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Antifungal Activity of Essential oil Extracted from *Kaempferia galanga* L. Rhizome against Fungal Phytopathogens

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ABSTRACT

Background: Fungal phytopathogens are one of the main causes of deterioration of plant growth and productivity. Reliability of farmers on chemical fungicides poses a threat to humans and the environment. Therefore, exploration and uses of green and biological alternatives should be encouraged in the field of agriculture in order to maintain the overall health of a country. Plant essential oil is one such alternative that is currently researched and holds promises in unlocking the solution to this problem.

Methods: Effect of the oil extracted from *K. galanga* by hydro-distillation against fungal phytopathogens isolated from different plant parts were tested using two different methods of inhibition *viz*., by direct contact method (M1) and volatile/fumigation method (M2). The minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) for both methods were also determined. Scanning electron microscopy (SEM) was also used to show the effect of oil on the physiology of the pathogens.

Result: MIC was in the range 1.5-6 µl mL^{-1} for M1, whereas for M2 the MIC was in the range 2-10 µl mL^{-1} air. The MFC for M1 ranges from 2-6 µl mL^{-1}, with the exception of *Fusarium* sp. and *Cercospora* sp. However for volatile/ fumigation assay, the MFC could not be determined. Thus the effectiveness of the oil for inhibiting the phytopathogens differ for different fungi and the methods used.

Key words: Antifungal activity, Essential oil, *Kaempferia galanga*, Phytopathogens.

INTRODUCTION

Plant diseases lead to enormous losses in yield and quality of field crops, resulting in reduction of food availability to man. One of the main causes of plant diseases are pathogenic fungi, which survive through the agency of soil, seed, or other plants and are dispersed by these and other environmental factors. They collectively cause more plant diseases than any other group of plant pests. Over 8000 species are shown to cause diseases in plants. They also exhibit a diversity of symptoms on infected plants. Their life cycles are complex involving two or more host plants. Not only do they reduce food availability but also cause serious consequences on human health (Al-Sadi, 2017; Gladieux *et al*., 2011; Singh, 2001). The ability of these pathogens to survive and spread by various modes of dispersal makes it hard to contain once the fungi enters the host plants (Singh, 2001).

Owing to the increase in population and limited resources, to get maximum production of food, the introduction of systemic fungicides that could penetrate tissue and work from within the plant was a major landmark in the history of fungicidal management of plant diseases. A large number of chemicals were employed and synthetic compounds were introduced in the market as fungicides to control and eliminate the spread of plant diseases (Singh, 2001). However, with time, it was found that these chemicals caused detrimental effects on plants, animals and the environment (Choudhary *et al*., 2018). To minimize and eliminate the use of synthetic fungicides, eco-friendly alternatives, especially plant-based fungicides have become a matter of priority among scientists globally (Reddy *et al.,* 2007). Plant essential oils which have been used for hundreds of years as natural medicines have also been found to combat a multitude of pathogens, including bacteria, fungi and viruses (Hammer *et al*., 1999). Interests in utilizing oil-yielding aromatic plants is because they have low mammalian toxicity, are environmentally friendly as they degrade easily and most importantly, the oil is easy to obtain and extract (Isman, 2000). The essential oil of many aromatic and medicinal plants have shown to inhibit pathogenic microbes *in vitro* condition (Mafakheri and Mirghazanfari, 2018).

Kaempferia galanga L. commonly known as resurrection lily, under the family Zingiberaceae is a short-stemmed herb which has flat, green, round leaves and bears white flowers with a purple marking. Locally known as *sying khmoh* in Meghalaya, its extracts have been found to have anti-inflammatory, analgesic, nematicidal, mosquito repellent,

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larvicidal, vasorelaxant, sedative, antineoplastic, antimicrobial, antioxidant, antiallergic and wound healing properties (Umar *et al*., 2011). Recent investigations revealed the potential antifungal, antibacterial, antibiofilm, antioxidant and antitumour activities of essential oil isolated from the rhizomes of this plant (Hertiani *et al*., 2010; Jantan *et al*., 2008; Zaeoung *et al*., 2005).

MATERIALS AND METHODS

Study area

The rhizomes of *K. galanga* were collected from Umsning, located in Ri-Bhoi district of Meghalaya from the months of November 2019 to February 2020. The experiment was conducted from July 2019 to November 2020 at North Eastern Hill University located in the East Khasi Hills District, Shillong.

Extraction of essential oil

The rhizomes were subjected to hydro-distillation using a Clevenger apparatus at 40°C. The oil collected was then stored at-20°C, until required for bioassay.

Isolation, Identification and culture of phytopathogens

Pathogens infecting various plant parts inducing characteristic visible symptoms like spots, blights, mildew *etc*., were collected. For the isolation of the pathogenic fungi, agar plate method (Muskett and Malone, 1941) was used. Identification was based on the cultural characteristics and direct microscopic observations of the fruiting bodies and spores using standard manuals (Barnett and Hunter, 1998; Sutton, 1980). Pure cultures of the fungi were cultured in Potato Dextrose Agar medium and stored at 4°C. DNA was extracted and purified using HiPurA™ Fungal DNA Purification kit from MolBio Himedia according to the manufacturer's instructions and amplication was done using ITS1 and ITS4 primers (Potshangbam *et al*., 2017). PCR products were sent to Eurofins for nucleotide sequencing and results obtained were matched using the Basic Local Alignment Search Tool (BLASTn) from the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cgi.) for identification. The sequences were then submitted to GenBank for obtaining the accession number. The pathogens name, its host plant and accession numbers were recorded.

Antifungal activity assay of the essential oil

Fungitoxicity of the essential oil was studied using direct contact (M1) and Vapour phase method (M2) *in vitro*. Direct Contact method was done according to the methods by Gakuubi *et al*. (2017) and Khammassi *et al*. (2018), where different concentrations of the essential oil was dissolved in Tween 20 (0.5% v/v) and added into 20 ml molten PDA.

Vapour phase method was done as described by Manssouri *et al*. (2016). The percentage inhibition of the mycelial growth of the test fungi by the essential oil was calculated according to Philippe *et al*. (2012).

The essential oil was then sent to CSIR-Central Institute of medicinal and Aromatic Plants (CIMAP) in Lucknow for GC-MS analysis of compounds present.

Determination of the hyphal morphology of fungi after treatment with the essential oil

The hyphal morphology of the fungi after oil treatment was observed using Scanning Electron Microscopy (SEM) in North Eastern Hill University-Sophisticated Analytical Instrument Facility (NEHU-SAIF). Methods were followed accordingly as provided by NEHU-SAIF. Mycelial cubes (0.5 cm) were fixed with 3% glutaraldehyde for 4 hrs at 4°C. They were then washed with 0.1M buffer for 3 changes of 15 mins each at 4°C, after which the samples were subjected to dehydration in a graded acetone series (30%, 50%, 70%, 80%, 90%, 95%, 100%) for two changes of 15 mins each at 4°C. Tetramethylsilane was then added at the last step for one change of 15 mins at room temperature and left to dry completely. The fixed materials were then mounted on stubs and placed in a high-vacuum chamber which were then coated with gold/palladium in a sputter coater system. The samples were finally examined under the JEOL JSM-6360 SEM model at 20kV.

Determination of minimum inhibitory concentration and minimum fungicidal concentration

Minimum inhibitory concentration (MIC) of a sample is determined when there is no growth occurring in the mycelial disc diameter after treatment of the fungi with the oil. For determining the Minimum Fungicidal Concentration (MFC), the treated fungal disc were re-inoculated into freshly prepared media and observed for growth after incubation for 7 days at 27°C, if no growth occurred then that concentration of the oil was taken as the MFC (Gakuubi *et al*., 2017).

Antioxidant activity of the essential oil of *Kaempferia galanga*

DPPH (2,2-Diphenyl-1-picrylhydrazyl) Free-Radical Scavenging Assay was done as described by Umaru *et al*. (2019) to determine the concentration of the sample required to inhibit 50% of the DPPH free radical which was represented as IC_{50}. Four series of prepared concentrations (5, 10, 15, 20 µl mL^{-1}) of sample solutions (1 ml) were done in four replicates and measured spectrophotometrically at 515 nm.

The per cent inhibition was plotted against concentrations and the equation for the line was used to obtain the IC_{50} value.

Data analysis

Data obtained were expressed as mean ± standard deviation of three replicates. Using the IBM SPSS 20 software, one way analysis of variance (ANOVA) was used to analyse the effect of the different concentrations and the methods used on the mycelial growth of the fungal pathogens (Huang *et al*., 2021).

Significant differences between the means were calculated using Tukey HSD post- hoc test at p≤0.05.

RESULTS AND DISCUSSION

Molecular identification of the phytopathogens

The molecular identification of the plant pathogens, their accession number and plant host isolated from different crop plants are listed in Table 1.

Antifungal activity of *K. galanga* essential oil

From the M1 test conducted, all the fungal pathogens showed inhibition percentages from 50%-100% at 2 µl essential oil mL^{-1} media with the exception of *Cercospora* sp. which showed only 23% inhibition. Complete inhibition was observed in *Epicoccum* sp. With the vapour phase test, 65%-100% growth reduction of fungal mycelia at 2 µl mL^{-1} air was observed. This range of inhibition of essential oils exhibiting antimicrobial activity was also reported by Huang *et al*. (2021); Jantan *et al*. (2008) and Khammassi *et al*. (2018). The growth inhibition of the pathogens by the two methods at different concentrations and one way ANOVA is depicted in Table 3. Graphical representation of the reduction in fungal colony is given in Fig 1.

From the GC-MS results, the major compounds identified were Heptadecane, 3-Carene, 1-Borneol, Camphene, Cymene, Ethyl p-methoxy cinnamate, D-Limonene and β-myrcene. This is in accordance with reports as cited in literature (AlSalhi *et al*., 2020; Raina and Abraham, 2015).

MIC/MFC of oil against fungal pathogens

M1 showed MIC from 1.5 µl - 6 µl mL^{-1} and MFC from 2 µl-6 µl mL^{-1}, with the exception of *Fusarium* sp. and *Cercospora* sp.

Table 1: Species identification of the phytopathogens.

Culture ID	Species identified	Plant host	Accession number
KW-P-01	*Alternaria alternate*	*Solanum tuberosum*	ON212478
KW-P-02	*Cercospora beticola*	*Tetragonia tetragonioides*	ON212479
KW-P-03	*Colletotrichum fructicola*	*Phaseolus vulgaris*	ON212480
KW-P-04	*Epicoccum sorghinum*	*Phaseolus vulgaris*	ON212481
KW-P-05	*Fusarium oxysporum*	*Solanum tuberosum*	ON212482
KW-P-06	*Rhizoctonia solani*	*Phaseolus vulgaris*	ON212483

Table 2: Absorbance of the oil at various concentrations and its Radical Scavenging (RS) activity percentage.

DPPH+Methanol	5 µl/ml	10 µl/ml	15 µl/ml	20 µl/ml
1.4811	1.1973	1.0334	0.6114	0.5696
1.4862	1.1340	0.7079	0.6947	0.5621
1.4791	1.0882	0.9472	0.5199	0.4208
1.4723	1.1881	0.6489	0.6083	0.4659
RS (%)	22.15	43.61	58.87	65.89

Table 3: Mycelial growth inhibition (%) of essential oil of *K. galanga* against the given phytopathogens.

Assay type	Fungal phytopathogens	Growth reduction (%)				
		0.125 µl ml^{-1}	0.25 µl ml^{-1}	0.5 µl ml^{-1}	1 µl ml^{-1}	2 µl ml^{-1}
Direct contact test	*Alternaria* sp.	22.81 ± 1.48^{ab}	32.19 ± 1.39^{a}	46.65 ± 0.34^{ab}	53.91 ± 0.75^{Aa}	56.25 ± 0.88^{Aa}
	Fusarium sp.	10.12 ± 0.73^{c}	19.94 ± 0.55^{bc}	30.64 ± 0.47^{c}	45.09 ± 0.33^{b}	50.87 ± 0.33^{a}
	Epicoccum sp.	32.94 ± 1.86^{Fd}	42.06 ± 3.91^{F}	53.53 ± 3.09^{ad}	65.59 ± 1.21^{c}	100 ± 0^{b}
	Cercospora sp.	12.90 ± 1.74^{Gce}	14.92 ± 1.79^{GHc}	18.15 ± 1.53^{GHBe}	19.76 ± 1.01^{HJ}	23.39 ± 0.47^{BJ}
	Colletotrichum sp.	19.16 ± 0.35^{ae}	24.55 ± 0.69^{ab}	29.34 ± 0.69^{c}	43.11 ± 0.35^{b}	67.66 ± 0.49^{c}
	Rhizoctonia sp.	29.17 ± 2.42^{bd}	53.06 ± 3.70	68.33 ± 0.32^{M}	75.28 ± 2.15^{MNd}	78.33 ± 1.32^{Nd}
Vapour phase test	*Alternaria* sp.	47.50 ± 1.44	66.56 ± 1.18	81.25 ± 0	91.88 ± 1.08	100 ± 0^{b}
	Fusarium sp.	16.19 ± 1.67^{Cac}	19.94 ± 0.55^{Cbc}	53.76 ± 3.40^{Dda}	59.83 ± 0.29^{DEac}	65.90 ± 0.33^{Ec}
	Epicoccum sp.	11.18 ± 1.02^{c}	14.12 ± 0.68^{c}	42.35 ± 0.68^{b}	55.88 ± 0.34^{a}	82.94 ± 0.34^{d}
	Cercospora sp.	2.42 ± 1.54^{Kf}	3.23 ± 1.74^{Kd}	16.94 ± 1.40^{e}	45.16 ± 3.72^{b}	77.82 ± 3.51^{d}
	Colletotrichum sp.	29.04 ± 0.57^{Lab}	32.04 ± 1.41^{La}	58.68 ± 0.35^{d}	65.27 ± 0.69^{c}	93.71 ± 2.26^{b}
	Rhizoctonia sp.	0.56 ± 0.32^{0f}	0.56 ± 0.32^{0d}	15 ± 2.47^{e}	74.44 ± 3.88^{d}	100 ± 0^{b}

Note: Means in the same row with the same upper case letter and means in the same column with the same lower case letter are not significantly different according to the Tukey HSD post-hoc test (p≤0.05). Values are means±standard error.

which showed no MFC even at concentration four times of its MIC. M2 showed MIC from 2 µl - 10 µl mL^{-1}, however no MFC was observed in any of the fungal pathogens even at concentration of 20 µl mL^{-1}. IC_{50} of direct method shows in between the range of 0.22-3.77 µl mL^{-1} liquid media. The vapour phase test showed IC_{50} in between the range of 0.14 -1.13 µl mL^{-1} air space. Laghchimi *et al.* (2014) and Manssouri *et* al. (2016) also reported the efficacy of oil in vapour phase in quickly inhibiting fungal plant pathogens at lower concentrations than in liquid phase, as evident from the IC_{50} of M2 which is much lower compared to M1. The ability of the oil in direct contact method to effectively kill the pathogens maybe due to the combine effect of the volatile and non-volatile compounds present in the oil. All the results of this test are depicted in Table 4.

Antioxidant activity of the essential oil of *Kaempferia galanga*

The absorbance was taken at 515 nm and results obtained were listed in Table 2. The radical scavenging activity of the essential oil at 50% was plotted in excel and found to be at approximately 11.8 µl mL^{-1}.

Scanning Electron Microscopy of some of the pathogens when treated with the essential oil of *K. galanga*

Different fungi were treated with different concentration of oil (1.5-2 µl mL^{-1}) for observation of the hyphal morphology. As seen in Fig 2, the hyphae of the control group were observed to be full and almost uniform in thickness. The observed shrinkage in some of the hyphae may be due to dehydration, however that said, such shrinkage is observed in only some hyphae and are almost negligible. The fungi under direct contact and vapour phase both shows severely shrunk, deformed and shrivelled hyphae. Indentations of the oil-treated hyphae were also prominent. The hyphae were also highly compacted and clumped in comparison to the control group. Spores in the treated group were also not visible. Similar observations were also reported by Umaru *et al.* (2019).

Table 4: MIC, MFC and IC_{50} of the phytopathogens by direct contact and vapour phase method.

Fungi	Direct method test			Vapour phase test		
	MIC	MFC	IC_{50}	MIC	MFC	IC_{50}
Alternaria	3	4	0.63	2	-	0.14
Fusarium	6	-	1.82	10	-	0.45
Epicoccum	1.5	2	0.41	7	-	0.74
Cercospora	6	-	3.77	7	-	1.13
Colletotrichum	4	6	1.27	3	-	0.39
Rhizoctonia	3	6	0.22	2	-	0.75

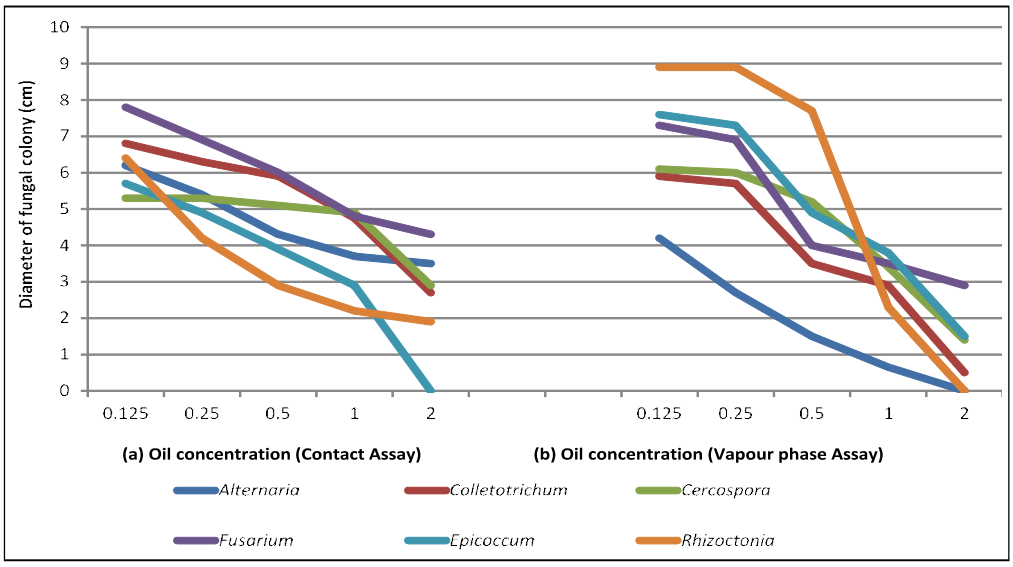

Fig 1: Graphical representation of fungal colony with increasing oil concentration.

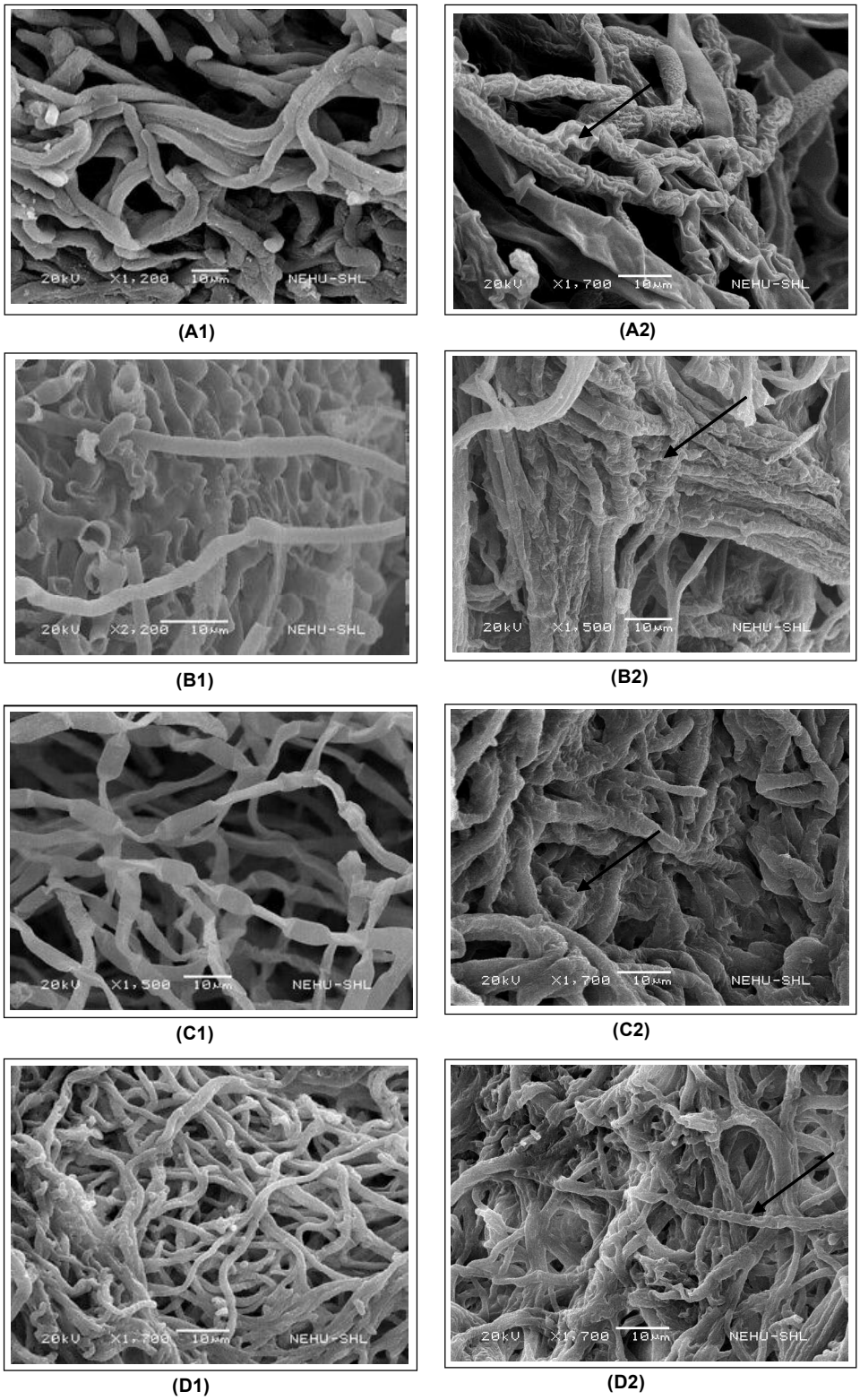

Fig 2: SEM micrographs of (A1) non-treated *Rhizoctonia*, (A2) oil treated *Rhizoctonia* by Contact method (CM), (B1) non-treated *Epicoccum*, (B2) oil treated *Epicoccum* by CM, (C1) non-treated *Alternaria*, (C2) oil treated *Alternaria* by Vapour Phase Method (VM), (D1) non-treated *Colletotrichum*, (D2) oil treated *Colletotrichum* by VM. Arrow marks indicate hyphal deformity.

CONCLUSION

From the two methods conducted, we can see that the vapour phase method shows a rapid decrease in fungal growth and the IC_{50} obtained is also at a relatively low concentration of the oil in comparison to the direct method. Though, the method fails to show any MFC to the pathogens, as compared to the direct method, yet the effectiveness in quickly inhibiting the phytopathogens may be due to the presence of many volatile compounds mostly of terpenes and alkanes in the oil. From the results, it is evident that the oil of *K. galanga* has the potential to inhibit or at least retard the growth of fungal phytopathogens as evident from the *in vitro* tests and SEM micrographs.

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Declaration of competing interest

Authors declare that there are no competing interests to this research.

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