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NOTE

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Antimicrobial activity of heartwood components of sugi (*Cryptomeria japonica*) against several fungi and bacteria

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Abstract Methanol extract of sawdust of sugi (*Cryptomeria japonica*) heartwood was fractionated with toluene and *n*-hexane to give solvent-soluble and solvent-insoluble fractions. The *n*-hexane-soluble fraction showed the most inhibition activity among the fractions against phytopathogenic microorganisms, namely *Fusarium oxysporum*, *Phytophthora capsici*, *Pythium splendens*, and *Ralstonia solanacearum*. Sandaracopimarinol and ferruginol, isolated from the *n*-hexane-soluble fraction, showed moderate antifungal activity against the three fungi and strong antibacterial activity against *R. solanacearum*. The content of sandaracopimarinol (7.07 g/kg based on the dried sawdust) in the heartwood was about twice that of ferruginol. Sandaracopimarinol and ferruginol strongly inhibited the growth of Gram-positive bacteria but did not show inhibitory action against Gram-negative bacteria except for *R. solanacearum*. The antibacterial effect of sandaracopimarinol was first found in the present study and was stronger than that of ferruginol.

Key words Sugi (*Cryptomeria japonica*) heartwood · Antimicrobial activity · Bacterium · Sandaracopimarinol · Ferruginol

Introduction

Chemical composition and biological activities of extracts and essential oils from different tissues of sugi (*Cryptomeria japonica*) have been reported. Morita et al.[1,2] reported antifungal activity of the hexane extract from yakusugi bogwood against *Penicillium italicum*, *Fusarium sporotorichioides*, *Aspergillus niger*, *Tyromyces palustris*, and *Coriolus versicolor*, and isolation of cryptomeridiol, sandaracopimarinal, and sandaracopimarinol from the distillation residue of the *n*-hexane extract. However, the antifungal activity of the isolated compounds was not demonstrated. Ferruginol included in the bark and the wood of *C. japonica* has been reported to be a strong inhibitor on mycelial growth of shiitake (*Lentinula edodes*).[3–5] Sandaracopimarinol isolated from the mixed sawdust of sapwood and heartwood of *C. japonica* was also found by us to inhibit the growth of *L. edodes*, although the 50% inhibition concentration (IC_{50}) value of sandaracopimarinol was tenfold lower than ferruginol.[5] Kofujita et al.[6,7] reported recently that ferruginol and cryptoquinone isolated from the bark of *C. japonica* had antifungal activity against several phytopathogenic fungi. Arihara et al.[8] reported that sugikurojinol B was isolated from the black heartwood of *C. japonica* as one of a series of new sesquiterpenes and its antibacterial activity against *Staphylococcus aureus* was as strong as hinokitiol. More recently, the essential oil from *C. japonica* heartwood was reported to have strong antifungal activity compared with the essential oils from the other tissues.[9] On the basis of this research, we further investigated the screening of antimicrobial components of the *C. japonica* heartwood against phytopathogenic fungi and bacteria in expectation of finding strong antimicrobial compounds.

Materials and methods

Materials and instruments

Cryptomeria japonica wood (around 30 years old) was collected from Takaoka-cho, Miyazaki, Japan, was air-dried for 3 months, and its heartwood part was crushed into sawdust. Potato dextrose agar (PDA), sucrose, and agar were purchased from Wako (Japan). Meuller Hinton agar (MHA) and Meuller Hinton broth were purchased from Difco (USA). Totarol was purchased from Sigma-Aldrich (USA) and used as a positive control for the antibacterial

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test. Silica gel BW-300 for column chromatography was purchased from Fuji Silysia (Japan) and silica gel 60 F254 TLC plates from Merck (Germany). The water content of the sawdust was determined by a Kett moisture meter F-1 (Kett Electronic Laboratory). Proton nuclear magnetic resonance (^{1}H-NMR) and ^{13}C-NMR spectra were recorded on an AC-250P spectrometer (Bruker, USA) in $CDCl_3$ solution with tetramethylsilane (TMS) as an internal standard. Infrared (IR) spectra were measured in $CHCl_3$ solution on a Hitachi IR 270-30 spectrometer (Japan). All purchased solvents were of high purity and were redistilled before use.

Extraction and separation of components of *Cryptomeria japonica* heartwood

The sawdust of *C. japonica* heartwood (319.2 g; moisture content 12.8%) was extracted with methanol (5.0 l) by Soxhlet extraction for 48 h to give 14.60 g of methanol extract S-2 (5.25% based on dry weight of the sawdust). The methanol extract S-2 (2.00 g) was fractionated stepwise with toluene and *n*-hexane according to our procedure described previously,[5] to afford toluene-insoluble fraction S-3 (0.72 g, 37.5% from S-2), *n*-hexane-insoluble fraction S-5 (0.20 g, 10.0% from S-2), and *n*-hexane-soluble fraction S-6 (0.96 g, 48.0% from S-2). Ten grams of fraction S-6 were collected by repeated fractionation. To a dichloromethane solution (100 ml) of fraction S-6 (4.81 g) was added silica gel (10 g), and the solution was evaporated. The fraction S-6 adsorbed on silica gel was loaded onto a silica gel column (100 g) and was separated by elution with, in turn, *n*-hexane–benzene [4 : 1 (500 ml), 3 : 1 (800 ml), 2 : 1 (750 ml), 1 : 1 (800 ml)], benzene (600 ml), and benzene–ethyl acetate [9 : 1 (250 ml), 6 : 1 (500 ml), 4 : 1 (300 ml), 2 : 1 (300 ml)], and ethyl acetate (300 ml) to give 108 fractions. The major components of each fraction were identified by IR and/or ^{1}H-NMR measurements; the fractions were grouped into group A (fr. 1–23, 871 mg), group B (fr. 24–28, 43.3 mg), group C (fr. 29–43, 203 mg), group D (fr. 44–62, 814 mg), group E (fr. 63–77, 242 mg), group F (fr. 78–93, 2398 mg), group G (fr. 94–99, 226 mg), and group H (fr. 100–108, 663 mg). β-Sitosterol (34 mg), sandaracopimarinal (162 mg), ferruginol (690 mg), and sandaracopimarinol (1350 mg) were isolated from the groups B, C, D, and F, respectively, by silica gel column chromatography under conditions similar to those described in our previous report.[5] Their spectral data coincided with the data reported previously for β-sitosterol,[10] sandaracopimarinal,[11] ferruginol,[6,12] and sandaracopimarinol.[13]

Bioassay

The phytopathogenic fungi tested for antifungal activity were *Fusarium oxysporum* OK1 provided by Professor K. Ogawa (Faculty of Agriculture, University of Miyazaki), *Phytophthora capsici* CAF892 by Dr. Y. Miyata (Rainbow Laboratory, Osaka), and *Pythium splendens* by Dr. N. Nishimura (Department of Vegetable and Flower Research, National Agricultural Research Center for Kyushu Okinawa Region). The phytopathogenic bacterium tested for antibacterial activity was *Ralstonia solanacearum* no. 8224 provided by Professor N. Matsuzoe (Prefectural University of Kumamoto). *Escherichia coli* NBRC 3301, *Proteus mirabilis* NBRC 13300, *Proteus vulgaris* NBRC 3851, *Pseudomonas fluorescens* NBRC 3757, *Achromobactor xylosoxidans* subsp. *xylosoxidans* NBRC 15126, *Bacillus subtilis* NBRC 13719, *Staphylococcus epidermidis* NBRC 12993, and *Micrococcus luteus* NBRC 3333 were provided by NITE Biological Resource Center, National Institute of Technology and Evaluation, Japan. Appropriate amounts of S-2, S-3, S-5, S-6, β-sitosterol, sandaracopimarinal, ferruginol, and sandaracopimarinol were dissolved in dimethyl sulfoxide, respectively, to afford sample solutions. Each sample solution was mixed with PDA, potato sucrose agar (PSA), or MHA by twofold dilution at 40°–50°C, and the mixture was cooled at room temperature to give the test plate. Antifungal assay was performed by a method similar to that described by Aoyama et al.[14] Each strain was preincubated on PDA in a petri dish at 25°C until the fungus covered most of the surface of the plate. Each preincubated culture was inoculated as a 5-mm-diameter agar disk on the test plate containing the sample in concentration from 630 to 10 000 μg/ml. Minimum inhibitory concentration (MIC) values were determined after 1 week of incubation at 25°C. Antibacterial assay was performed using the agar dilution method according to the standard MIC determination method of the Japan Society of Chemotherapy.[15] *Ralstonia solanacearum* was preincubated in potato sucrose broth at 30°C for 48 h. The preincubated culture was adjusted to approximately 10^6 CFU/ml (CFU: colony-forming units) with sterile and buffered saline (pH 7.0) according to McFarland turbidity standards and was streaked on the test plate containing the sample in concentration from 630 to 10 000 μg/ml. After 48 h of incubation at 30°C, MIC values were determined. Because ferruginol and sandaracopimarinol inhibited the growth of *R. solanacearum* below 630 μg/ml, the antibacterial activity was examined again in the concentration range of 1–1024 μg/ml by twofold dilution. The preincubated cultures of *E. coli*, *P. mirabilis*, *P. vulgaris*, *P. fluorescens*, *A. xylosoxidans*, *B. subtilis*, *S. epidermidis*, and *M. luteus* in Meuller Hinton broth at 30°C for 48 h were adjusted, respectively, to approximately 10^6 CFU/ml with sterile and buffered saline (pH 7.0) and were streaked on the test plates containing sandaracopimarinol, ferruginol, or totarol in concentration from 1 to 1024 μg/ml. After 48 h of incubation at 30°C, MIC values were determined.

Results and discussion

The methanol extract S-2 from sawdust of the *Cryptomeria japonica* heartwood was fractionated with toluene and *n*-hexane under conditions similar to those described previously (Fig. 1).[5] Toluene-insoluble fraction S-3, *n*-hexane-insoluble fraction S-5, and *n*-hexane-soluble frac-

Table 1. Amounts of methanol extract S-2 and solvent-fractionated fractions from S-2, and their antimicrobial activity against phytopathogenic microorganisms

Fraction	Amount[a] (g/kg)	MIC (μg/ml)			
		Fusarium oxysporum[b]	*Phytophthora capsi*[b]	*Pythium splendens*[b]	*Ralstonia solanacearum*[c]
S-2	52.5	>10 000	>10 000	>10 000	10 000
S-3	19.7	>10 000	>10 000	>10 000	10 000
S-5	5.3	>10 000	10 000	10 000	5 000
S-6	25.2	5 000	2 500	5 000	2 500

MIC, minimum inhibition concentration; S-2, methanol extract; S-3, toluene-insoluble fraction; S-5, *n*-hexane-insoluble fraction; S-6, *n*-hexane-soluble fraction
[a] Based on 1 kg of dry heartwood sawdust
[b] Antifungal assay was performed using potato dextrose agar at 25°C for 1 week
[c] Antibacterial assay was performed using potato sucrose agar at 30°C for 48 h

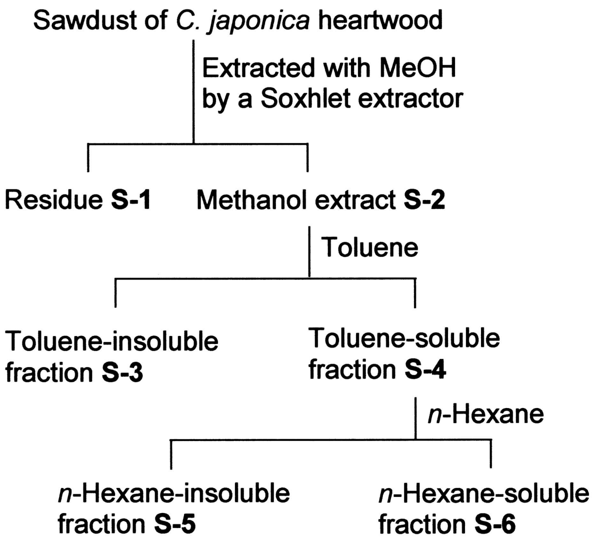

Fig. 1. Methanol extraction followed by solvent-fractionation of sawdust of *Cryptomeria japonica* heartwood

tion S-6 were obtained (Table 1). Antimicrobial activities of the fractions S-2, S-3, S-5, and S-6 were investigated against three phytopathogenic fungi *Phytophthora capsici*, *Fusarium oxysporum*, and *Pythium splendens*, and one phytopathogenic and Gram-negative bacterium *Ralstonia solanacearum*. The MIC values of the solvent-extracted fractions are summarized in Table 1. The methanol extract S-2, the toluene-insoluble fraction S-3, and the *n*-hexane-insoluble fraction S-5 showed no or very weak antimicrobial activity, but the *n*-hexane-soluble fraction S-6 showed moderate activity. Because the amount of fraction S-6 (25.2 g/kg; 48.0% from S-2) was the largest among the three fractions obtained, fraction S-6 seemed to contain strong and/or a large amount of antimicrobial components.

Fraction S-6 was separated by silica gel chromatography using solvent systems of *n*-hexane–benzene and benzene–ethyl acetate into 108 fractions, and IR and/or ^{1}H-NMR spectra of each fraction were collected. The 108 fractions were grouped into eight groups, A–H, on the basis of the spectral data of major components, and antibacterial activity of each group was evaluated against *R. solanacearum* (Table 2). Groups D and F were found to have the strongest

Table 2. Amounts of fraction groups A–H separated from the *n*-hexane-soluble fraction S-6 by silica gel column chromatography and their antibacterial activity against *Ralstonia solanacearum*

Group	Solvent ratio of column eluent	Amount[a] (g/kg)	Major component[b]	MIC[c] (μg/ml)
A	H:B (4:1–3:1)	4.56	Hydrocarbons	>10 000
B	H:B (3:1)	0.22	β-Sitosterol	5 000
C	H:B (2:1)	1.06	Sandaracopimarinal	5 000
D	H:B (1:1)	4.26	Ferruginol	<630
E	B	1.27	Complex mixture	1 250
F	B:EA (9:1–6:1)	12.56	Sandaracopimarinol	<630
G	B:EA (4:1)	1.18	Complex mixture	1 250
H	B:EA (2:1–0:1)	3.47	Complex mixture	1 250

H, *n*-Hexane; B, benzene; EA, ethyl acetate
[a] Based on 1 kg of dry sawdust
[b] Identified from infrared and proton nuclear magnetic resonance spectra
[c] Antibacterial assay performed by the same method as described in Table 1

activity: each MIC value was below 630 μg/ml. Groups E, G, and H had relatively strong activity (MIC 1250 μg/ml) but groups A, B, and C had very weak or no activity.

The major components of groups B, C, D, and F were further purified by silica gel chromatography, respectively, to afford β-sitosterol, sandaracopimarinal, ferruginol, and sandaracopimarinol (Fig. 2). The amounts and the antimicrobial activity of the isolated compounds are shown in Table 3. Totarol (Fig. 3) was used as a positive control for the antibacterial assay, because it has been shown to strongly inhibit the growth of several bacteria, and, in particular, Gram-positive bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA).[16–18] The content of sandaracopimarinol in the *n*-hexane-soluble fraction S-6 was the highest among the four terpenes and was about twice that of ferruginol. The antifungal and/or antibacterial activities of β-sitosterol and sandaracopimarinal were very weak. Sandaracopimarinol and ferruginol showed considerably strong activity against the three fungi, and the MIC values of sandaracopimarinol were approximately equal to those of ferruginol. Kofujita et al.[6] reported that the antifungal activity of the *n*-hexane extract of the bark of *C. japonica* was examined using phytopathogenic fungi, namely *Alternaria alternaria*, *Pyricularia oryzae*, *Rhizoctonia solani*, and *Fusarium oxysporum cucumerinum*, and that the bark contained 0.4%–1.0% of ferruginol, which played

Table 3. Amounts of isolated terpenes from the *n*-hexane-soluble fraction S-6 and their antimicrobial activities against phytopathogenic microorganisms

Isolated compound	Amount[a] (g/kg)	MIC[b] (μg/ml)			
		F. oxysporum	*P. capsici*	*P. splendens*	*R. solanacearum*
β-Sitosterol	0.15	>5000	–[c]	–	>5000[d]
Sandaracopimarinal	0.85	>5000	5000	>5000	2500
Ferruginol	3.62	1250	2500	2500	32
Sandaracopimarinol	7.07	2500	1250	1250	8
Totarol[d]		–	–	–	4

[a] Based on the dry sawdust
[b] Antimicrobial assay performed by the same method as described in Table 1
[c] Not examined
[d] Totarol was used as a positive control for antibacterial assay

Table 4. Antibacterial effects of sandaracopimarinol and ferruginol

Type	Bacterium	MIC (μg/ml)		
		Sandaracopimarinol	Ferruginol	Totarol[b]
Gram-negative	*Escherichia coli*	>256	>256	>256
	Proteus vulgaris	>256	>256	>256
	Pseudomonas fluorescens	>256	>256	>256
Gram-positive	*Bacillus subtilis*	8	16	2
	Staphylococcus epidermidis	8	64	2
	Micrococcus luteus	8	16	2

Antibacterial assay was performed using Meuller Hinton agar at 30°C for 48 h
[a] Totarol was used as a positive control for antibacterial assay

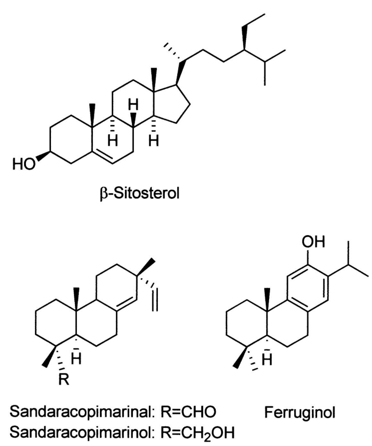

Fig. 2. Isolated terpenes from the *n*-hexane–toluene-soluble fraction S-6

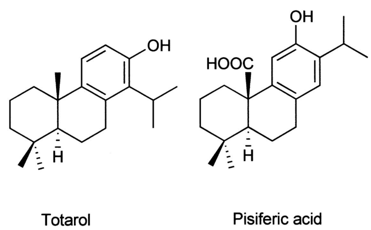

Fig. 3. Diterpenes having antibacterial activity against several bacteria

an important role in the antifungal activity of the bark. The present antifungal effect of ferruginol is in fair agreement with that reported by Kofujita et al.[6] On the other hand, sandaracopimarinol was only reported to have antifungal activity against *Lentinula edodes*.[4,5] In this work, sandaracopimarinol was ascertained to be one of the antifungal components in *C. japonica* heartwood for the phytopathogenic fungi. The bark was reported to contain a very low content of sandaracopimarinol as compared with the wood of *C. japonica*.[19] For this reason, it seems that sandaracopimarinol was not found as the antifungal component in *C. japonica* bark.[6] Besides, sandaracopimarinol and ferruginol showed very strong antibacterial activity against *R. solanacearum*. Ferruginol has been already well known to have antibacterial activities,[20–22] but antibacterial effect of sandaracopimarinol has not been reported. The antibacterial activity of sandaracopimarinol against *R. solanacearum* was four times stronger than ferruginol and was nearly comparable with that of totarol.

Because the strong inhibition of *R. solanacearum* with sandaracopimarinol was of interest, further antibacterial tests against several Gram-negative and Gram-positive bacteria were performed. The results are summarized in Table 4. Sandaracopimarinol always showed strong antibacterial activity compared with ferruginol against the Gram-positive bacteria (the order of activity; totarol > sandaracopimarinol > ferruginol). On the contrary, these three compounds had no or weak activity against the Gram-negative bacteria. It has been known that the inhibitory activity of ferruginol and totarol against Gram-negative bacteria was considerably weaker than that against Gram-positive bacteria and the phenolic hydroxy group in their structures has been found to be an important factor for the inhibitory activity.[16–18,20–22] Moreover, it has already been demonstrated that totarol inhibited bacterial respiration as an electron transport inhibitor[17,18] and the pisiferic acid stucturally related to ferruginol (Fig. 3) selectively inhibited bacterial peptidoglycan synthesis in *Bacillus subtilis*.[20] It is interesting that sandaracopimarinol bearing no phenolic hydroxy group was found to possess potent activity against Gram-positve bacteria. A study of the relationship between the sturucture and the antimicrobial activity of sandaracopimarinol and its derivatives is now in progress.

Conclusions

The antifungal and antibacterial components of the heartwood of *Cryptomeria japonica* were studied and the results are summarized as follows:

1. The *n*-hexane-soluble fraction from the methanol extract of *C. japonica* heartwood had antimicrobial effect, and sandaracopimarinol and ferruginol were isolated from the *n*-hexane-soluble fraction as major diterpenes.
2. Sandaracopimarinol and ferruginol had moderate antifungal activity against three phytopathogenic fungi and very strong antibacterial activity against phytopathogenic and Gram-negative bacterium *Ralstonia solanacearum*.
3. The antibacterial activity of sandaracopimarinol against Gram-positive bacteria was always stronger than that of ferruginol, but both sandaracopimarinol and ferruginol had no activity against Gram-negative bacteria except for *R. solanacearum*.
4. This study is the first to report sandaracopimarinol as one of the major antimicrobial components of *C. japonica* heartwood.

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