Composition analysis of exudates produced by conifers grown in Taiwan and their antifungal activity

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Abstract
Exudates are involved in the defense mechanism of trees; they could work against insects or microorganisms through a physical or chemical system. The main components of exudates are terpenoids. This study identified the main compounds of exudates from 13 conifers of Taiwan using gas chromatogram–mass spectrometry (GC–MS) and spectroscopic analysis. The results revealed that the main volatiles were α-pinene, β-ocimene, β-pinene, sabinene, and caryophyllene. On the other hand, the main nonvolatile compounds were diterpenoids, which were classified into three skeletons (abietane-, labdane-, and pimarane-types). Among these, abietane-type presented in Pinaceae and in most of Cupressaceae; labdane-type presented in Pinaceae and in all of Cupressaceae and Araucariaceae; pimarane-type existed in both Pinaceae and Cupressaceae. Furthermore, the epigenetics of conifers analysis results by GC–MS and heteronuclear single quantum coherence (HSQC) of nuclear magnetic resonance (NMR) fingerprints were similar to traditional taxonomy classification; it indicated that exudates chemotaxonomy by using GC–MS and HSQC profiling is a useful technology to classify the conifers. Besides, the exudates of Pinus elliottii, Pinus taiwanensis, Calocedrus macrolepis and Chamaecyparis formosensis possessed the strong antifungal activity. For white-rot fungus, Trametes versicolor, Pinus morrisonicola, Chamaecyparis obtusa, and Araucaria heterophylla exhibited the higher antifungal index. For brown-rot fungus, Laetiporus sulphureus, Pinus elliottii, Pinus morrisonicola, and Chamaecyparis formosensis revealed a good antifungal activity.

Keywords: Diterpenoids, GC–MS, HSQC, Metabolomics

Introduction
Exudates are a constitutive defense system of conifers against insects or pathogens [1, 2]. The main compositions of resin are sesquiterpenoids and diterpenoids in conifers [3–7]. The resin will exude when conifers attacked by insects; it repels insects by intoxication and obstruction mechanism. On the other hand, volatile compounds of exudates contribute the indirect defenses against herbivores, and nonvolatile diterpenoids provide direct protection through the formation of lasting physical barriers at the point of insect attack [8, 9]. Regarding the classification of tree exudates, which could be classified into resin, gum, kino, latex, manna, amber, balsam, maple sugar, and crystalline compounds [10]. Conifers contain richness resins in the resin ducts or blisters; it secretes after mechanical injures or attacked by insects or pathogens [11–13].

Because tree exudates have high application value in medicine and industry, the bioactivities investigation for exudates is also a fascinating topic attracting many researchers. Several bioactivities of exudates have been proved, for example, anti-oxidative and anti-inflammatory activities, cytotoxicity, antimicrobial activity, central nervous system diseases regulation, cardiovascular diseases prevention, α-glucosidase inhibitory activity,
wound-healing activity, gastroprotective, antifungal activity, and hepatoprotective activity [14–35]. Some tree exudates possessed unique flavors, such as frankincense and myrrh, which are famous fragrances since ancient times [36].

In this study, the metabolite fingerprints of the conifer exudates were established by gas chromatogram–mass spectrometry (GC–MS) and nuclear magnetic resonance (NMR) analysis to characterize the composition and classification of exudates of 13 conifers grown in Taiwan. Although NMR or GC–MS techniques have been used to analyze the composition of exudates, it did not obtain enough data to decipher their chemotaxonomy as well as the major compounds in the exudates [37–41]. Our research revealed that epigenetics of conifers analysis by metabolomic analysis strategy and proved that exudates chemotaxonomy by using GC–MS and 2D-NMR metabolites profiling is a useful technique to classify the conifers. It is the first time to identify the significant compounds of the conifers and classify them using two-dimensional (2D) heteronuclear single-quantum coherence (HSQC) spectral analysis. In the meantime, the antifungal activity of exudates was also examined in this study.

Materials and methods

Plant materials

Exudates of conifers were collected at the Campus of National Chung Hsing University (NCHU) and Huisun Experimental Forest Station, located at the central of Taiwan, in July 2018. Totally 13 exudates of conifers were collected in this study, including Pinus elliottii (P. elliottii), Pinus insularis (P. insularis), Pinus taiwanensis (P. taiwanensis), Pinus morrisonicola (P. morrisonicola), Pseudotsuga wilsoniana (P. wilsoniana), Cunninghamia lanceolata (C. lanceolata), Cunninghamia konishii (C. konishii), Chamaecyparis formosensis (C. formosensis), Chamaecyparis obtusa (C. obtusa), Calocedrus macrolepis (C. macrolepis), Agathis dammarra (A. dammarra), Araucaria cunninghamii (A. cunninghamii), and Araucaria heterophylla (A. heterophylla). One exudates of broadleaf tree Liquidambar formosana (L. formosana). All the trees were identified by Prof. Yen-Hsueh Tseng (Department of Forestry, NCHU), and they were healthy trees. The conifers sampled are all planted trees, with an age of 30–40 years and the DBH were between 18 and 62 cm in diameter (Table 1).

The exudates were collected by first creating a 5-cm wounding site on the tree trunk with a scraping cutter, the exudates that naturally exuded from wounding site after 1 day, and then was collected directly in a sample vial, and then dissolved in ethyl acetate (EA), then used the 0.45-micrometer filter to remove any particulate matter. The exudates were dried by N2, and stored at dark and frozen stored (4 °C) until study.

### Table 1 The information of exudate-sampling trees in this study

| Tree          | Location      | Age (years) | DBH (cm) |
|---------------|---------------|-------------|----------|
| Pinus elliottii | Campus⁣       | 30          | 28       |
| Pinus insularis | Campus        | 35          | 58       |
| Pinus taiwanensis | Experimental forest⁣ | 35          | 62       |
| Pinus morrisonicola | Experimental forest⁣ | 35         | 60       |
| Pseudotsuga wilsoniana | Experimental forest⁣ | 35         | 61       |
| Cunninghamia lanceolata | Experimental forest⁣ | 35         | 45       |
| Cunninghamia konishii | Experimental forest⁣ | 35         | 43       |
| Chamaecyparis formosensis | Experimental forest⁣ | 40        | 37       |
| Chamaecyparis obtusa | Experimental forest⁣ | 40        | 38       |
| Calocedrus macrolepis | Campus | 30          | 18       |
| Agathis dammarra | Campus       | 30          | 45       |
| Araucaria cunninghamii | Campus | 30          | 49       |
| Araucaria heterophylla | Campus | 30          | 48       |
| Liquidambar formosana | Experimental forest⁣ | 30         | 48       |

⁣ a Campus: The main campus of National Chung-Hsing university, the location is at Taichung City, Taiwan
⁣ b Experimental Forest: Huisun Experimental Forest Station of National Chung-Hsing University, which located at the central of Taiwan, the altitude is about 800 m

Headspace solid-phase microextraction/GC–MS analysis

To obtain and analyze of complete volatile compounds of exudates, the solid-phase microextraction (SPME) technique was performed to collect the volatile compounds. A SPME holder and carboxen-polydimethylsiloxane (75 μm) were purchased from Supelco Co. (Bellefonte, USA). Before using, SPME fibers were conditioned by heating in a hot injection port of a GC at 250 °C for 20 min to remove contaminants. The exudates were placed into a 20-μL sample vial sealed with the parafilm. The volatile compounds were analyzed by ITQ 900 mass spectrometer coupled with DB-5MS column. The temperature program was as follows: 40 °C for 2 min, then increased by 4 °C min⁻¹ to 100 °C and then increased 10 °C min⁻¹ to 280 °C hold for 5 min. The other parameters were injection temperature, 240 °C; ion source temperature, 200 °C; EI, 70 eV; carrier gas, He 1 mL min⁻¹ and mass scan range 40–600 m/z. The volatile compounds were identified by Wiley/NBS Registry of Mass spectral databases (V. 8.0), National Institute of Standards and Technology (NIST) Ver. 2.0 GC–MS libraries, and the Kovats indices were calculated for all volatile constituents using a homologous series of n-alkanes C₉–C₂₄. The major components were identified by co-injection with standards (wherever possible).
Extraction and isolation
The exudates of *A. cunninghamii*, *C. macrolepis*, *C. formosensis*, and *P. taiwanensis* were selected to purify and identify the major compounds in exudates. The exudates were chromatographed over silica gel eluted with *n*-hexane and gradient of *n*-hexane–EtOAc (ethyl acetate). The eluent was collected in constant volumes, and combined into five fractions based on the results of thin-layer chromatography (TLC) analysis. Each fraction was further separated by high-performance liquid chromatography (HPLC) (1100 Series, Agilent, Santa Clara, California, U.S.) using a normal-phase column (250 × 10 mm, 5 μm, Phenomenex Co., Washington, D.C., U.S.). The structures of compounds were elucidated and confirmed by spectroscopic analysis. The 1H-NMR spectra of compounds 1–22 are shown in the supplementary data (Additional file 1: Fig. S1–S22).

NMR analysis
The NMR spectra were obtained using a Bruker Avance III-400 NMR spectrometer. For HSQC, approximately 20 mg of the exudate was dissolved in 600 μL of CDCl₃ containing tetramethylsilane (TMS), and the solution was loaded into 5-mm NMR tube. Spectra were recorded with standard pulse sequences of the instrument at 300 K without spinning. On the X-axis (¹H axis) of HSQC was divided into 10 divisions from 10 to 1 ppm named 1 to 10; Y-axis (¹³C axis) was divided 16 divisions from 0 to 160 ppm named A to P. Thus, the HSQC spectrum was divided into A1 to P10 areas (Fig. 1).

Antifungal assay
The antifungal assay was performed in this study to evaluate the antifungal activity of exudates and ferruginol. Two fungal strains were used, namely *Trametes versicolor* (L. ex Fr.) Quel. (BCRC 35253, Bioresource Collection and Research Center, Hsinchu city, Taiwan) and *Laetiporus sulphureus* (B. ex Fr.) Bond (BCRC 35305). Antifungal assays were performed three times and the data were averaged. Exudates were added to sterilized potato dextrose agar (PDA) to give 200 ppm concentrations of extractives. The testing plates were incubated at 27 ± 2 °C. When the mycelium of fungi reached the edge of the control plate the antifungal index was calculated as follows: antifungal index (%) = (1 − Da/Db) × 100, where Da: diameter of growth zone in the experimental dish (cm), Db: diameter of growth zone in the control dish (cm).

Data analyses
Cluster analyses and principal components analyses (PCA) were performed with MVSP (Multi-Variate Statistical Package, V3.13 m) program to evaluate the similarity of fragrance compounds emitted from coniferous exudates.

Results and discussion
Yield of exudate
Exudates of Pinaceae and *C. macrolepis* were light-yellow liquid, Araucariaceae and genus *Cunninghamia* were milky white liquid, genus *Chamaecyparis* and *L. formosana* were light-yellow solid. Exudates of Araucariaceae, genus *Cunninghamia* and *Pinus* could be collected more than 1000 mg/day, Cupressaceae and *P. wilsoniana* were 300–500 mg/day.

Volatile compounds identification in exudates
Table 2 shows the analysis results of volatiles emitted from the conifer exudates. Although contents and compositions of volatiles were various in different conifers, the dominant compounds were monoterpenoids and sesquiterpenoids. Based on the results obtained in this study, the most abundant volatiles in conifer exudates were α-pinene, β-ocimene, β-pinene, sabinene, and caryophyllene. The abundant volatiles in Pinaceae were α-pinene and sabinene; however, α-pinene and β-pinene were the abundant compounds in Cupressaceae. Regarding Araucariaceae, the molecular weight of volatiles emitted from *A. heterophylla* was higher than others; most of them were sesquiterpenoids. However, the dominant volatiles in *A. cunninghamii* and *A. dammara* were α-pinene and β-ocimene. Caryophyllene was abundant in genus *Cunninghamia*. Multivariate statistical analysis was...
performed to compare the degrees of similarity of the volatile composition from conifer (Fig. 2). Exception was C. obtusa, which was classified into the Araucaria group by volatiles, because it had higher β-ocimene. Results from other tree species analysis are consistent with the traditional (morphology) taxonomy results. The results

| Constituent | KI | Contents (%) | Identification |
|-------------|----|--------------|----------------|
| Tricyclene  | 906 | 0.1          | -              |
| α-Thujene   | 921 | 0.1          | -              |
| α-Pinene    | 935 | 0.1          | -              |
| Camphene    | 950 | 0.1          | -              |
| Sabinene    | 977 | 0.1          | -              |
| β-Pinene    | 989 | 0.1          | -              |
| α-Pheollandrene | 1004 | 0.1  | - |
| 3-Carene    | 1007| 0.1          | -              |
| α-Terpine   | 1016| 0.1          | -              |
| p-Cymene    | 1023| 0.1          | -              |
| β-Ocimene   | 1029| 0.1          | -              |
| β-Pheollandrene | 1031 | -  | - |
| γ-Terpine   | 1057| 0.1          | -              |
| Terpinolene | 1084| 0.1          | -              |
| p-Cymenene  | 1087| 0.1          | -              |
| Estragole   | 1196| 0.1          | -              |
| Bornel      | 1284| 0.1          | -              |
| δ-Elemene   | 1336| 0.1          | -              |
| α-Cubebeke  | 1350| 0.1          | -              |
| Ylangene    | 1372| 0.1          | -              |
| α-Copaene   | 1380| 0.1          | -              |
| β-Cubebeke  | 1389| 0.1          | -              |
| α-Gurjunene | 1404| 0.1          | -              |
| Caryophyllene | 1412 | t  | - |
| β-Gurjunene | 1435| 0.1          | -              |
| Aromadendrene | 1441 | t  | - |
| α-Guaine    | 1448| 0.1          | -              |
| Alloaromadendrene | 1453 | t  | - |
| γ-Murrolene | 1462| 0.1          | -              |
| Germacrene D | 1469 | t  | - |
| β-Chamigrene | 1475 | -  | - |
| Valencence  | 1480| 0.1          | -              |
| γ-Cadinene  | 1517| 0.1          | -              |
| δ-Cadinene  | 1521| 0.1          | -              |
| L-Calamenene | 1525| 0.1          | -              |
| α-Cadinene  | 1537| 0.1          | -              |

Total identified (%) | 95.0 | 39.9 | 83.1 | 99.1 | 97.5 | 94.3 | 98.8 | 97.9 | 95.2 | 99.9 | 64.6 | 99.8 | 98.4

**Table 2** The volatile organic compounds of exudates

A1 Agathis dammara, A2 Araucaria cunninghamii, A3 Araucaria heterophylla, C1 Calocedrus macrocarpa, C2 Chamaecyparis formosensis, C3 Chamaecyparis obtuse, C4 Cunninghamia konishii, C5 Cunninghamia lanceolata, P1 Pinus elliottii, P2 Pinus insularis, P3 Pinus mironnicola, P4 Pinus taiwanaensis, P5 Pseudotsuga wilsoniana

KI Kovats retention index on DB-5MS column in reference to n-alkanes

MS the mass spectrum in NIST and Wiley libraries and in the literature

ST standard compounds

t trace, concentration < 0.1%
obtained from this study show that chemotaxonomy has considerable reference value.

**Identification of nonvolatile compounds in exudates**

To understand the skeletons of the main constituents in the exudates, four conifers, including *A. cunninghamii*, *C. macrolepis*, *C. formosensis*, and *P. taiwanensis*, with higher among of exudates were selected for the separation and identification of the compounds. After spectral analysis, 22 compounds (Fig. 3) were identified from exudates of *A. cunninghamii*, *C. macrolepis*, *C. formosensis*, and *P. taiwanensis*. All of these compounds of exudates were diterpenoids, including isocupressic acid (1), acetyl isocupressic acid (2), 15-hydroxy-8,13-labdadien (3) [42], 15-hydroxy-8,13-labdadien-19-carbonsaeure (4), 15-acetoxy-8,13-labdadien-19-oic acid (5), 8,13-labdadien-15,19-diol (6), 15-hydroxy-8,13-labdadien-19-ol (7), 15-hydroxy-8,13-labdadien-19-al (8), 15-acetoxy-8,13-labdadien-19-al (9) [43], ferruginol (10) [44], 6α-hydroxysugirol (11) [45], *trans*-communnic acid (12) [44], isopimarol (13) [46], agathadiol (14) [44], 13-*epi*-cupressic acid (15) [47], 8,15-isopimaradien-19-al (16), 8,15-isopimaradien-19-oic acid (17), 8,15-isopimaradien-19-ol (18) [48], *trans*-communal (19), *trans*-communol (20), dehydroabietic acid (21) [44], isopimaric acid (22), respectively. The $^1$H-NMR spectra of compounds 1 to 11 are shown in Additional file 1: Figs. S1–S22. Regarding the skeletons of the compounds identified from exudates, they could be classified into three types, namely abietane-, labdane-, and pimarane-types. The compound 1–9 were isolated from the exudates of *A.c* (Araucariaceae); compounds 10–13 were isolated from the exudate of *C. macrolepis* (Cupressaceae); compounds 1, 2, 5, 14, and 15 were isolated from the exudate of *C. formosensis* (Cupressaceae); compounds 12 and 16–20 were isolated
from the exudate of *C. lanceolate* (Cupressaceae); compounds 21 and 22 isolated from the exudate of *P. taiwanensis* (Pinaceae).

**HSQC analysis of exudates of conifer**

The main compounds of conifer exudates are diterpenoids [3–7], and most of them belong to abietane-, labdane-, and pimarane-types diterpenoids. The diterpenoids of the above three types of skeletons have their own special signals in the NMR spectrum. For the abietane-type diterpenoid, the NMR signals are at δ_C 120–150 ppm and δ_H 6.0–8.0 ppm, represented the carbon and proton characteristic signals at benzene ring; and signals at δ_C 20–30 ppm and δ_H 3.0–3.5 ppm is an isopropyl absorption peaks at C15 of the benzene ring. According to the above information, special cross-peak signals of abietane-type in HSQC spectrum were at M3-4, N3-4 or O3-4 coupled with C7. Consideration of labdane-type diterpenoids, two double bonds are at C-8 and C-13, which NMR signals were at δ_C 100–150 ppm and δ_H 4.0–7.0 ppm. According to the above information, cross-peak signals of labdane-type were shown at E-F6 coupled with L-M5 or N5 coupled with O4, and sometimes the signals were presented at K-L6. The third skeleton, pimarane-type diterpenoids, an end double bond is at C-16, the NMR signal are at δ_C 100–120, 140–150 ppm and δ_H 4.0–6.0 ppm. According to the above information, characteristic cross-peak signals of pimarane-type diterpenoids are at K-L6 coupled with O5.

The HSQC analysis spectra are shown in Additional file 1: Figs. S23–S36. The results revealed that the exudates of *P. elliottii*, *P. insularis*, *P. morrisonicola*, *P. taiwanensis*, *P. wilsoniana*, *C. obtusa*, *C. formosensis*, and *C. macrolepis* contained the abietane-type diterpenoids; the exudates of *P. elliottii*, *P. insularis*, *C. obtusa*, *C. formosensis*, *C. macrolepis*, *C. lanceolate*, *C. konishii*, *A. cunninghamii*, *A. heterophylla*, and *A. dammara* contained the labdane-type diterpenoids; the exudates of *P. elliottii*, *P. insularis*, *P. taiwanensis*, *C. obtusae*, *C. formosensis*, *C. macrolepis*, *C. lanceolate*, *C. konishii*, *A. cunninghamii* and *A. heterophylla* contained the pimarane-type diterpenoids. Moreover, cross peaks in the HSQC spectra of exudates from 13 conifers were the useful chemotaxonomic index for conifers classification. The cross peaks at C8, D8, K6, M3, M4, and M5 were presented in family Pinaceae (Fig. 4); H3, K4, K6, M5, N3, N5, O4, and O5 were in Cupressaceae (Fig. 5); and C8, F7, K6, L5, L6, M5, N4, N5, and O4 were presented in Araucariaceae (Fig. 6). PCA and cluster analysis were performed to detect the degrees of similarity of the compositions of the exudates analyzed. Three different groups can be identified in the loading plots of PCA 1 and PCA 2 (Fig. 7), i.e., Pinaceae (blue triangle), Cupressaceae (green inverted triangle), and Araucariaceae (light blue square); an angiosperm species, *L. formosana* (Hamamelidaceae), was used as an outer group. Figure 8 shows the results of cluster analysis, it is obviously that all *Pinus* species were closer than others. Both species of *Araucaria* were similarly, and they were close to *A. dammara*, all of them are Araucariaceae. *C. obtusa*,
C. formosensis, and C. macrolepis. were very close to each other. C. lanceolate and C. konishii were similarly, but they were closer to Araucariaceae than C. obtusa, C. formosensis, and C. macrolepis. (Cupressaceae). It might be the abietane type of diterpenoids were in C. obtusa, C. formosensis, C. macrolepis, but not in C. lanceolate, C. konishii. Besides, only P. elliottii and P. insularis (Pinaceae) had labdane-type diterpenoids, both of them were similar in cluster analysis.

Evaluation of antifungal activities
In the growth test of the white-rot fungus, T. versicolor, the mycelial growth period was 7 days after the inoculum. As the results show in Table 3, C. obtusa, P. taiwensis, and P. elliottii exhibited the stronger antifungal activity. On the other hand, the exudates of P. wilsoniana, P. elliottii, and C. formosensis presented better antifungal activity against L. sulphureus (brown-rot fungus) than others. The results indicated Pinaceae and Cupressaceae had better antifungal activities, and according to HSQC analysis this two families had abietane-type diterpenoids, but not in others. The conclusion that abietane-type diterpenoids have good antifungal activities is supported by some studies [49–52].

Conclusion
This study could provide reference information on conifers’ epigenetics and evolutionary classification, and to explore whether their exudates have antifungal activity. The degrees of similarity of the volatile composition from conifers were like the morphology taxonomy. And we could know the dominant volatile compounds in exudates were α-pinene, β-ocimene, β-pinene, sabinene, and caryophyllene. The molecular weight volatiles were larger in Araucariaceae compared to the other two families. However, using GC–MS was still not good to classify exudates of conifers, because C. obtusa was classified under the Araucaria group. To improve the method, we chose to classify exudates of conifers using HSQC. The result of spectral analysis was consistent with the morphology taxonomy. It also could quickly identify the kind of diterpenoid skeleton (abietane-, labdane-, and pimarane-types). Finally, the exudates of conifers revealed
the antifungal activity. Exudates from *C. obtusa*, *P. taiwanensis*, and *P. elliottii* exhibited the stronger against white-rot fungal activity; *P. wilsoniana*, *P. elliottii*, and *C. formosensis* presented better antifungal activity against *L. sulphureus* (brown-rot fungus). The results of antifungal activities and HSQC analysis showed that abietane-type diterpenoids have good antifungal activity.

**Table 3** Antifungal index of exudates of conifers grown in Taiwan

| Conifers* | Antifungal index (%) | Trametes versicolor | Laetiporus sulphureus |
|-----------|----------------------|---------------------|-----------------------|
| A1        | 28.27                | 33.24               |
| A2        | 39.02                | 20.83               |
| A3        | 41.19                | 12.07               |
| C1        | 19.46                | 11.65               |
| C2        | 42.42                | 38.07               |
| C3        | 61.74                | 34.28               |
| C4        | 35.09                | 37.50               |
| C5        | 34.23                | 29.83               |
| P1        | 52.08                | 46.40               |
| P2        | 44.60                | 30.11               |
| P3        | 40.53                | 43.75               |
| P4        | 53.13                | 35.23               |
| P5        | 36.07                | 31.25               |

* A1: *Agathis dammara*; A2: *Araucaria cunninghamii*; A3: *Araucaria heterophylla*; C1: *Calocedrus macrolepis*; C2: *Chamaecyparis formosensis*; C3: *Chamaecyparis obtusa*; C4: *Cunninghamia konishii*; C5: *Cunninghamia lanceolata*; P1: *Pinus elliottii*; P2: *Pinus insularis*; P3: *Pinus morrisonicola*; P4: *Pinus taiwanensis*; P5: *Pseudotsuga wilsoniana*

**Supplementary Information**

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**Fig. S8** Epigenetics of conifers relationship of 13 conifers grown in Taiwan by HSQC analysis of exudates
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Author contributions
NW and YC performed the experiments and analyses, and wrote of this manuscript. YH and SC analyzed the data. SY designed and oversaw this study. All authors have read and approved the submitted manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations
Competing interests
The authors declare that they have no competing interests.

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