Tsao *et al. Journal of Wood Science* (2022) 68:46
https://doi.org/10.1186/s10086-022-02056-z

Journal of Wood Science

ORIGINAL ARTICLE Open Access

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,

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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.

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

Tree	Location	Age (years)	DBH (cm)
Pinus elliottii	Campus[a]	30	28
Pinus insularis	Campus	35	58
Pinus taiwanensis	Experimental forest[b]	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 dammara	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

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. lanceolate*), *Cunninghamia konishii* (*C. konishii*), *Chamaecyparis formosensis* (*C. formosensis*), *Chamaecyparis obtusa* (*C. obtusa*), *Calocedrus macrolepis* (*C. macrolepis*), *Agathis dammara* (*A. dammara*), *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 between18 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 N_2, and stored at dark and frozen stored (4 °C) until study.

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-mL 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 C_9–C_{24}. 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 ^{1}H-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 $CDCl_3$ 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 (^{1}H axis) of HSQC was divided into 10 divisions from 10 to 1 ppm named 1 to 10; Y-axis (^{13}C 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.

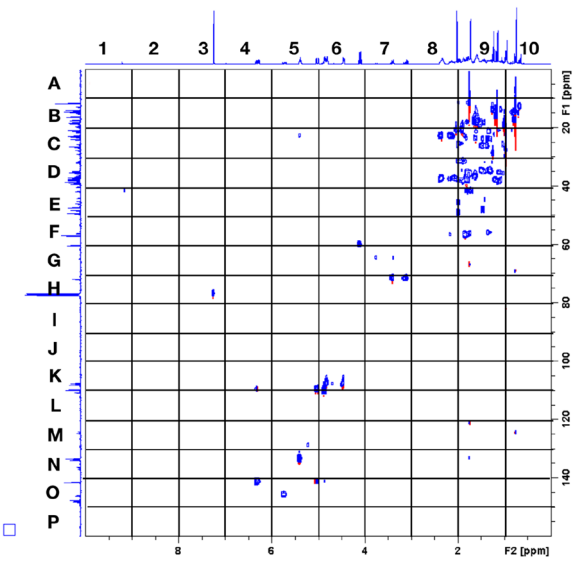

Fig. 1 HSQC NMR spectrum of the *Cunninghamia konishii* Hayata exudates

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

Table 2 The volatile organic compounds of exudates

Constituent	KI	Contents (%)													Identification
		Araucariaceae			Cupressaceae					Pinaceae					
		A1	A2	A3	C1	C2	C3	C4	C5	P1	P2	P3	P4	P5	
Tricyclene	906	-	-	-	0.1	-	-	-	-	-	-	t	-	-	MS, KI
α-Thujene	921	2.7	0.2	-	0.3	0.1	t	0.1	0.1	0.1	0.3	t	0.1	0.6	MS, KI, ST
α-Pinene	935	44.0	21.7	0.2	65.7	74.7	2.8	54.1	64.0	50.7	59.6	38.7	88.6	70.0	MS, KI, ST
Camphene	950	2.1	0.4	-	2.6	0.4	0.1	0.4	0.4	1.0	1.0	0.7	1.5	2.1	MS, KI, ST
Sabinene	977	4.6	1.5	t	1.3	2.2	0.2	1.6	1.7	34.2	1.7	15.3	4.0	2.4	MS, KI
β-Pinene	989	1.9	0.6	-	5.9	9.1	36.3	4.7	12.4	1.9	1.1	3.7	1.0	2.0	MS, KI, ST
α-Phellandrene	1004	-	0.3	-	-	0.2	0.2	-	-	0.1	1.1	0.1	0.1	0.1	MS, KI, ST
3-Carene	1007	-	-	-	11.4	0.1	-	0.3	0.4	0.1	1.1	0.1	0.1	0.1	MS, KI, ST
α-Terpinene	1016	0.2	0.1	-	0.1	0.1	1.3	0.1	0.1	t	0.2	0.2	t	0.1	MS, KI, ST
p-Cymene	1023	0.4	0.5	-	0.4	0.6	2.1	0.5	0.9	0.1	0.2	0.1	t	0.2	MS, KI, ST
β-Ocimene	1029	37.8	4.0	t	2.8	3.1	19.3	0.8	2.1	1.8	-	1.6	1.0	7.8	MS, KI, ST
β-Phellandrene	1031	-	-	-	1.0	2.2	-	1.3	2.4	1.6	33.0	0.7	0.7	-	MS, KI, ST
γ-Terpinene	1057	0.3	-	-	0.1	0.1	1.0	0.1	0.2	t	0.1	0.1	t	0.1	MS, KI
Terpinolene	1084	0.4	0.1	-	0.8	2.0	25.0	0.1	0.3	0.2	0.2	0.2	0.1	0.4	MS, KI, ST
p-Cymenene	1087	0.2	-	-	0.2	0.3	3.9	t	0.1	t	t	-	t	t	MS, KI
Estragole	1196	0.1	-	-	0.1	0.1	-	-	-	0.4	0.9	t	-	0.1	MS, KI
Borneol	1284	0.1	-	-	0.2	0.7	0.3	0.2	0.5	-	t	-	-	2.8	MS, KI, ST
δ-Elemene	1336	-	-	0.9	t	-	-	-	-	-	-	-	-	t	MS, KI
α-Cubebene	1350	0.1	-	1.4	2.2	-	-	t	-	-	t	-	0.1	0.1	MS, KI
Ylangene	1372	-	-	1.6	0.1	-	t	t	-	-	-	-	t	0.1	MS, KI
α-Copaene	1380	0.1	4.6	49.0	1.7	-	0.1	t	-	-	-	t	0.1	0.5	MS, KI, ST
β-Cubebene	1389	-	-	0.9	0.1	-	-	0.5	t	-	-	-	-	0.2	MS, KI, ST
α-Gurjunene	1404	-	-	3.1	t	-	-	0.1	t	-	-	-	t	-	MS, KI
Caryophyllene	1412	t	-	-	-	-	-	26.8	11.2	2.4	-	-	2.4	3.0	MS, KI, ST
β-Gurjunene	1435	-	1.8	0.3	-	-	-	0.1	-	-	-	-	-	-	MS, KI
Aromadendrene	1441	-	1.0	-	-	-	-	0.2	-	-	-	-	-	0.1	MS, KI
α-Guaiene	1448	-	0.1	0.7	0.1	1.5	0.1	0.6	0.3	-	-	-	-	0.6	MS, KI
Alloaromadendrene	1453	-	0.8	-	-	-	0.1	-	-	0.5	-	-	-	0.2	MS, KI
γ-Murrolene	1462	-	0.1	5.1	0.1	-	0.2	0.5	0.1	-	-	-	-	0.3	MS, KI, ST
Germacrene D	1469	t	0.1	2.0	-	-	0.2	-	-	-	-	-	-	2.5	MS, KI
β-Chamigrene	1475	-	0.6	-	-	-	-	2.8	0.3	-	-	-	-	0.2	MS, KI
Valencene	1480	-	1.0	2.7	0.3	-	0.3	2.8	0.4	t	-	-	-	0.4	MS, KI
γ-Cadinene	1517	-	0.1	1.4	0.2	-	0.1	-	-	-	-	-	-	0.3	MS, KI
δ-Cadinene	1521	-	0.2	10.1	0.7	-	0.4	-	-	0.1	-	-	-	0.7	MS, KI
L-Calamenene	1525	-	0.1	2.2	0.4	-	0.3	0.1	t	-	-	-	-	0.3	MS, KI
α-Cadinene	1537	-	-	1.5	0.2	-	t	-	-	-	-	0.1	-	0.1	MS, KI, ST
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	

A1 *Agathis dammara,* A2 *Araucaria cunninghamii*, A3 *Araucaria heterophylla*, C1 *Calocedrus macrolepis*, C2 *Chamaecyparis formosensis*, C3 *Chamaecyparis obtuse*, C4 *Cunninghamia konishii*, C5 *Cunninghamia lanceolata*, P1 *Pinus elliottii*, P2 *Pinus insularis*, P3 *Pinus morrisonicola*, P4 *Pinus taiwanensis*, 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%

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

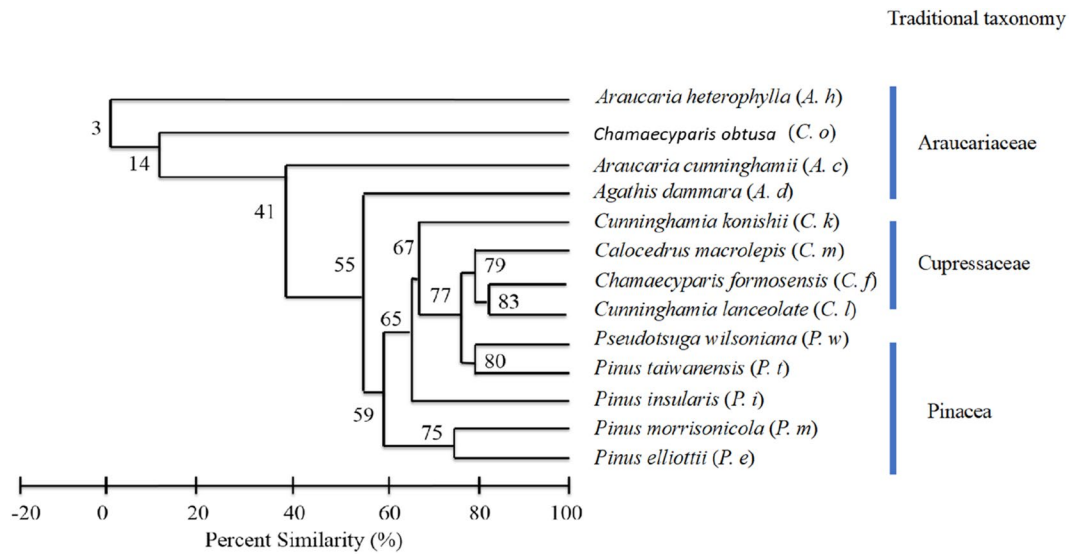

Fig. 2 Epigenetics of conifers relationship of 13 conifers grown in Taiwan by GC–MS analysis of volatile constituents from exudates

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-hydroxysugiol (**11**) [45], *trans*-communic 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

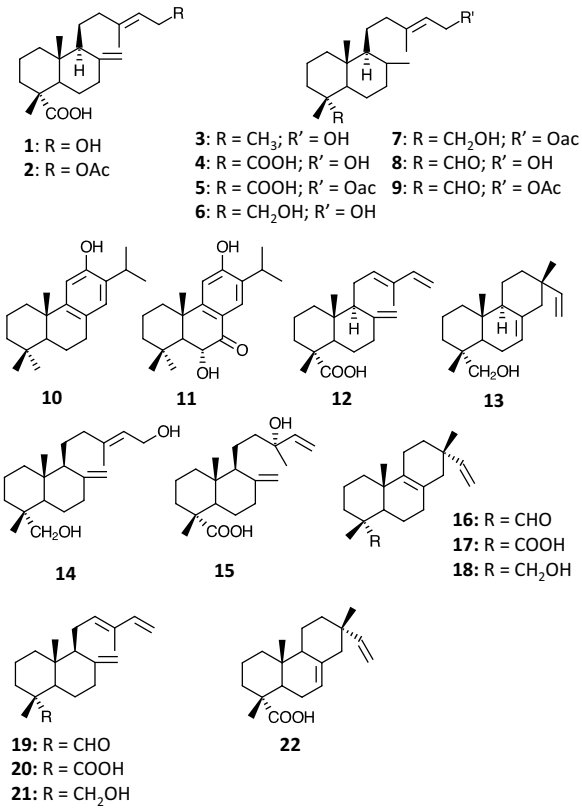

Fig. 3 Compounds identified from exudates of conifers in this study

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. obtuse*, *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*,

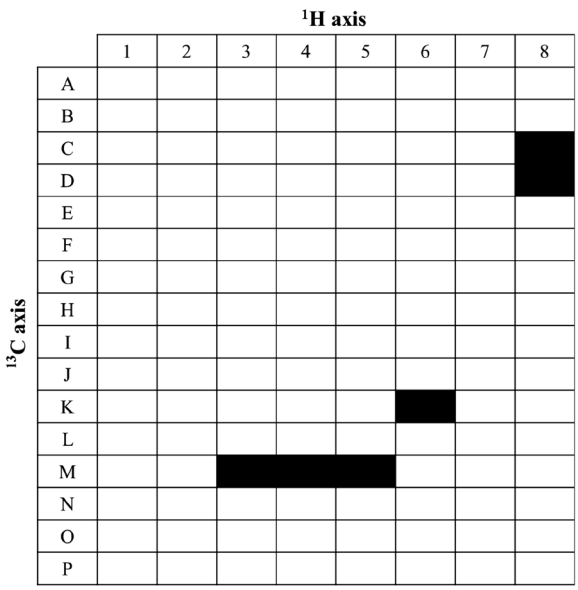

Fig. 4 Cross-peaks region of Pinaceae exudate in HSQC spectrum

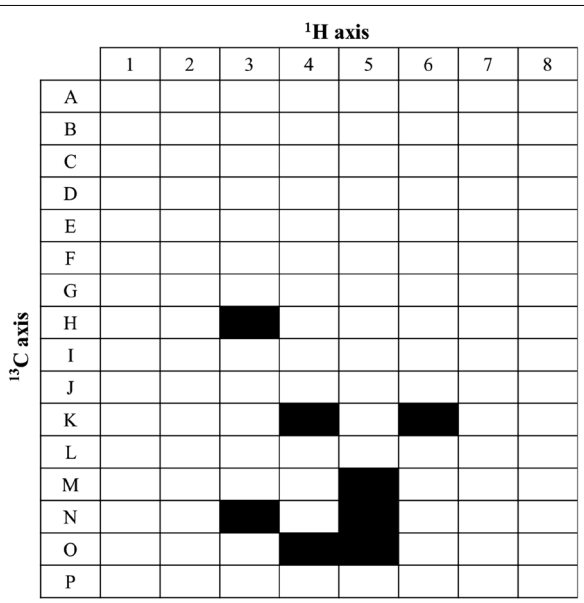

Fig. 5 Cross-peaks region of Cupressaceae exudate in HSQC spectrum

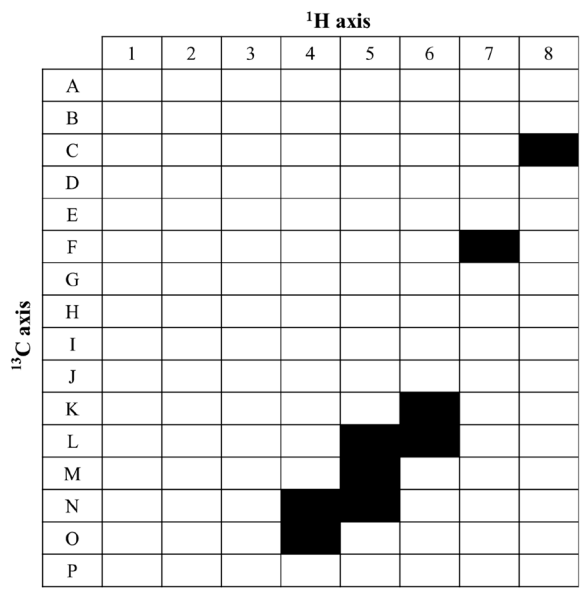

Fig. 6 Cross-peaks region of HSQC Araucariaceae exudate in HSQC spectrum

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. taiwanensis*, 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

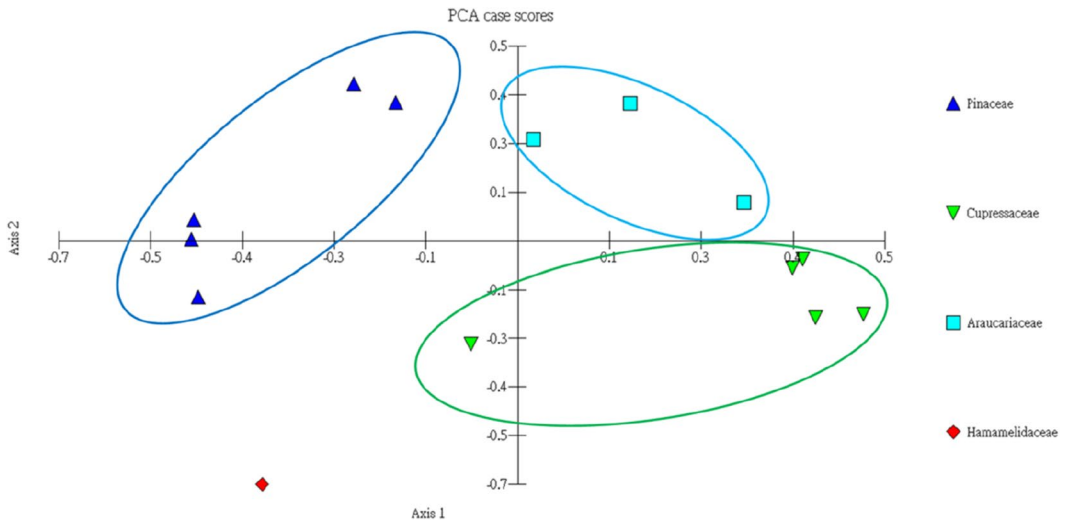

Fig. 7 PCA score plot based on signal regions of HSQC and grouped by families

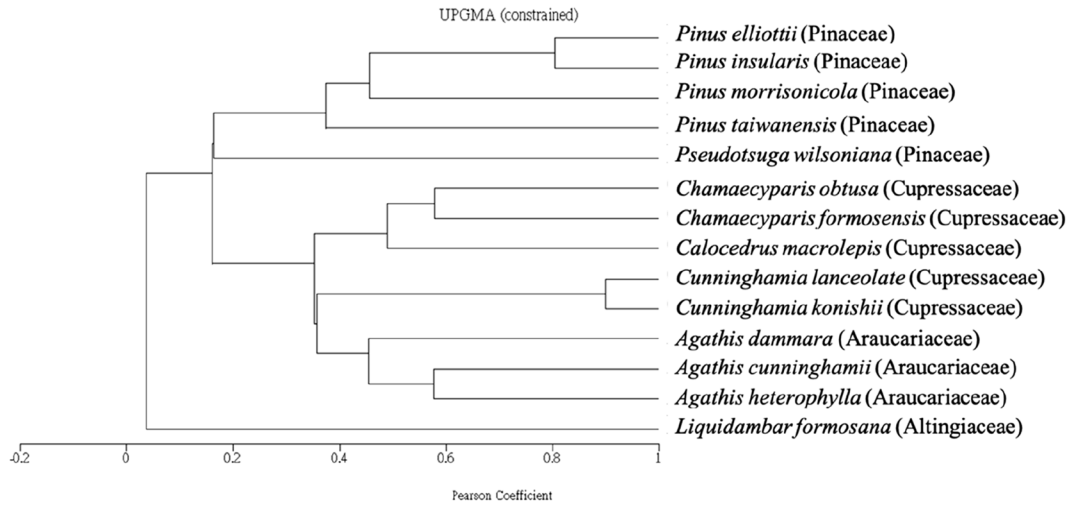

Fig. 8 Epigenetics of conifers relationship of 13 conifers grown in Taiwan by HSQC analysis of exudates

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*

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.

Abbreviations

A. cunninghamii: *Araucaria cunninghamii*; *A. dammara*: *Agathis dammara*; *A. heterophylla*: *Araucaria heterophylla*; *C. formosensis*: *Chamaecyparis formosensis*; *C. konishii*: *Cunninghamia konishii*; *C. lanceolate*: *Cunninghamia lanceolata*; *C. macrolepis*: *Calocedrus macrolepis*; *C. obtusa*: *Chamaecyparis obtuse*; EA: Ethyl acetate; HSQC: Heteronuclear single quantum coherence; GC–MS: Gas chromatogram–mass spectrometry; *T. versicolor*: *Trametes versicolor*; *L. sulphureus*: *Laetiporus sulphureus*; NMR: Nuclear magnetic resonance; PCA: Principal components analyses; PDA: Potato dextrose agar; *P. elliottii*: *Pinus elliottii*; *P. insularis*: *Pinus insularis*; *P. morrisonicola*: *Pinus morrisonicola*; *P. taiwanensis*: *Pinus taiwanensis*; *P. wilsoniana*: *Pseudotsuga wilsoniana*; SPME: Solid-phase microextraction.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s10086-022-02056-z.

Additional file 1: Fig. S1. Proton spectrum of isocupressic acid (1). **Fig. S2**. Proton spectrum of acetylisocupressic acid (2). **Fig. S3**. Proton spectrum of 15-hydroxy-8,13-labdadien (3). **Fig. S4**. Proton spectrum of 15-hydroxy-8,13-labdadien- 19-carbonsaeure (4). **Fig. S5**. Proton spectrum of 15-acetoxy-8,13-labdadien-19-oic acid (5). **Fig. S6**. Proton spectrum of 8,13-labdadien-15,19-diol (6). **Fig. S7**. Proton spectrum of 15-hydroxy-8,13-labdadien-19-ol (7). **Fig. S8**. Proton spectrum of 15-hydroxy-8,13-labdadien-19-al (8). **Fig. S9**. Proton spectrum of 15-acetoxy-8,13-labdadien-19-al (9). **Fig. S10**. Proton spectrum of ferruginol (10). **Fig. S11**. Proton spectrum of 6α-hydroxysugiol (11). **Fig. S12**. Proton spectrum of trans-communic acid (12). **Fig. S13**. Proton spectrum of isopimarol (13). **Fig. S14**. Proton spectrum of agathadiol (14). **Fig. S15**. Proton spectrum of 13-epi-cupressic acid (15). **Fig. S16**. Proton spectrum of 8,15-isopimaradien-19-al (16). **Fig. S17**. Proton spectrum of 8,15-isopimaradien-19-oic acid (17). **Fig. S18**. Proton spectrum of 8,15-isopimaradien-19-ol (18). **Fig. S19**. Proton spectrum of trans-communal (19). **Fig. S20**. Proton spectrum of trans-communol (20). **Fig. S21**. Proton spectrum of dehydroabietic acid (21). **Fig. S22**. Proton spectrum of isopimaric acid (22). **Fig. S23**. HSQC spectrum of Pinus elliottii. **Fig. S24**. HSQC spectrum of Pinus insularis. **Fig. S25**. HSQC spectrum of Pinus taiwanensis. **Fig. S26**. HSQC spectrum of Pinus morrisonicola. **Fig. S27**. HSQC spectrum of Pseudotsuga wilsoniana. **Fig. S28**. HSQC spectrum of Cunninghamia lanceolata. **Fig. S29**. HSQC spectrum of Cunninghamia konishii. **Fig. S30**. HSQC spectrum of Chamaecyparis formosensis. **Fig. S31**. HSQC spectrum of Chamaecyparis obtusa. **Fig. S32**. HSQC spectrum of Calocedrus macrolepis. **Fig. S33**. HSQC

spectrum of Agathis dammara. **Fig. S34**. HSQC spectrum of Araucaria cunninghamii. **Fig. S35**. HSQC spectrum of Araucaria heterophylla. **Fig. S36**. HSQC spectrum of Liquidambar formosana.

Acknowledgements
We thank National Chung Hsing University and Huisun Experimental Forest Station for obtaining exudates.

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.

Funding
This study was financially supported by the Ministry of Science and Technology of Taiwan (103-2313-B-005-037-MY3).

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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Received: 7 April 2022 Accepted: 31 July 2022
Published online: 12 August 2022

References

1. Trapp S, Croteau R (2001) Defensive resin biosynthesis in conifers. Annu Rev Plant Physiol Plant Mol Biol 52:689–724. https://doi.org/10.1146/annurev.arplant.52.1.689
2. Zeneli G, Krokene P, Christiansen E, Krekling T, Gershenzon J (2006) Methyl jasmonate treatment of mature Norway spruce (*Picea abies*) trees increases the accumulation of terpenoid resin components and protects against infection by *Ceratocystis polonica*, a bark beetle-associated fungus. Tree Physiol 26:977–988. https://doi.org/10.1093/treephys/26.8.977
3. Abdel-Sattar E, Monem AR, Ezzat SM, El-Halawany AM, Mouneir SM (2009) Chemical and biological investigation of *Araucaria heterophylla* Salisb. resin. Z Naturforsch C 64:819–823. https://doi.org/10.1515/znc-2009-11-1211
4. Keeling CI, Bohlmann J (2006) Diterpene resin acids in conifers. Phytochemistry 67:2415–2423. https://doi.org/10.1016/j.phytochem.2006.08.019
5. Lu Y, Hautevelle Y, Michels R (2013) Determination of molecular signature of fossil conifers by experimental palaeochemotaxonomy—part 1: the Araucariaceae family. Biogeosciences 10:1943–1962. https://doi.org/10.5194/bg-10-1943-2013
6. Marynowski L, Otto A, Zaton M, Philippe M, Simoneit BR (2007) Biomolecules preserved in ca. 168 million year old fossil conifer wood. Naturwissenschaften 94:228–236. https://doi.org/10.1007/s00114-006-0179-x
7. Popova MP, Chinou IB, Marekov IN, Bankova VS (2009) Terpenes with antimicrobial activity from Cretan propolis. Phytochemistry 70:1262–1267. https://doi.org/10.1016/j.phytochem.2009.07.025
8. Keeling CI, Bohlmann J (2006) Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. New Phytol 170:657–675. https://doi.org/10.1111/j.1469-8137.2006.01716.x
9. Phillips MA, Croteau RB (1999) Resin-base defenses in conifers. Trends Plant Sci 4:184–190. https://doi.org/10.1016/S1360-1385(99)01401-6
10. Hillis WE (ed) (1987) Heartwood and tree exudates. Springer-Verlag, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-72534-0
11. Klepziq KD, Kruqer EL, Smalley EB, Raffa KF (1995) Effect of biotic and abiotic stress on induced accumulation of terpenes and phenolics in red pines inoculated with bark beetle-vectored fungus. J Chem Ecol 21:601–626. https://doi.org/10.1007/BF02033704
12. Lewinsohn E, Gijzen M, Croteau R (1991) Defense mechanisms of conifers: differences in constitutive and wound-induced monoterpene biosynthesis among species. Plant Physiol 96:44–49. https://doi.org/10.1104/pp.96.1.44
13. Tomlin ES, Antonejevic E, Alfaro RI, Borden JH (2000) Changes in volatile terpene and diterpene resin acid composition of resistant and susceptible white spruce leaders exposed to simulated white pine weevil damage. Tree Physiol 20:1087–1095. https://doi.org/10.1093/treephys/20.16.1087
14. Romero-Estrada A, Maldonado-Maqaña A, González-Christen J, Bahena SM, Garduño-Ramírez ML, Rodríguez-López V, Alvarez L (2016) Anti-inflammatory and antioxidative effects of six pentacyclic triterpenes isolated from Mexican copal resin of *Bursera copallifera*. BMC Complement Altern Med 16:422. https://doi.org/10.1186/s12906-016-1397-1
15. Nalbantsoy A, Kariş M, Karakaya L, Akgul Y (2016) Antioxidant, cytotoxic and iNOS activity of *Liquidambar orientalis* Mill. Resin extracts Turk J Biochem 41:198–205. https://doi.org/10.1515/tjb-2016-0030
16. Cheng SS, Chang ST (2014) Bioactivity and characterization of exudates from *Cryptomeria japonica* bark. Wood Sci Technol 48:831–840. https://doi.org/10.1007/s00226-014-0644-1
17. Yamassaki FT, Lenzi RM, Campestrini LH, Bovo F, Seyfried M, Soldera-Silva A, Stevan-Hancke FR, Zawadzki-Baqqio SF, Pettolino FA, Bacic A, Maurer JB (2015) Effect of the native polysaccharide of cashew-nut tree gum exudate on murine peritoneal macrophage modulatory activities. Carbohydr Polym 125:241–248. https://doi.org/10.1016/j.carbpol.2015.02.041
18. Simirgiotis MJ, Quispe C, Bórquez J, Mocan A, Seqúlveda B (2016) High resolution metabolite fingerprinting of the resin of *Baccharis tola* Phil. from the Atacama Desert and its antioxidant capacities. Ind Crop Prod 94:368–375. https://doi.org/10.1016/j.indcrop.2016.08.037
19. Messina F, Curini M, Di Sano C, Zadra C, Giqliarelli G, Rascón-Valenzuela LA, Robles Zepeda RE, Marcotullio MC (2015) Diterpenoids and triterpenoids from the resin of *Bursera microphylla* and their cytotoxic activity. J Nat Prod 78:1184–1188. https://doi.org/10.1021/acs.jnatprod.5b00112
20. Ansari MJ, Al-Ghamdi A, Al-Waili NS, Adgaba N, Khan KA, Amro A (2016) Antimicrobial activity of *Dracaena cinnabari* resin from Soqotra Island on multi drug resistant human pathogens. Afr J Tradit Complem Altern Med 13:123–127. https://doi.org/10.4314/ajtcam.v13i1.17
21. Paetz C, Hammerbacher A, Menezes RC, Feistel F, Weigel C, Voigt K, Schneider B (2016) Chemical composition and antimicrobial activity of *Populus nigra* shoot resin. Nat Prod Commun 11:989–992. https://doi.org/10.1177/1934578X1601100730
22. Von Rhein C, Weidner T, Henß L, Martin J, Weber C, Sliva K, Schnierle BS (2016) Curcumin and *Boswellia serrata* gum resin extract inhibit chikungunya and vesicular stomatitis virus infections *in vitro*. Antiviral Res 125:51–57. https://doi.org/10.1016/j.antiviral.2015.11.007
23. Habibi Najafi MB, Hajimohamadi Farimani R, Tavakoli J, Madayeni S (2014) GC-MS analysis and antimicrobial activity of the essential oil of trunk exudates of *Pistacia atlantica* var. mutica. Chem Nat Compd 50:376–378. https://doi.org/10.1007/s10600-014-0959-z
24. Dimkić I, Ristivojević P, Janakiev T, Berić T, Trifković J, Milojković-Opsenica D, Stanković S (2016) Phenolic profiles and antimicrobial activity of various plant resins as potential botanical sources of Serbian propolis. Ind Crop Prod 94:856–871. https://doi.org/10.1016/j.indcrop.2016.09.065
25. Adhami HR, Fitz V, Lubich A, Kaehlig H, Zehl M, Krenn L (2014) Acetylcholinesterase inhibitors from galbanum, the oleo gum-resin of *Ferula gummosa* Boiss. Phytochem Lett. https://doi.org/10.1016/j.phytol.2014.08.023
26. Rueda DC, Raith M, De Mieri M, Schöffmann A, Hering S, Hamburger M (2014) Identification of dehydroabietc acid from *Boswellia thurifera* resin as a positive $GABA_A$ receptor modulator. Fitoterapia 99:28–34. https://doi.org/10.1016/j.fitote.2014.09.002
27. Hsieh YH, Chen KJ, Chien SC, Cheng WL, Xiao JH, Wang SY (2012) ACAT inhibitory activity of exudates from *Calocedrus macrolepis* var. *formosana*.

Nat Prod Commun 7:1573–1574. https://doi.org/10.1177/1934578X1200701206
28. Xie Y, Zhang J, Liu W, Xie N, Feng F, Qu W (2016) New urushiols with platelet aggregation inhibitory activities from resin of *Toxicodendron vernicifluum*. Fitoterapia 112:38–44. https://doi.org/10.1016/j.fitote.2016.05.001
29. Bisht S, Kant R, Kumar V (2013) α-D-glucosidase inhibitory activity of polysaccharide isolated from *Acacia tortilis* gum exudate. Int J Biol Macromol 59:214–220. https://doi.org/10.1016/j.ijbiomac.2013.04.057
30. Li JH, Pan JT, Yin YQ (2016) Two novel resin glycosides isolated from *Ipomoea cairica* with α-glucosidase inhibitory activity. Chin J Nat Med 14:227–231. https://doi.org/10.1016/S1875-5364(16)30020-6
31. Yaseen Khan M, Ali SA, Pundarikakshudu K (2016) Wound healing activity of extracts derived from *Shorea robiusta* resin. Pharm Boil 54:542–548. https://doi.org/10.3109/13880209.2015.1052886
32. Simas-Tosin FF, Barraza RR, Maria-Ferreira D, Werner MF, Baggio CH, Wagner R, Smiderle FR, Carbonero ER, Sassaki GL, Iacomini M, Gorin PA (2014) Glucuronoarabinoxylan from coconut palm gum exudate: chemical structure and gastroprotective effect. Carbohydr Polym 107:65–71. https://doi.org/10.1016/j.carbpol.2014.02.030
33. Wang YG, Ren J, Wang AG, Yang JB, Ji TF, Ma QG, Tian J, Su YL (2013) Hepatoprotective prenylaromadendrane-type diterpenes from the gum resin of *Boswellia carterii*. J Nat Prod 76:2074–2079. https://doi.org/10.1021/np400526b
34. Wang YG, Ma QG, Tian J, Ren J, Wang AG, Ji TF, Yang JB, Su YL (2016) Hepatoprotective triterpenes from the gum resin of *Boswellia carterii*. Fitoterapia 109:266–273. https://doi.org/10.1016/j.fitote.2015.12.018
35. Chien SC, Xiao JH, Tseng YH, Kuo YH, Wang SY (2013) Composition and antifungal activity of balsam from *Liquidambar formosana* Hance. Holzforschung 67:345–351. https://doi.org/10.1515/hf-2012-0086
36. Rijkers T, Ogbazghi W, Wessel M, Bongers F (2006) The effect of tapping for frankincense on sexual reproduction in *Boswellia papyrifera*. J Appl Ecol 43:1188–1195. https://doi.org/10.1111/j.1365-2664.2006.01215.x
37. Lambert JB, Kozminiski MA, Fahlstrom CA, Santiago-Blay JA (2007) Proton nuclear magnetic resonance characterization of resins from the family Pinaceae. J Nat Prod 70:188–195. https://doi.org/10.1021/np060486i
38. Lambert JB, Kozminski MA, Santiago-Blay JA (2007) Distinctions among conifer exudates by proton magnetic resonance spectroscopy. J Nat Prod 70:1283–1294. https://doi.org/10.1021/np0701982
39. Lambert JB, Heckenbach EA, Hurtley AE, Wu Y, Santiago-Blay JA (2009) Nuclear magnetic resonance spectroscopic characterization of legume exudates. J Nat Prod 72:1028–1035. https://doi.org/10.1021/np900188j
40. Lambert JB, Donnelly EW, Heckenbach EA, Johnson CL, Kozminiski MA, Wu Y, Santiago-Blay JA (2013) Molecular classification of the natural exudates of the rosids. Phytochemistry 94:171–183. https://doi.org/10.1016/j.phytochem.2013.06.013
41. Isidorov VA, Szczepaniak L, Wróblewska A, Pirożnikow E, Vetchinnikova L (2014) Gas chromatographic-mass spectrometric examination of chemical composition of two Eurasian birch (*Betula* L.) bud exudates and its taxonomical implication. Biochem Syst Ecol 52:41–48. https://doi.org/10.1016/j.bse.2013.12.008
42. Arima Y, Kinoshita M, Akita H (2007) Natural product synthesis from (8aR)- and (8aS)-bicyclofarnesols: synthesis of (+)-wiedendiol A, (+)-norsesterterpene diene ester and (−)-subersic acid. Tetrahedron-Asymmetr 18:1701–1711. https://doi.org/10.1016/j.tetasy.2007.07.010
43. Caputo R, Dovinola V, Mangoni L (1974) New diterpenes from *Araucaria cunninghami*. Phytochemistry 13:475–478. https://doi.org/10.1016/S0031-9422(00)91236-6
44. Chiang YM, Liu HK, Lo JM, Chien SC, Chan YF, Lee TH, Su JK, Kuo YH (2003) Cytotoxic constituents of the leaves of *Calocedrus formosana*. J Chin Chem Soc 50:161–166. https://doi.org/10.1002/jccs.200300022
45. Naman CB, Gromovsky AD, Vela CM, Fletcher JN, Gupta G, Varikuti S, Zhu X, Zywot EM, Chai H, Werbovetz KA, Satoskar AR, Kinghorn AD (2016) Antileishmanial and cytotoxic activity of some highly oxidized abietane diterpenoids from the bald cypress, *Taxodium distichum*. J Nat Prod 79:598–606. https://doi.org/10.1021/acs.jnatprod.5b01131
46. Ravu RR, Jacob MR, Jeffries C, Tu Y, Khan SI, Agarwal AK, Guy RK, Walker LA, Clark AM, Li XC (2015) LC-MS- and ^{1}H NMR spectroscopy-guided identification of antifungal diterpenoids from *Sagittaria latifolia*. J Nat Prod 78:2255–2259. https://doi.org/10.1021/acs.jnatprod.5b00470
47. Woo KW, Choi SU, Park JC, Lee KR (2011) A new lignan glycoside from *Juniperus rigida*. Arch Pharm Res 34:2043–2049. https://doi.org/10.1007/s12272-011-1206-9
48. Xu M, Hillwig ML, Lane AL, Tiernan MS, Moore BS, Peters RJ (2014) Characterization of an orphan diterpenoid biosynthetic operon from *Salinispora arenicola*. J Nat Prod 77:2144–2147. https://doi.org/10.1021/np500422d
49. Kusumoto N, Ashitani T, Murayama T, Ogiyama K, Takahashi K (2010) Antifungal abietane-type diterpenes from the cones of *Taxodium distichum* Rich. J Chem Ecol 36:1381–1386. https://doi.org/10.1007/s10886-010-9875-2
50. Kofujita H, Fujino Y, Ota M, Takahashi K (2006) Antifungal diterpenes from the bark of *Cryptomeria japonica* D. Don Holzforschung 60:20–23. https://doi.org/10.1515/HF.2006.004
51. Tsujimura M, Goto M, Tsuji M, Yamaji Y, Ashitani T, Kimura KI, Ohira T, Kofujita H (2019) Isolation of diterpenoids from sugi wood-drying byproducts and their bioactivities. J Wood Sci 65:19. https://doi.org/10.1186/s10086-019-1787-3
52. Zhang MW, Guo K, Zhang Y, Teng LL, Huang QP, Liu Y, Li SH (2021) Leucoabietenes A and B, rearranged abietane and ent-abietane diterpene hydrocarbons against resistant infectious fungus and bacterium from the leaves of *Leucosceptrum canum*. Tetrahedron Lett 81:153356. https://doi.org/10.1016/j.tetlet.2021.153356

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