Journal of Wood Science

ORIGINAL ARTICLE

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Isolation of diterpenoids from sugi wood-drying byproducts and their bioactivities

Maiko Tsujimura[1,2], Manami Goto[3], Minori Tsuji[3], Yoshie Yamaji[3], Tatsuya Ashitani[2,4], Ken-ichi Kimura[2,3], Tatsuro Ohira[5] and Hisayoshi Kofujita[2,3]*

Abstract

Diterpenoids are the main components of the byproducts discharged from sugi wood-drying process (BWP), making BWP a source of these useful compounds. Since there is no method for purifying these compounds from BWP, we sought to develop one. In this study, seven diterpenoids compounds were isolated: abietatriene (I), phyllocladenes (II-i and II-ii; a complex of isophyllocladene and phyllocladene), abietadiene (III), sandaracopimarinal (IV), 6,7-dehydroferruginol (V), ferruginol (VI), and sandaracopimarinol (VII) using medium-pressure liquid chromatography (MPLC) and high-performance liquid chromatography (HPLC). We tested the biological activities of these diterpenoids. Compounds V and VI showed antioxidant activity in the DPPH radical scavenging method, β-carotene bleaching method, and linoleic acid method. Furthermore, compounds V–VII showed antimicrobial activity against *Propionibacterium acnes*, and III, VI, and VII showed antifungal activity against *Trichophyton mentagrophytes* and *Trichophyton rubrum*. These results contribute to the accumulation of knowledge on diterpenoids and will help in developing new ways of utilizing BWP, a currently unused woody biomass.

Keywords: *Cryptomeria japonica*, Terpenoids, Biological activities

Introduction

Recently, due to environmental protection efforts in Southeast Asia and changes in the price of wood on the world market, domestic conifer woods have become the main materials for plywood veneer sheets in the Japanese wood industry, replacing imported lumber such as lauan. In plywood factories, green veneer sheets are dried in a boiler at around 170 °C. Because the exhaust steam from the drying process contains a large amount of volatile organic compounds, it is collected by cooling to avoid its release outdoors. After collecting this exhaust steam, a viscous tar-like sediment is also collected, especially from conifer woods like *Cryptomeria japonica* (sugi), which has a rich resin. Ohira reported that 50 tons of sediment discharged during the drying process is collected per year [1]. Therefore, development of a way to utilize these byproducts is urgently needed.

Our previous studies demonstrated that the main components of the byproducts discharged from sugi wood-drying process (BWP) are diterpenoids, such as ferruginol and sandaracopimarinol [1], and it could remove nitrogen dioxide when they come into contact with nitrogen dioxide gas [1]. BWP also exhibited the antimicrobial activity [2].

We also analyzed the composition of the main diterpene components [3], which in order of abundance were sandaracopimarinol, ferruginol, 6,7-dehydroferruginol, abietadiene, and sandaracopimarinal.

These diterpene compounds have many known bioactivities. For example, sandaracopimarinol has antimicrobial activity [4] and termiticidal activity [5]. Ferruginol has antimicrobial activity [4, 6], antioxidant activity [7], gastroprotective and ulcer healing effects [8], termite resistance effects [9], and growth inhibition activity against *Heterosigma akashiwo* [10]. Moreover, there is a combined inhibitory effect of sandaracopimarinol and ferruginol on shiitake (*Lentinula edodes*) mycelial growth [11]. 6,7-Dehydroferruginol, which is a derivative of ferruginol, also shows antimicrobial activity [12].

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Abietadiene can remove nitrogen dioxide upon contact with nitrogen dioxide gas [1] and affects human electroencephalograms when the subjects smelled the compound that dissolved in ethanol by a filter paper [13]. Thus, since BWP is a source of useful compounds, the aims of this study were to accumulate knowledge about the functions of these terpenoids that will help in developing new ways of utilizing BWP and to give them added value.

In this study, seven diterpenoids compounds were isolated from BWP: abietatriene (I), phyllocladenes (phyllocladane (II-i) and isophyllocladene (II-ii)), abietadiene (III), sandaracopimarinal (IV), 6,7-dehydroferruginol (V), ferruginol (VI), and sandaracopimarinol (VII). In our previous report [3], these compounds in BWP are already identified. Then, we examined their antioxidant, antibacterial and antifungal activities to estimate the practical availability. Particularly, the application as the antioxidant for lipid and the therapeutic drug for skin diseases was discussed in this study.

Materials and methods

Test microorganisms
Propionibacterium acnes (NBRC 107605), *Trichophyton mentagrophytes* (NBRC 6174), and *Trichophyton rubrum* (NBRC 5467) were ordered from the National Institute of Technology and Evaluation (NITE, Kisarazu, Japan). Culture conditions are described below.

Chemicals
Palladium-activated carbon (10% Pd–C), Wakogel C-300HG, butylated hydroxytoluene (BHT), linoleic acid, Tween 40, β-carotene, methyl linoleate, and miconazole nitrate were obtained from Wako Pure Chemical Industries., Ltd. (Osaka, Japan). Cellulose powder was obtained from Toyo Roshi Kaisha., Ltd. (Tokyo, Japan). Chloroform-d [99.8% atom% D, containing 0.03% (v/v) TMS] was obtained from Sigma-Aldrich (Tokyo, Japan). 1,1-Diphenyl-2-picrylhydrazyl was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). GAM broth was obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan). Benzalkonium chloride was obtained from MP Biomedicals, LLC (Santa Ana, CA, USA). Peptone was obtained from Nihon Pharmaceutical Co., Ltd. (Tokyo, Japan).

Isolation of diterpenoids
BWP was distilled by solvent-assisted distillation (SAD) using a method developed in our previous research [3] to obtain a water distillate (WD) fraction and a triethylene glycol distillate (TGD) fraction.

Then, the TGD fraction was separated by medium-pressure liquid chromatography (MPLC) and high-performance liquid chromatography (HPLC) as shown in Fig. 1. The TGD fraction was separated into three fractions by MPLC using a normal phase column (Sepacore Silica, 220 g, BÜCHI, Germany). Toluene was used as a first mobile phase for collecting Fraction 1 and Fraction 2. Then, the mobile phase was changed to ethanol for collecting Fraction 3. The chromatogram was checked by RI Monitor RI-10 (EYELA, Tokyo, Japan), Ceramic Pump VSP-3050 (EYELA) was equipped with this fractionation system.

Each collected fraction was evaporated and weighed by drying under reduced pressure. The quantitative analysis was done by gas chromatography with flame ionization detection (GC-FID), and the chemical composition of each collected fraction was confirmed by gas chromatography–mass spectrometry (GC–MS). The analysis conditions of GC-FID and GC–MS are written in the later passage.

Based on GC-FID and GC–MS analyses, compounds I, II-i, II-ii, and III were contained in Fr. 1; compounds IV, V and VI were in Fr. 2; and compound VII was in Fr. 3. The eluted fractions were further fractionated by HPLC using a reversed-phase column (Inertsil ODS-3 20 mm i.d. ×250 mm, GL Science, Tokyo, Japan), a Shimadzu Liquid Chromatograph LC-6AD pump (Shimadzu, Kyoto, Japan), and a Shodex RI-72 (Showa Denko K.K., Tokyo, Japan) or Shimadzu SPD-20A (UV) detector (Shimadzu). The isolation scheme of diterpenoids from TGD fraction is shown in Fig. 1.
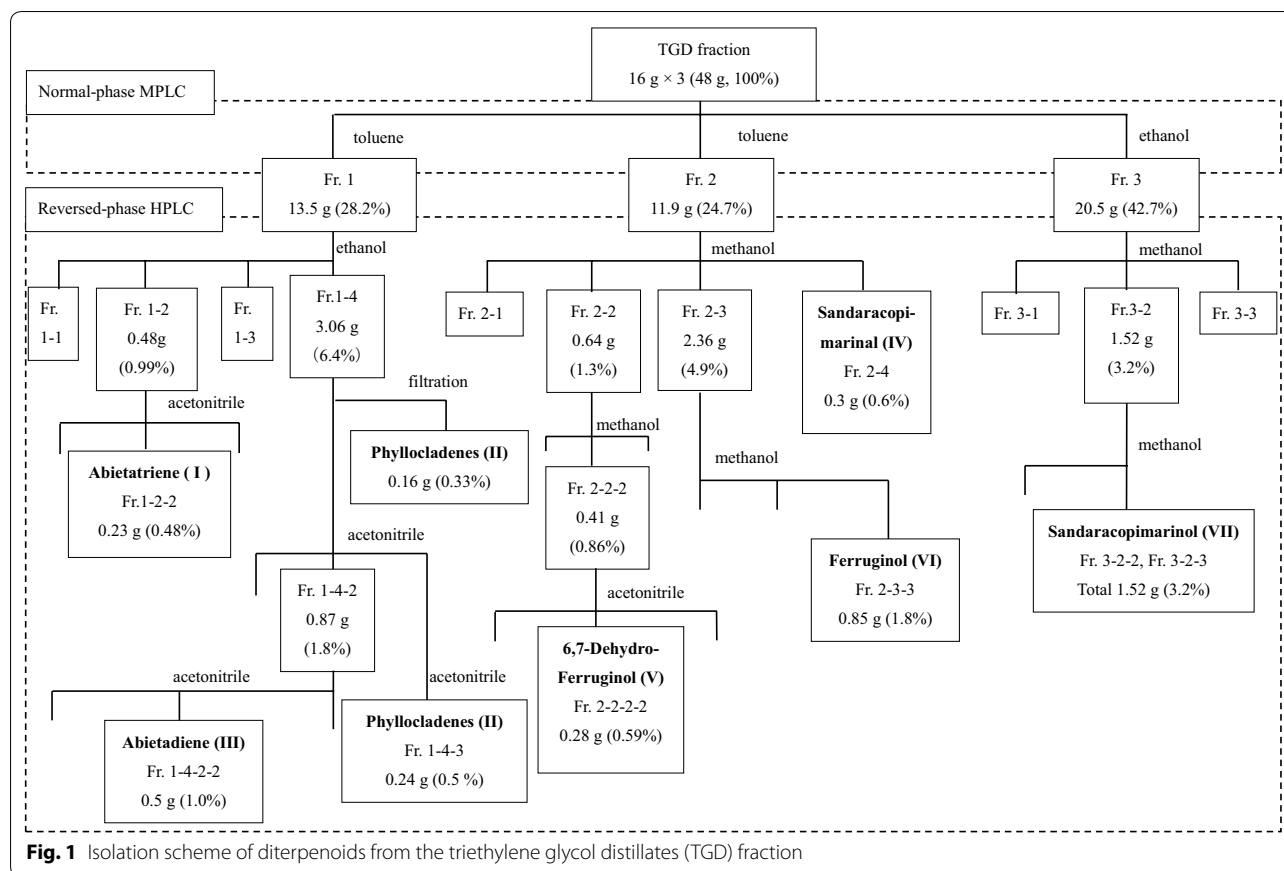
The collected fractions were evaporated and dried under reduced pressure. Then, the yield of each compound (based on the TGD fraction) was calculated as I (0.48%), II-i and II-ii (0.33%), III (1.0%), IV (0.6%), V (0.59%), VI (1.8%), and VII (3.2%). The analysis and confirmation of the chemical composition of each purified fraction were carried out by GC-FID and GC–MS using an HP-5 column. Finally, ^1H and ^{13}C NMR were used for identification of the isolated diterpenoids.

Reduction of sandaracopimarinal (IV) and 6,7-dehydroferruginol (V)
Because there are no detailed data about NMR analysis for IV and V in literature, we confirmed the structure of IV and V by reduction; IV and V was changed to VII and VI, respectively. The condition of each reaction was written in the following passage.

Sandaracopimarinal (IV)
Isolated compound IV (27.4 mg) was dissolved in methanol, then $NaBH_4$ was added gradually (total amount of $NaBH_4$, 611.4 mg). After the reaction, the solution (methanol; 20 ml) was mixed with *n*-hexane (50 ml) and purified water (30 ml), and then the organic phase was collected. The

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Fig. 1 Isolation scheme of diterpenoids from the triethylene glycol distillates (TGD) fraction

organic phase was dried under reduced pressure. The quantity collected was 20.9 mg, and the yield was 76.3%.

6,7-Dehydroferruginol (V)

According to our previous report [3], isolated compound V (35.0 mg) was dissolved in ethyl acetate and 10% Pd–C (14.0 mg) was added. The gas phase used in this study was H_2, and the reaction was done under room temperature. The reaction product was filtered under reduced pressure using a separation funnel containing cellulose powder (40–100 mesh) and Wakogel C-300HG (40–60 μm). Then, the filtrate was concentrated and dried under reduced pressure. The quantity collected was 30.9 mg, and the yield was 88.3%.

Antioxidant activity

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity [14], with some modifications

Each diterpenoid sample was dissolved in ethanol at a concentration of 10 mM. BHT was used as a positive control. A 0.2 ml aliquot of sample solution, 1.0 ml 0.2 M DPPH, 0.2 ml 0.1 M Tris–HCl (pH 7.4), and 0.6 ml ethanol were mixed in a screw-cap tube. The tubes were shaken and the contents allowed to react at room temperature in the dark. After 30 min, the absorbance was measured at 517 nm by a UV mini-1240 spectrophotometer (Shimadzu, Japan).

β-carotene bleaching assay [15]

60 μl of each sample solution (5 mM in dichloromethane) or dichloromethane (control), and 2940 μl of an emulsion of linoleic acid, β-carotene and Tween 40 in sodium phosphate buffer (pH 6.8) were collected in an opaque screw-cap tube and mixed well. Each sample was then incubated at 50 °C, and the absorbance measured at 470 nm by a UV-mini 1240 spectrophotometer after 0, 15, 30, 45, 75, 105, 135, 165, and 195 min.

Linoleic acid method [16], with some modifications

150 μl methyl linoleate solution (0.05 mM in ethyl acetate) and 100 μl of each sample (1×10^{-3} mM; 2% mol equivalent weight of methyl linoleate) were mixed in a 4 ml vial. After removing ethyl acetate, the vials were heated at 85 °C in an oven. Heating time was set to 0, 6, 12, and 24 h. After heating, the vials were cooled in a desiccator. Then, 1.2 ml ethyl acetate was poured into each vial. Samples were analyzed by GC-FID to calculate the

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residual rate of methyl linoleate by the internal standard method using heptadecane as internal standard.

Antibacterial activity against *P. acnes*

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values were determined for *P. acnes* (NBRC 107605). *P. acnes* was pre-incubated in GAM liquid medium (pH 7.3) at 37 °C for 72 h under anaerobic conditions. The number of bacteria was counted by the McFarland turbidimetry method; turbidity corresponded to the No. 5 standard test tube (15×10^8/ml) [17]. Isolated diterpenoids were dissolved in ethanol, and twofold serial dilutions of each sample from 5 mg/ml were prepared. Benzalkonium chloride was used as a positive control, and the solution was prepared in the same manner as the isolated terpenoids. To a 50 μl aliquot of each sample solution, 10 ml GAM liquid medium was added in a screw-cap test tube and mixed well. The final concentrations of purified diterpenoids and benzalkonium chloride were twofold serial dilutions from 25 μg/ml. Then, inoculum was added to each screw-cap test tube ($n = 3$), and the tubes were incubated at 37 °C for 72 h under anaerobic conditions. The concentrations showing no visually detectable bacterial growth were determined to be the MIC value. A 1 ml aliquot of the culture medium collected from test tubes with no visually detectable bacterial growth was inoculated in 10 ml of fresh GAM liquid medium. As done for MIC, the concentration at which there was no bacterial growth after incubation was determined to be the MBC.

Antifungal activity against *Trichophyton* species [18]

Trichophyton mentagrophytes (NBRC 6174) and *T. rubrum* (NBRC 5467) were pre-incubated in Sabouraud agar medium (pH 6.0) at 25 °C for about 2 weeks. The concentration of isolated diterpenoids was adjusted to 4 mg/ml by acetone. Miconazole nitrate (positive control) was prepared twofold serial dilutions from 50 to 3.13 μg/ml. Then, 2 ml molten Sabouraud agar medium was poured into a sterilized Petri dish (35 mm diameter, Falcon), and 100 μl of sample solution was spread on the surface of the medium, for a final concentration of each isolated diterpenoid of 400 μg, with the miconazole nitrate concentration ranging from 5 to 0.313 μg. After removing acetone by air-drying, the agar blocks with hyphae on the surface were punched out by a cork borer (5 mm in diameter), and then placed in the center of Petri dishes. The Petri dishes were incubated at 25 °C for 10 days. The average diameter of hyphae for three test specimens was measured by a caliper, and the area in which mycelia grew (mm^2) was calculated to evaluate the antifungal activity of test samples.

$$\begin{aligned} \text{Area in which mycelia grew} &= (\text{Total area occupied by mycelia,} \\ &\quad \text{calculated from average diameter}) \\ &\quad - (\text{Agar block area}) \end{aligned}$$

GC-FID

GC-FID was performed on a 7890B GC System (Agilent Technologies, Santa Clara, CA, USA) equipped with an HP-5 column (30 m × 0.25 mm i.d., 0.25 μm film thickness, Agilent Technologies). The oven temperature started at 60 °C and increased at 3.0 °C/min to at 240 °C. The temperature of both the injector and the detector was 250 °C. Flame ionization was used for detection. The volume of injected sample was 1 μl, and the split ratio was 1:4. The carrier gas was N_2 and the flow rate was 1.3 ml/min. When we analyzed the mixture, 0.2 mg of heptadecane was added for 10 mg of sample. On the other hand, when pure substance or a few yield samples were analyzed, 1 mg of heptadecane was added to 1 mg of sample. The total volume was adjusted to 1 ml using *n*-hexane or ethyl acetate.

The conditions for the linoleic acid method were an initial oven temperature of 180 °C, which was increased at 10 °C/min to at 280 °C. The temperature of both the injector and detector was 280 °C. Other parameters were the same as above.

GC–MS

GC–MS was performed on a GCMS-QP2010 instrument (Shimadzu) equipped with an HP-5 column. The initial oven temperature was at 60 °C, which increased at 3.0 °C/min to 240 °C. The injector and the detector temperatures were both 250 °C. The injected sample volume was 1 μl and split rate was 1:40. The carrier gas was He and the flow rate was 1.61 ml/min. Electron ionization was used and the mass range was from 40 to 500 m/z. Mass spectra were taken at 70 eV and the ion source temperature was 250 °C. The GC–MS samples were prepared using the same procedure used for GC-FID.

NMR

NMR spectra (^{1}H: 400 MHz, ^{13}C: 100 MHz) were recorded on a JEOL AL400 FT-NMR spectrometer. Each purified diterpenoid (50 mg) was dissolved in 0.7 ml chloroform-*d*. The experimentally measured spectra were compared with data from the literature [6, 19–23].

Statistical analysis

All biological activity tests were performed in triplicate. The results were evaluated by a Tukey–Kramer test, and were expressed as mean ± standard deviation (SD) for each measurement. The *p* values were

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set at < 0.05. Statcel 3 (4 Steps Excel-Statistics, OMS Publishing Ltd, Saitama, Japan) was used as statistical software.

Results and discussion

Isolation and identification of diterpenoids

From the TGD fraction, seven diterpenoids compounds were isolated by MPLC, and HPLC. We could purify these diterpenoids more easily from the BWP than the plant materials such as wood, branches and leaves, because the content rate of diterpenoids of BWP was higher than that of the plant materials. Furthermore, these diterpenoids were fractionated in the TGD fraction from BWP by SAD.

The yield of each fraction was based on the TGD fraction (48 g). The structure and purity of each terpenoid were confirmed by NMR. However, there were no NMR data that the ^{13}C signals have been assigned for IV or V in published reports. Thus, the isolated samples were reduced to form VII and VI, and then identified by ^{1}H and ^{13}C NMR analyses. We also obtained the DEPT, HMBC and HMQC data of IV and V, and assigned the ^{13}C NMR signals based on these data. The chemical shifts of the diterpenoids are in Tables 1 and 2, and the structures of the diterpenoids purified from the TGD fraction are shown in Figs. 2 and 3. These major components of the TGD fraction were all known compounds. In this study, the ^{13}C NMR signals of IV and V were assigned for the first time.

Antioxidant activities

To investigate the antioxidant activities of the purified terpenoids, three assays, a DPPH radical scavenging

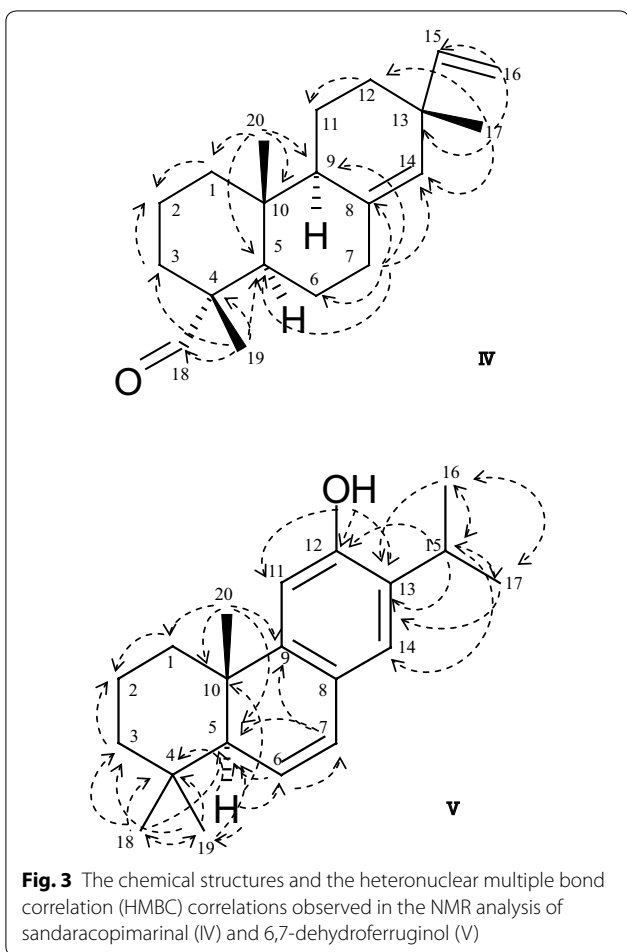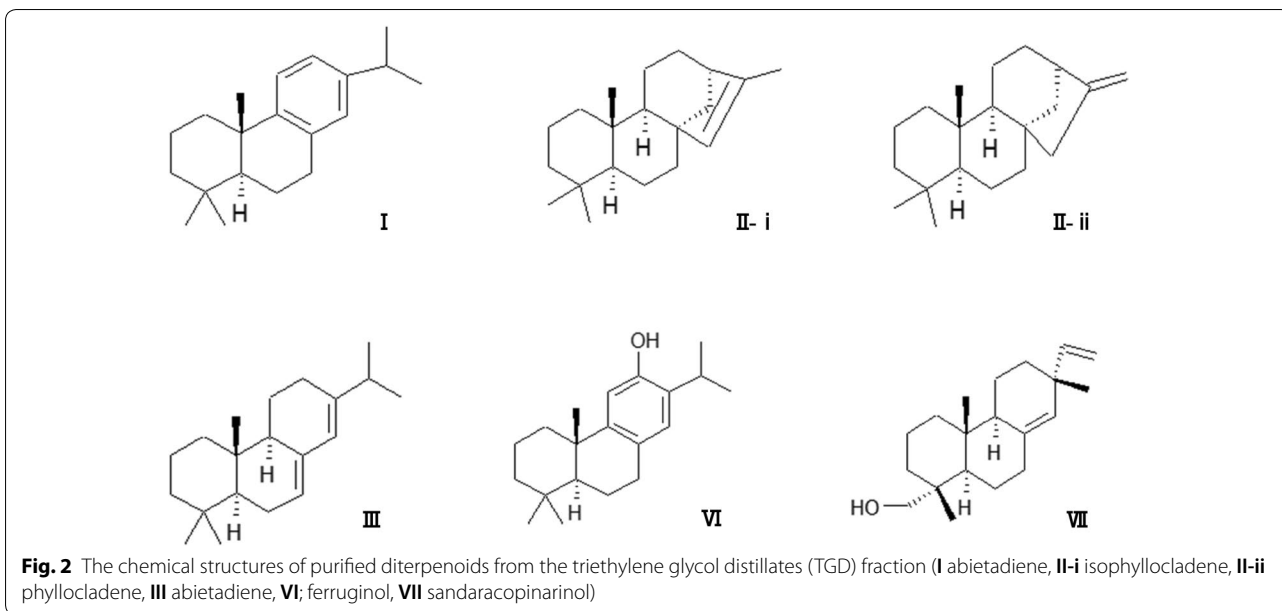**Table 2** ^{1}H and ^{13}C data for compound IV and compound V in CDCl$_3$-d

Carbon number[a]	IV: Sandaracopimarinal		V:6,7-Dehydroferruginol	
	^{1}H	^{13}C	^{1}H	^{13}C
1	1.08 s	38.3	1.54 m	36.0
	1.80 d (J = 4.52)			
2	1.62 m	17.4	1.69 sept (J = 6.8)	19.0
3	1.28 d (J = 6.8)	32.4	1.50 m	41.0
4		49.7		32.8
5	1.55 d (J = 2.68)	46.8	2.05 m	51.0
	1.62 m			
6	1.42 m	24.7	5.87 dd (J = 9.6, 2.7)	127.5
7	2.10 m	35.4	6.49 dd (J = 9.6, 3.0)	127.2
	2.21 dd (J = 14.2, 4.8)			
8		136.3		126.3
9	1.81 s	50.3		147.5
10		37.2		37.7
11	1.46 m	18.6	6.57 s	109.5
12	1.36 m	34.4		152.2
	1.51 m			
13		37.4		130.9
14	5.24 s	129.5	6.88 s	124.5
15	5.77 d (J = 17.2, 10.8)	148.7	3.16 sept (J = 6.8)	26.7
16	4.90 dd (J = 17.4, 10.8)	110.2	1.22 d (J = 6.8)	22.4
17	1.04 s	26.0	1.26 d (J = 6.8)	22.5
18	9.23 s	206.5	0.96 s	32.6
19	1.09 s	14.6	1.04 s	22.8
20	0.86 s	15.4	1.01 s	20.1

Compound IV: sandaracopimarinal; compound V: 6,7-dehydroferruginol

[a] Numbers correspond to the numbers in Fig. 3

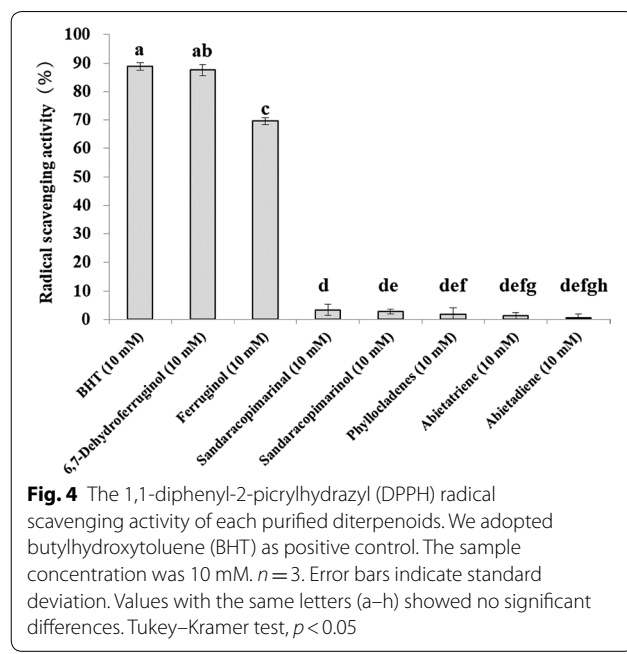**Table 1** ^{13}C chemical shifts of purified diterpenoids from the TGD fraction

	No.	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10
Abietatriene	I	38.9	19.3	41.8	33.5	50.4	19.1	30.5	134.9	147.6	37.5
Isophyllocladene	II-i	19.0	18.6	42.2	33.2	56.0	20.3	37.8	47.9	53.3	37.3
Phyllocladene	II-ii	39.5	18.5	42.0	33.2	56.6	20.3	41.1	43.7	57.0	37.9
Abietadiene	III	39.3	18.9	42.4	34.9	50.3	24.0	121.5	135.4	51.0	32.9
Ferruginol	VI	38.8	19.3	41.6	33.4	50.3	19.2	29.7	127.0	148.5	37.4
Sandaracopimarinol	VII	38.8	18.3	34.5	37.3	47.8	22.3	35.7	136.9	50.5	37.7

	No.	C-11	C-12	C-13	C-14	C-15	C-16	C-17	C-18	C-19	C-20	Reference no.
Abietatriene	I	124.3	123.8	145.4	126.8	33.4	24.0	24.0	33.3	21.6	24.9	[19]
Isophyllocladene	II-i	19.2	24.6	43.3	55.0	140.6	129.3	15.5	33.7	21.9	15.0	[20]
Phyllocladene	II-ii	19.0	34.0	42.7	50.4	157.6	41.5	102.3	33.7	22.0	15.1	[21]
Abietadiene	III	22.7	27.6	144.9	122.5	34.9	21.4	21.9	33.3	20.8	13.6	[22]
Ferruginol	VI	110.9	150.8	131.5	126.5	26.7	22.5	22.7	33.3	21.5	24.7	[6, 23]
Sandaracopimarinol	VII	18.7	35.4	38.1	128.6	149.0	110.0	25.9	72.1	17.9	15.5	[6]

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Fig. 2 The chemical structures of purified diterpenoids from the triethylene glycol distillates (TGD) fraction (**I** abietadiene, **II-i** isophyllocladene, **II-ii** phyllocladene, **III** abietadiene, **VI**; ferruginol, **VII** sandaracopinarinol)

Fig. 3 The chemical structures and the heteronuclear multiple bond correlation (HMBC) correlations observed in the NMR analysis of sandaracopimarinal (IV) and 6,7-dehydroferruginol (V)

assay, a β-carotene bleaching assay and the linoleic acid method, were adopted. BHT was used as the positive control.

DPPH radical scavenging activity

The results are shown in Fig. 4. The activity of VI was 69.6%. It has already been reported that VI has a radical

Fig. 4 The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity of each purified diterpenoids. We adopted butylhydroxytoluene (BHT) as positive control. The sample concentration was 10 mM. $n = 3$. Error bars indicate standard deviation. Values with the same letters (a–h) showed no significant differences. Tukey–Kramer test, $p < 0.05$
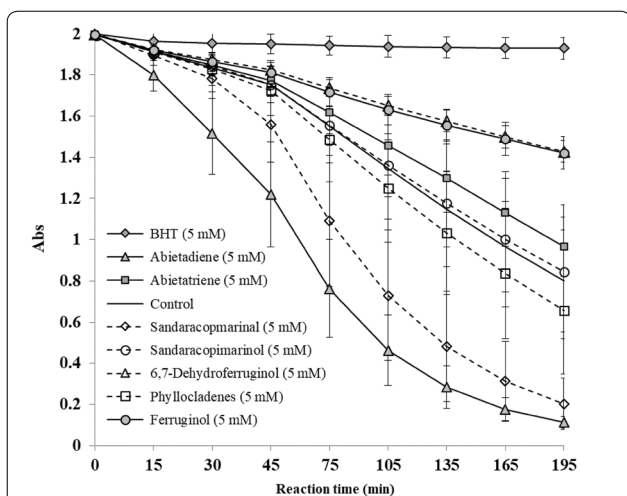
Fig. 5 The antioxidant activity of purified diterpenoids evaluated by the β-carotene bleaching method. The sample concentration was 5 mM. $n = 3$. Error bars indicate standard deviation. Tukey–Kramer test, $p < 0.05$

Fig. 6 The antioxidant activity of purified terpenoids evaluated by the linoleic acid method. The sample concentration was 1×10^{-3} mM. $n = 3$. Error bars indicate standard deviation. Tukey–Kramer test, $p < 0.05$

capturing function [7]. Also, it has been reported that VI showed 53% of inhibition ratio at 100 μg/ml, but the activity of VII was less than 10% at 100 μg/ml in DPPH radical scavenging activity assay [24]. Our results supported this knowledge. The activity of V was 87.6% (10 mM), which was comparable of the activity of BHT (88.8%, 10 mM). Moreover, though there were no previous data in the literature about the DPPH radical scavenging activity of V, it was stronger than that of VI. However, the other terpenoids did not show any DPPH radical scavenging activity.

β-Carotene bleaching activity

The results are shown in Fig. 5. After 195 min of reaction, BHT (5 mM) maintained high antioxidant activity, inhibiting the bleaching of β-carotene. Compared with BHT, VI and V (at 5 mM) showed weak antioxidant activity, with the degree of activity of VI and V almost equal. Other purified terpenoids did not show antioxidant activity. Our results supported those of a previous study [16], in which VI showed antioxidant activity based on a β-carotene bleaching assay.

Linoleic acid method

The results are shown in Fig. 6. After 24 h of heating, BHT maintained high antioxidant activity. Among the purified terpenoids, VI and V had antioxidant activity. After 6 h of heating, both compounds showed the same activity as BHT. However, their activity weakened slowly, and the amount of methyl linoleate almost disappeared after 24 h of heating. It may be that the chemical composition of
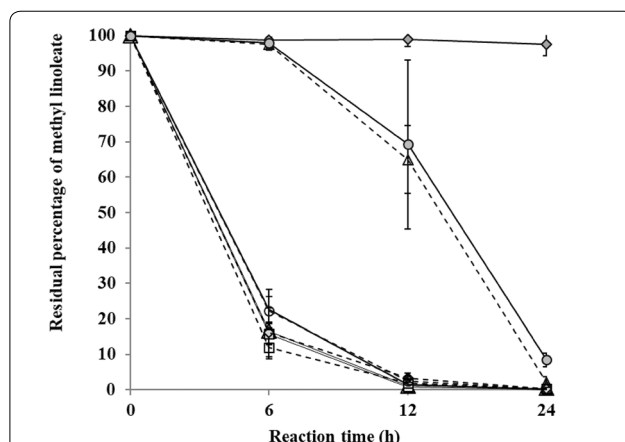
these terpenoids was changed by heating [16]. The relationship between the change in chemical composition and antioxidant activity is not clear; so detailed investigations should be carried out. Other purified terpenoids showed the same tendency as the negative control; so we could not confirm antioxidant activities.

VI and V showed antioxidant activity in every assay. There are literature data for evaluating the bioactivities of 11 essential oils [25]. Based on these results, *Thymus vulgaris* essential oil, thymol chemotype, shows the highest activities in the DPPH assay and β-carotene bleaching test. The substructures of VI and V are common to the structure of thymol. We currently consider that the key to their activity is their structure.

Antibacterial and antifungal activities

In commercial use of terpenoids, mixing them with an ointment is better than a water-based solution like skin lotion because they are hydrophobic compounds. Data are, therefore, needed on how diterpenoids affect microbes that cause skin diseases. Thus, we chose *P. acnes*, *T. mentagrophytes* and *T. rubrum* as the test microorganisms because they are epidermal bacteria.

Antibacterial activity against *P. acnes*

The results are shown in Table 3. The antimicrobial activity against *P. acnes* was evaluated by MIC and MBC values. Benzalkonium chloride was adopted as a positive

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Table 3 The growth inhibition activity of purified diterpenoids against Propionibacterium acnes is shown as MIC and MBC values

Compound	Growth inhibition against *P. acnes*	
	MIC (µg/ml)	MBC (µg/ml)
Benzalkonium chloride	1.56	3.13
Abietatriene (I)	6.25	25≦
Phyllocladenes (II)	12.5	25≦
Abietadiene (III)	12.5	25≦
Sandaracopimarinal (IV)	12.5	25≦
6,7-Dehydroferruginol (V)	3.13	6.25
Ferruginol (VI)	3.13	3.13
Sandaracopimarinol (VII)	6.25	12.5

Benzalkonium chloride was used as positive control. Cultures and test sections were grown at 37 ℃ for 72 h

control. VI, V and VII showed antimicrobial activities based on both MIC and MBC. VI exhibited the strongest activity of the purified terpenoids, and the activity of V, an analog of VI, was the next strongest. VII exhibited the third strongest activity, but IV, an analog of VII, had no detectable MBC value. There is interest in a patent for treatment of acne that uses the essential oil or the *epi*-cubenol and isopropylmethylphenol collected from Japanese cedar wood or barrels [26]. Though the method was not the same as ours, there is, thus, a possibility that terpenoids originating from *C. japonica* may be useful in the treatment of acne. *P. acnes* are anaerobic Gram-positive bacteria. In previous study, ferruginol showed antimicrobial activity toward Gram-positive bacteria [6]. However, the MBC value of the other purified terpenoids (I, II, III, IV) could not be detected.

Antifungal activity against Trichophyton *species*

Trichophyton mentagrophytes and *T. rubrum* were used in this test. The results are shown in Figs. 7 and 8. III, VII, and VI inhibited the fungal growth. Compound III showed the highest growth inhibition, which was stronger than that of oxygen-containing diterpenoids like VI and VII. Interestingly, abietatriene (I) did not show any inhibition, though its structure is similar to that of III: both I and III are hydrocarbon abietane-type diterpene, the A ring structure is common. And V, an analog of VI, and IV, an analog of VII, showed no activity too. Generally, III showed weak bioactivity, such as antimicrobial activity. It was considered that the degree of volatility and the difference in permeability of these compounds in agar medium affected the growth of *Trichophyton* species. A previous study reported that essential oils have anti-*Trichophyton* activity, and it was found that essential oils show antimicrobial activity according to a process: first
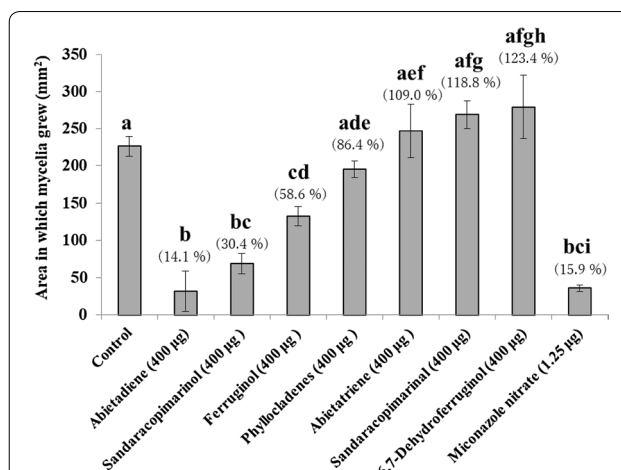

Fig. 7 The growth inhibitory activity of purified diterpenoids for *Trichophyton mentagrophytes*. $n = 3$. Error bars indicate standard deviation. Values with the same letters (a–i) showed no significant differences. Tukey–Kramer test, $p < 0.05$. The ratio of each test section's area in which mycelia grew for that of control was written on this graph
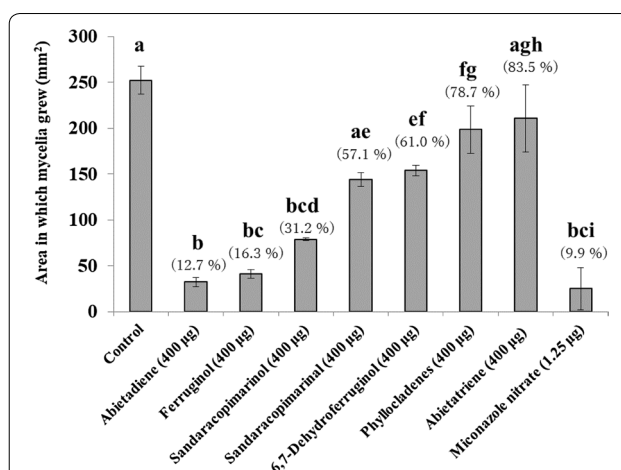

Fig. 8 The growth inhibitory activity of purified diterpenoids for *Trichophyton rubrum*. $n = 3$. Error bars indicate standard deviation. Values with the same letters (a–i) showed no significant differences. Tukey–Kramer test, $p < 0.05$. The ratio of each test section's area in which mycelia grew for that of control was written on this graph

they volatilize and diffuse in the air, and then they are absorbed by the agar medium [27]. In that study [27], the essential oil compounds that were used as experimental materials were mainly monoterpenoids and sesquiterpenoids. The behavior of diterpenoids was not the same as that of monoterpenoids or sesquiterpenoids, but it could be that the volatility of compounds affects their antimicrobial activity. Also, it was considered that the structure of diterpenoids could have been modified over the 10-day incubation term. In any case, the reason for changes in

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activity over time was not clear; so we would like to consider this subject for future analysis.

Conclusions

Seven diterpenoids compounds were isolated: I, II, III, IV, V, VI, and VII, from the TGD fraction collected by SAD using BWP as the raw material. We also tried to measure several kinds of assays to investigate each potency of purified diterpenoids. From these results, V and VI were showed antioxidant activity using the three antioxidant assays. Then, VI, V, and VII showed antimicrobial activity to *P. acnes*. And III, VI, and VII showed growth inhibition activity toward *T. mentagrophytes* and *T. rubrum*. These results indicate the potentials of the diterpenoids as the antioxidant for lipid and the therapeutic drug for skin diseases. This study has contributed to accumulation of knowledge about diterpenoids derived from *C. japonica* and to expansion of the possibilities for development of the practical uses for BWP.

Author details

[1] Institute of Wood Technology, Akita Prefectural University, 11-1 Kaieizaka, Noshiro, Akita 016-0876, Japan. [2] The United Graduate School of Agricultural Science, Iwate University, 18-8 Ueda 3-chome, Morioka, Iwate 020-8550, Japan. [3] Faculty of Agriculture, Iwate University, 18-8 Ueda 3-chome, Morioka, Iwate 020-8550, Japan. [4] Faculty of Agriculture, Yamagata University, 1-23 Wakaba-machi, Tsuruoka, Yamagata 997-8555, Japan. [5] Forestry and Forest Products Research Institute (FFPRI), 1, Matsunosato, Tsukuba, Ibaraki 305-8687, Japan.

Competing interests

The authors declare that they have no competing interests.

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