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GC-MS Analysis of Phytocomponents and Antifungal Activities of *Zanthoxylum acanthopodium* DC. Collected from Manipur, India

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Authors' contributions

This work was carried out in collaboration between all authors. Authors OZD, AB and RW performed the whole work. Author OZD wrote the first draft of the paper. Authors KSR and OMS read and approved the final draft.

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ABSTRACT

Aims: The aim of this study was to evaluate the antifungal activity and phytochemical analysis of *Zanthoxylum acanthopodium* to validate the folklore use of this plant.
Study Design: The present study was designed to evaluate the antifungal activity in different crude extracts of the *Z. acanthopodium* leaves followed by the characterization of chemical constituents in the extract which possess highest antifungal activity by using gas chromatography-mass spectrometry (GC-MS).
Place and Duration of Study: Department of Botany, University of Delhi and Institute of Bioresources of Sustainable Development, Takyelpat Institutional Area, Imphal between December 2013 and May 2014.
Methodology: The antifungal activity of *Z. acanthopodium* leaf extracts in three organic solvents

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viz. petroleum ether, ethyl acetate and chloroform were investigated against 5 strains of fungi- *Aspergillus flavus, A. fumigates, A. niger, Candida albicans* and *C. krusei* by following agar well diffusion method. The characterization of bioactive compounds in petroleum ether extract was performed using GC-MS by electron ionization in scan mode.

Results: The extracts exhibited antifungal activity on two fungal species, namely *C. albicans* and *C. krusei* and the petroleum ether extract proved to possess high antifungal activity compared to the rest of the solvent extracts. The GC-MS analysis of petroleum ether extract of *Z. acanthopodium* leaves identified forty three compounds.

Conclusion: GC-MS study highlights the existence of various bioactive compounds in *Z. acanthopodium*. Paulownin is found as the major compound having highest peak area and it is reported to possess antifungal property which proves that petroleum ether crude extract of *Z. acanthopodium* could be used as a natural antifungal.

Keywords: Zanthoxylum acanthopodium DC.; antifungal activity; GC-MS; phytocomponents.

1. INTRODUCTION

Z. acanthopodium is commonly known as prickly ash, toothache tree, lemon pepper tree, yellow wood, suterberry and in Manipur, it is locally known as Mukthrubi. Manipur, a border state in the North Eastern corner of India is known for its richness in medicinal plants. The fruits, seeds and leaves of the plant are traditionally used in chronic fever, cholera, dyspepsia, dysentery, stomachache, toothache, cough, bronchitis and hair diseases [1,2]. The plant extract is used in the preparation of insecticides.

The chemical studies carried out on *Zanthoxylum* species have revealed the occurrence of alkaloids, lignans, steroids, terpenes, amides and coumarins, of which some shows fungitoxic potential [3]. Extracts of some *Zanthoxylum* species, namely *Z. chiloperone* [4], *Z. monophyllum* [5], *Z. fagara, Z. elephantiasis* and *Z. martinicense* [6], *Z. americanum* [7] and *Z. armatum* [8] have shown significant antifungal activity. In the case of *Z. acanthopodium*, recently Ishwori et al. [9] have reported the antibacterial activity in the methanolic leaf extracts of *Z. acanthopodium* and its antifungal activity is not yet reported.

The literature survey reveals that there are an increasing number of studies focussing the phytochemicals of *Z. acanthopodium* in India. Virendra & Blazquez reported the chemical analysis of *Z. acanthopodium* leaf oil and the major compounds are linalool (14.3%), 9, 12-octadecadien-ol (8.4%), 1, 8-cineole (7.7%), 2-undecanone (7.3%), farnesol (3.6%), 9,12,15-octadecatrien-1-ol (3.2%) and β-caryophyllene (3.0%) [10]. The chemical constituents of essential fruit oil from *Z. acanthopodium* growing in Meghalaya have been determined [11,12] and the main components are eucalyptol (36.563%), limonine (16.903%), δ-3-carene (13.525%) and methyl-cinnamate (9.366%).The flavonoids such as herbacetin-8,4'-dimethyl ether [13,14] and 7-O-α-D-glucosyl-3,8-dihydroxy-2-(3-hydroxy-4-methoxy phenyl)-5-methoxy-4*H*-1-benzopyran-4-one [15] have been isolated from the *Z. acanthopodium* fruits collected from Shillong. Tapan Seal has analyzed the nutritive value, mineral contents and antioxidant activity of the plant growing in Meghalaya, India [16]. In the international arena, there are reports for chemical analysis of *Z. acanthopodium* growing in Brazil [17] and Indonesia [18-20].

The literature search reveals that so far no study has attempted to work on the evaluation of antifungal activity on Z. acanthopodium crude extracts and also the chemical analysis has been performed only on essential oil of leaf and fruit, not on crude extracts. Therefore, the aim of the present study is to determine the antifungal activity of the *Z. acanthopodium* leaf extracts in petroleum ether, chloroform and ethyl acetate; and characterization of the phytocomponents in the extract which shows maximum antifungal activity by GC-MS technique.

2. MATERIALS AND METHODS

2.1 Chemicals

All solvents used in this study such as petroleum ether, chloroform and ethyl acetate were obtained from Merck, Mumbai, India. Nutrient agar, potato-dextrose Agar, sabouraud dextrose agar and amphotericin-B were obtained from Hi-Media. The other chemicals used were of analytical grade.

2.2 Plant Sample

Z. acanthopodium was collected from Thoubal district, Manipur, India. Its identification was clarified by the staff member of Medicinal Plants and Horticultural Resources Division, Institute of Bioresources of Sustainable Development (IBSD), Manipur, India after comparison with the herbarium sample (specimen voucher no. 2114) in the department. All plant leaves were washed with fresh water and dried under the shade at room temperature. The dried leaves were powdered using a grinder machine and stored in a sterile container for further use.

2.3 Sample Preparations

A mass of the dried powdered leaves was placed in a Soxhlet apparatus and extracted separately with petroleum ether, ethyl acetate and chloroform for 12 hrs. The crude extracts were filtered to obtain particle-free crude extract and the filtered extracts were concentrated on a rotary evaporator. Some parts of the crude extracts were analyzed for antifungal activity. The petroleum ether extract showed maximum antifungal activity compare to other two extracts, hence, the petroleum ether extract was subjected to GC-MS analysis. GC-MS sample was prepared by dissolving 50 mg of the petroleum ether crude extract in 2 mL of petroleum ether and filtered using whatman no. 1. filter paper to obtain a clear sample.

2.4 Evaluation of Antifungal Activity

The antifungal activity was assessed by agar well diffusion method [21] using 20 mL of sterile nutrient agar, potato-dextrose agar and sabouraud dextrose agar. The test fungi are *Aspergillus flavus, A. fumigates*, *A. niger*, *Candida albicans* and *C. Krusei*. The test cultures were swabbed on the top of the solidified media and allowed to dry for 10 min. Sterile 6 mm diameter cork borer was pierced in the agar. Each compound was diluted to 15 mg/mL. 40 µL of each diluted compound were deposited on the inoculated well and left for 10 min at room temperature for the compound diffusion. Negative control was prepared using dimethyl sulphoxide (DMSO). Amphotericin-B was served as positive control. The plates, which are inoculated with fungi were incubated at 30ºC for 24-48 hr. The experiment was repeated thrice and the average results were recorded. The antifungal activity was determined by measuring the diameter of the inhibition zone (mm) around the well.

The susceptibility of microbial was determined by minimum inhibitory concentration determination method [22]. The minimum inhibitory concentrations (MICs) of the compounds were determined by serial dilution against the micro-organisms. The minimum concentrations at which no visible growth were observed were defined as the MICs, which were expressed in mg/mL.

2.5 GC-MS Analysis

The petroleum ether crude extract from the leaves of *Z. acanthopodium* was analyzed by using GC clarus 500 Perkin Elmer system comprising a AOC-20I auto sampler and gas chromatograph interfaced to a mass spectrophotometer instrument equipped with column Elite-1 fused silica capillary column (30 mm x 0.25 mm I. D x 1µM df, composed of 5% diphenyl/95% dimethyl polysiloxane), operating in the electron impact mode at 70 ev. Inert gas, helium was used as carrier gas at a constant flow rate of 1.21 ml/min and an injection volume of 1 µl was employed (split ratio of 10:1); injector temperature and ion-source temperature was set at 250^{o} C and 230^{o} C, respectively. The oven temperature was programmed from 100^{o}C (isothermal for 2 min), with an increase of 10^{o} C/min to 280^{o} C, ending with a 9 min isothermal at 280 ^{o} C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was 46 min. The percentage composition of the crude extract constituents was expressed as a percentage of the peak area.

2.6 Identification of Components

The identification of chemical compounds in the crude extract was based on similarity of GC retention time and mass spectra (%) with the standards. The mass spectra of the individual components were compared with those of standards stored in NIST (National Institute of Standards and Technology, U.S. Department of Commerce) and Wiley (John Wiley & Sons Ltd) libraries. The details about their name, molecular formula, molecular weight and structure were ascertained.

3. RESULTS

3.1 Antifungal Activity

The crude extracts of *Z. acanthopodium* exhibited moderate antifungal activity against two fungi namely *Candida albicans* and *C. Krusei* as compared to the standard Amphotericin-B and the results were presented in Table 1. The zone of inhibition is higher in *C. Krusei* (18 mm) as compared to *C. albicans* (10 mm). The petroleum ether extract was found to have great antifungal activity (18 mm) in comparison to rest of the solvent extracts as shown in Fig. 1. The susceptibility of the microbial was also determined by calculating minimum inhibitory concentration (MIC) of the extracts against all the five fungi as shown in Table 2. In all the extracts, the minimum MIC was found against *Candida krusei* and the lowest MIC (< 0.9375 mg/mL) was found in the petroleum ether extract.

3.2 GC-MS Analysis

The petroleum ether crude extract of *Z. acanthopodium* leaves were analysed by GC-MS which leads to the identification of 43 organic compounds. The chemical compounds were identified according to their retention time on a fused silica capillary column. The list of the compounds with their retention time, molecular formula, molecular weight, area and area (%) were presented in Table 3. The chromatogram of the identified compounds presented in Fig. 2 showed 6 prominent peaks. They are paulownin (24.31%), a lignan with retention time of 31.494, 1*H*,3*H*-furo[3,4-*c*]furan, 1,4-bis(3,4-dimethoxyphenyl)tetrahydro-(16.49%) or eudesmin, a lignan with retention time of 32.521, stigmast-5-en-3-ol, (3-β)- or β-sitosterol, a steroid (10.74%) with retention time of 33.910, 4-tert-butylcalix[4]arene (6.56%), a polycyclic aromatic hydrocarbon with retention time of 43.925, *n*-hexadecanoic acid (3.28%), a fatty acid with retention time of 15.299 and phytol (2.71%), a fatty alcohol with retention time of 16.742.

Table 1. Antifungal activity (zone of inhibition) of different extracts of *Z. acanthopodium* against fungi

Plant extracts	Zone of inhibitions (mm)				
	1	2	3	4	5
Petroleum Ether (15 mg/mL)	-	-	-	10	18
Chloroform (15 mg/mL)	-	-	-	10	14
Ethyl Acetate (15 mg/mL)	-	-	-	12	16
Amphotericin-B (16 µg/mL)	32	34	38	38	40

1- Aspergillus flavus; 2 - A. Fumigates; 3 - A. Niger; 4 – Candida albicans; 5 - C. krusei, Values are average of three determinations

Table 2. MIC for antifungal activity

Chemical	fungal	MIC (mg/mL)
Petroleum Ether	1	>15
	2	>15
	3	>15
	4	3.75
	5	<0.9375
Chloroform	1	>15
	2	>15
	3	>15
	4	>3.75
	5	1.875
Ethyl Acetate	1	>15
	2	>15
	3	>15
	4	<1.875
	5	>0.9375

1- Aspergillus flavus; 2 - A. Fumigates; 3 - A. Niger; 4 – Candida albicans; 5 - C. krusei

Table 3. Components identified in the petroleum ether extract of *Z. acanthopodium* leaves

Sl. no.	R. time	Area	Area%	Name of the compound
1	8.720	207059	0.32	Dodecane
2	10.061	181875	0.28	Hexadecane
3	10.507	70906	0.11	1-methyl-4-(5-methyl-1-methylene-4-hexenyl) or bisabolene
4	10.878	156168	0.24	Dodecanoic acid
5	11.385	226271	0.35	(-)-5-oxatricyclo[8.2.0.0(4,6)]dodecane,,12-trimethyl-9-methylene-,
6	13.193	483198	0.74	Tetradecanoic acid
7	14.025	174975	0.27	2,6,10-Trimethyl,14-ethylene-14-pentadecne
8	14.097	480360	0.74	2-Pentadecanone, 6,10,14-trimethyl-
9	14.421	456225	0.70	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
10	14.899	294569	0.45	1,2-Benzenedicarboxylic acid, diundecyl ester
11	15.299	2128648	3.28	n-Hexadecanoic acid
12	15.578	306617	0.47	1,2-Benzenedicarboxylic acid, bis(2-methoxyethyl) ester
13	16.201	319489	0.49	(Z)6,(Z)9-Pentadecadien-1-ol
14	16.448	313589	0.48	1-Hexadecanol
15	16.742	1759863	2.71	Phytol
16	17.026	674775	1.04	Z,Z-8,10-Hexadecadien-1-ol
17	17.654	429033	0.66	bis[(2E)-Dec-2-en-1-yloxy](dimethyl)silane
18	18.502	271068	0.42	Ethanol, 2-[2-[4-(1,1,3,3-tetramethylbutyl)phenoxy]ethoxy]-
19	19.000	393284	0.61	4,8,12,16-Tetramethylheptadecan-4-olide
20	25.677	867113	1.34	Furan, 4,5-diethyl-2,3-dihydro-2,3-dimethyl-
21	25.938	612653	0.94	Furan, 4,5-diethyl-2,3-dihydro-2,3-dimethyl-
22	26.326	244098	0.38	3,11-Dioxatricyclo[5.4.0.0(2,4)]undecane, 1,6,6,10-tetramethyl-, (1R,2R,4R,7R),
23	26.600	214100	0.33	Neryl linalool isomer
24	28.867	941942	1.45	Hexatriacontane
25	28.998	1081496	1.67	Octacosanol
26	29.256	678505	1.04	Stigmast-5-en-3-ol, oleat
27	29.652	245943	0.38	Cholesterol
28	29.785	366073	0.56	Vitamin E
29	30.436	1427453	2.2	2,6-Bis(3,4-methylenedioxyphenyl)-3,7-dioxabicyclo(3.3.0)octane
30	30.967	275412	0.42	Pluviatilol .gamma.,.gamma.-dimethylallyl ether
31	31.115	1094328	1.68	Fargsin
32	31.494	15792931	24.31	Paulownin
33	31.799	1424984	2.19	Ergost-5-en-3-ol, (3.BETA.,24R)-
34	32.239	1889297	2.91	1H,3H-Furo[3,4-c]furan, 1,4-bis(3,4-dimethoxyphenyl)tetrahydro-,
35	32.521	10710370	16.49	1H,3H-Furo[3,4-c]furan, 1,4-bis(3,4-dimethoxyphenyl)tetrahydro-,
36	32.891	1158005	1.78	Hexatriacontane
37	33.910	6974051	10.74	Stigmast-5-en-3-ol, (3.BETA.)-
38	34.840	597660	0.92	4,4,6A,6B,8A,11,11,14B-OctamethyL-1,4,4A,5,6,6A,6B,7,8,8A,9,10,11,12,12A,
39	35.433	23546	1.58	Methyl commate D
40	36.101	1575055	2.42	Lup-20(29)-en-3-yl acetate
41	37.273	815119	1.25	Vitamin E
42	37.736	1353250	2.08	Stigmast-4-en-3-one
43	43.925	4260826	6.56	Pentacyclo[19.3.1.1(3,7).1(9,13).1(15,19)]octacosa-1(25),3,5,7(28),9,11,13(27), or 4-tert-Butylcalix[4]arene

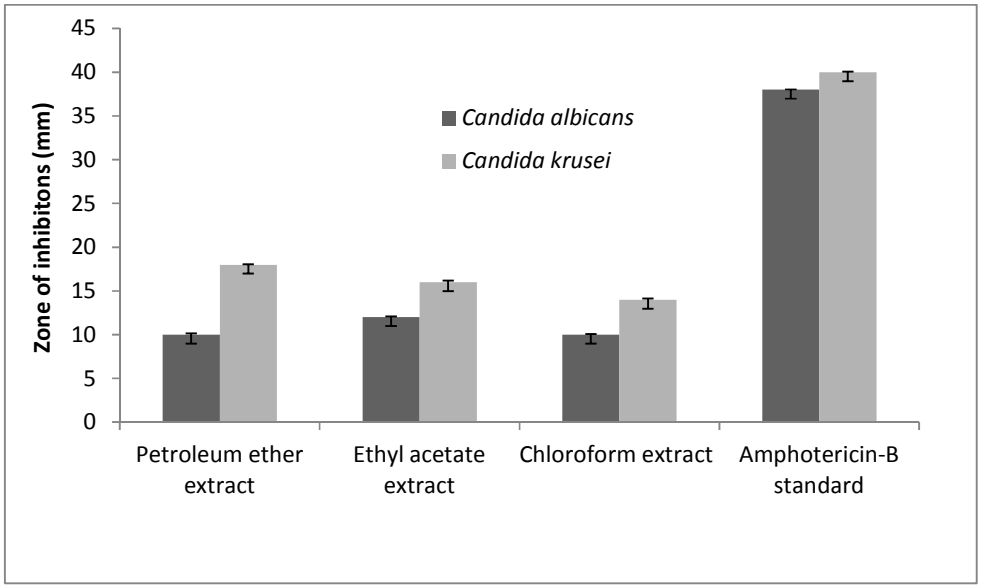

Fig. 1. Antifungal activity of different extracts of *Z. acanthopodium leaves*

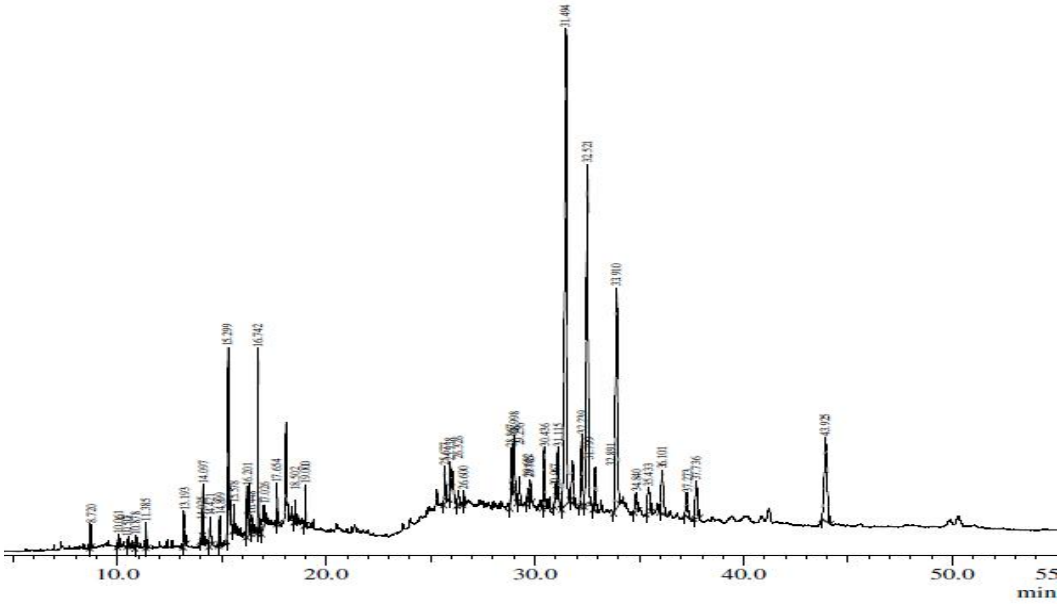

Fig. 2. Chromatogram of the phytochemicals in petroleum ether extract of *Z. acanthopodium* leaves

4. DISCUSSION

The entire unknown constituents of *Z. acanthopodium* were extracted with common solvents in the order of increasing polarity (petroleum ether, chloroform and ethyl acetate) and all the extracts were subjected to antifungal assay. The petroleum ether crude extract of *Z. acanthopodium* showed to possess maximum antifungal activity and this extract was analyzed for chemical constituents by GC-MS. The major classes of bioactive compound found in the extract are lignan, terpenens, steroid, fatty acid, fatty alcohol and hydrocarbons. Literature search reveals that most of the predominant compounds identified in the extract are biologically active molecules [23-33] and some have antifungal activity [23-32]. Table 4 listed the biological activity of the major compounds.

Table 4. Biological activity of major compounds identified in the petroleum ether extract of *Z. acanthopodium* leaves

Sl. No.	Name of the compound	Biological activity
1.	β-sitosterol	Anticancer,anti-hypercholesterolemic, anti-inflammatory, antibacterial, antifungal, and anti-hyperlipoproteinemic activities
2.	Hexadecanoic acid or palmitic acid	Antifungal activity, antioxidant
3.	Phytol	Antifungal, anticancer, anti-inflammatory, anti-diuretic, immunostimulatory and anti-diabetic
4.	Paulownin	Exhibits insecticide enhancing activity & antifungal activity
5.	1H,3H-furo[3,4-c]furan, 1,4-bis(3,4-dimethoxyphenyl)tetrahydro-, [1r-(1.alpha.,3a.alpha.,4.alpha.,6a.alpha.)]- or eudesmin	Antifungal, potent platelet activating factor antagonist, antioxidant, inhibitor of camp phosphodiesterase and antitumor
6.	4-tert-butylcalix[4]arene	No report on its activity

Among the five lignans (paulownin, eudesmin, fargesin, sesamin and pluviatilol.gamma.gamma.-dimethylallyl ether) detected in the extract, paulownin and eudesmin possess antifungal properties [26,27]. Paulownin which is the major compound in the extract also exhibited insecticide enhancing activity [28]. Other compounds like β-sitosterol [29], fatty acids such as dodecanoic acid, tetradecanoic acid and n-hexadecanoic acid [30,31] and fatty alcohols such as phytol and octacosanol also exhibited antifungal property [24,32,33]. Fig. 1 in supplementary material showed the structures of the major constituents of *Z. acanthopodium* leaf extract *viz*: paulownin, eudesmin, sitosterol, n-hexadecanoic acid, phytol and 4-tert-Butylcalix[4]arene. Among the major constituents of *Z. acanthopodium* leaf extract, eudesmin, β-sitosterol and n-hexadecanoic acid have been reported from other zanthoxylum species such as *Z. armatum* [34], *Z. oxyphyllum* [35], *Z. culantrillo* [36], *Z. limonella* [37,38], *Z. bungeanun* [39] and *Z. rhetsa* [40]. However, this is the first report for the identification of paulownin and 4-tert butyl calix in zanthoxylum family. The identification of many important compounds from *Z. acanthopodium* thus provides a significant reference point for the therapeutic use of this plant. However, the isolation of individual active compounds and the study of their biological activity will give a fruitful result.

5. CONCLUSION

The petroleum ether extract of *Z. acanthopodium* exhibited maximum antifungal activity by inhibiting the growth of *C. albicans* and *C. krusei.* The GC-MS analysis of the petroleum ether extract of *Z. acanthopodium* revealed the presence of many bioactive compounds. Among the identified compounds, paulownin, eudesmin, β-sitosterol, dodecanoic acid, tetradecanoic acid, n-hexadecanoic acid, phytol and octacosanol have the property of antifungal activity as reported by earlier worker. Paulownin is found as a major compound in the petroleum ether extract of *Z. acanthopodium.* To our knowledge, this report is the first report to investigate antifungal activity as well as the chemical composition of plant crude extracts of *Z. acanthopodium* by GC–MS. Hence, it could be concluded that the petroleum ether extract of *Z. acanthopodium* can be used for antifungal activity. Further studies are needed for the isolation and identification of individual compounds from the plant crude extracts of *Z. acanthopodium* and also *in vivo* studies are needed for better understanding of their mechanism of action as antifungal.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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