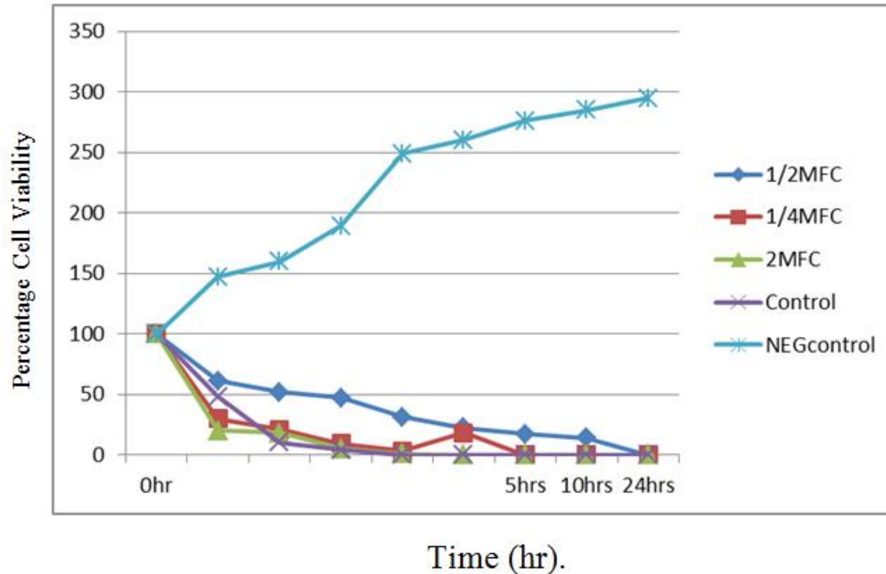


Fig. 5. Kill kinetics of *Cola nitida* seed on *Trichophyton rubrum*

Although *in-vitro* test do not confirm that plant extracts are effective but it provides a basic understanding of the plant efficacy which could lead to a search for new active substances. Investigating the pharmacological activity of extract is a less expensive therapeutic agent that can be used in economical less privileged region [18]. The plant used in this experiment is relatively affordable and available, so a candidate in our region for treating the mentioned microorganisms. The potential of *Cola nitida* in the treatment of oral and skin infection were elucidated. To establish the therapeutic applicability of this plant in the treatment of the infections, investigation of its toxicity, *in vivo*, antimicrobial effects and bioactive compounds is on-going in our laboratory.

The use of herbs for the treatment of infection and disease has over time proven to be effective as an alternative treatment, therefore, it should be promoted with scientific standardization and studies should be carried out to identify the most active phytochemical(s).

5. CONCLUSION

In conclusion, this study has shown that the methanol extract of *Cola nitida* possesses both antidermatophytic and anticandidiasis effects against the tested pathogens but at varied concentrations. The Minimum fungicidal concentration (MFC) and minimum inhibitory concentration (MIC) values of the extract against these microbes ranged from 0.0625 mg/ml to 1.000 mg/ml. The Time-kill kinetic studies indicated that the extract exhibited both fungistatic and fungicidal effects against the tested pathogens. This preliminary study therefore suggests *Cola nitida* may be a good source of antidermatophytic and anticandidiasis agents.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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