〔Original〕

Phytoconstituent profile and biological activity of wood chips of Aomori Hiba (*Thujopsis dolabrata* SIEB. *et* ZUCC. var. *hondai* MAKINO)

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Abstract

We obtained essential oil from wood chips of the Aomori hiba tree by steam distillation. Component analysis of the essential oil and extract of distilled water identified 25 kinds of components, equivalent to 75% of the total amount of essential oil. The main component was thujopsene 35.6%, followed by *epi*-cedrol 15.9%.

In addition, 32 ingredients were identified from a distilled water extract of these wood chips. The main component was *epi*-cedrol 16.1%, followed by terpinen-4-ol 14.8% and thujopsene 10.3%.

Among other constituents, hinokitiol showed antimicrobial activity against all bacteria tested, namely *Escherichia coli*, EHEC (enterohemorrhagic *E. coli*), *Pseudomonas aeruginosa*, *Candida albicans*, and MRSA (methicillin-resistant *Staphylococcus aureus*), while cedrol showed activity against all but *P. aeruginosa*. While an aged sample of essential oil did not show antimicrobial activity, a newly obtained sample showed activity against Gram-positive bacteria (*S. aureus*, MRSA) and Gram-negative bacteria *E. coli*, *Shigella sonnei*).

Key words: wood chips of Aomori hiba; essential oil; steam distillation; distilled water; biological activity

1. Introduction

The Hiba tree of Aomori Prefecture is regarded as one of three most beautiful forest tree species in Japan, alongside “Kiso Hinoki” in Nagano Prefecture and “Akita Sugi (Cedar)” in Akita Prefecture. Called “Hinoki Asunaro” in Japanese, about 80% of Japanese hiba (*Thujopsis dolabrata* Sieb. *et* Zucc. var. *hondai* Makino) are found in the forests of Aomori, where the tree has been selected as the “Prefectural tree”.

Despite declining resources, Aomori prefecture has recently devoted substantial efforts to restore its hiba forests. A restoration project team was established this fiscal year. “Aomori hiba” has excellent durability as residential building wood, and also has an insecticide effect against termites and some other insects. The wood is also said to have deodorant and relaxant effects [1–5].

As part of our efforts to study the components of fruits commonly grown in Aomori, we have examined the essential oil and distilled water components of the leaves of apple, Steuben grape and cassis plants, for which Aomori Prefecture has the highest production in Japan [6–8]. We previously reported the essential oil and distilled water constituents of the leaves of Aomori Hiba and their antioxidant activity as a non-fruit plant produced in Aomori Prefecture [9].

In the present paper, we investigated the essential oil and distilled water components of wood chips of Aomori hiba and their antimicrobial activity as a non-fruit Aomori Prefecture-derived plant.

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2. Materials and Methods

2.1. Plant materials

800 g of wood chips of "Aomori hiba" obtained from March to April, were used.

2.2. Reagents

Hinokitiol and cedrol were purchased from Tokyo Chemical Industry Co., Ltd. (Japan). Common reagents of biochemistry grade or highest quality such as hexane and ethanol were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Paper discs of (Φ) 6 mm diameter were purchased from Advantec (Toyo Roshi Kaisha, Ltd., Tokyo, Japan).

2.3. Steam distillation and extraction of distilled water

Steam distillation was performed over about 4 to 6 hours using wood chips (800 g) of Aomori hiba. A 9.8 g (1.2%) amount of volatile oil and approximately 2 L of distilled water were obtained from the wood chips. Distilled water was then extracted four times with hexane, and yielded 0.52 g (0.065%) of extract.

2.4. Analysis

Volatile oil and distilled water extract were analyzed by flame ionization detector-gas chromatography (GC-FID) using a Hitachi Gas Chromatograph G-5000A (Hitachi, Tokyo, Japan). Nitrogen was passed through a poly (alkylene glycol) column (30 m × 0.25 mm i.d.) at a carrier flow rate of 60 ml/min using a 10 °C/min gradient, initiated with a 5-min hold at 40 °C and ending upon reaching 200 °C. Injector and detector temperatures were 150 °C and 250 °C, respectively. Mass spectrometer-gas chromatography (GC-MS) analysis was performed on a JEOL Q1000GC-Mk II Mass Spectrometer (Japan Electron Optics Laboratory Co. Ltd., Tokyo, Japan), consisting of an HP-5 column (30 m × φ0.32 mm × 0.25 μm film thickness; coated with 5%-phenyl-95% dimethyl polysiloxane) with a 1 ml/min helium carrier flow rate. Temperature was programmed to deliver a 15 °C/min gradient initiated with a 4.7 min hold at 50 °C and terminated with a 2-min hold at 280 °C. Injector and GC-transfer line temperatures were both set at 200 °C.

Separated components were measured under non-isothermal conditions and identified by comparison with the RIs calculated from the Van den Dool-Kratz equation and the MS coincidence rate [10, 11]. Component ratios were computed from the peak area ratio of GC-MS. Oil-drilling rate and extractability were calculated from the sample weight and extraction yield.

2.5. Assay of antimicrobial activity

Antimicrobial activity was screened by the sensitive disc method using two kinds of reagent (cedrol and hinokitiol) and negative control. A sample of 15 μl of stock solution was adsorbed on a Φ 6 mm paper disc, which was used as a sensitive disc.

Antibacterial activity testing was done using *Escherichia coli* (*E. coli*, ATCC 25922), enterohemorrhagic *Escherichia coli* (EHEC), *Pseudomonas aeruginosa*, *Candida albicans* and methicillin-resistant *Staphylococcus aureus* (MRSA). After culture of the sensitive disks for 16 h, the size of the inhibition circle was measured and the antibacterial activity was qualitatively determined.

The antibacterial test was done using *S. aureus* 209P, *E. coli* IFO-3806, and *Shigella sonnei* (*S. sonnei*) by a component of the MIC (minimum inhibitory concentration) method.

3. Results and Discussion

3.1. Essential oil components

Analysis of the essential oil revealed 25 components corresponding to 75.0% of the total oil, as shown in Table 1.

The essential oil of wood chips of hiba contained thujopsene at 35.6%, followed by *epi*-cedrol at 15.9%. Interestingly, most of the essential oil components of these wood chips were sesquiterpenes (C15), whereas most of those of the leaves were monoterpenes (C10) [9]. This tendency is also seen in Hinoki (Cupressaceae) species and the plants of Pinaceae family [12], likely because these trees easily accumulate large volatile monoterpenes in the leaves and sesquiterpenes in the xylem.

The essential oil of hiba is also known to contain hinokitiol, which has high antibacterial activity [13]. Here, however, we did not detect hinokitiol by our usual method.

3.2. Components of distilled water extract.

Distilled water of wood chips of hiba was extracted

with hexane to obtain 0.52 g of residue. The results of these component analyses are shown in Table 2.

In distilled water of these wood chips, *epi*-cedrol accounted for the highest amount, at 16.1%, which was closely similar to its concentration in the essential oil. In contrast, thujopsene, which accounted for 35.6% in essential oil, was only 10.3%.

Conversely, the concentration of terpinen-4-ol was only 0.5% in essential oil, versus 14.8% in distilled water.

In the essential oil, only two monoterpenes were found, namely terpinen-4-ol and carvacrol. In this distilled water, in contrast, 17 monoterpene species were identified, mainly compounds with a *p*-menthane skeleton.

3.3. *Assay of antimicrobial activity*

Antimicrobial testing of hinokitiol, cedrol and negative control was carried out using the sensitive disc method. The results are shown in Table 3 and Photo 1.

Table 1. Component essential oil contained in wood chips of Aomori hiba

No.	RT	RI	compound	rate %	CI	No.	RT	RI	compound	rate %	CI
1	10:54	1177	terpinen-4-ol	0.5	○	14	15:33	1500	α-selinene	0.1	-
2	12:52	1301	carvacrol	0.5	○	15	15:37	1505	β-himachalene	3.5	-
3	14:10	1394	β-elemene	0.2	-	16	15:41	1511	cuparene	5.1	-
4	14:16	1401	α-funebrenes	0.2	-	17	15:53	1527	δ-cadinene	0.8	-
5	14:28	1417	α-cedrene	0.7	-	18	16:09	1549	β-sesquiphellandrene	1.4	-
6	14:35	1426	β-cedrene	0.4	-	19	16:44	1597	cedrol	1.6	-
7	14:43	1436	thujopsene	**35.6**	-	20	16:53	1610	*epi*-cedrol	**15.9**	-
8	14:47	1441	elixene	0.3	-	21	17:10	1635	δ-cadinol	1.1	-
9	15:00	1458	aromadendrene	0.2	-	22	17:32	1667	alloaromadendrene oxide	0.6	-
10	15:09	1469	aromadendr-9-ene	0.4	-	23	17:47	1688	α-bisabolol	2.1	-
11	15:15	1477	patchoulene	0.2	-	24	17:57	1703	*cis*-*Z*-α-bisabolene epoxide	2.0	-
12	15:20	1483	β-chamigrene	0.9	-	25	18:31	1755	γ-costol	0.7	-
13	15:26	1491	β-eudesmene	0.2	-				Total	75.0	

RT: Retention Time RI: Retention Index CI: Co-injection with authentic sample

Table 2. Components in distilled water of wood chips of Aomori hiba

No.	RT	RI	compound	rate %	CI	No.	RT	RI	compound	rate %	CI
1	7:57	1027	*p*-cymene	tr	○	18	12:54	1304	carvacrol	8.0	○
2	8:03	1030	limonene	tr	○	19	13:02	1313	citronellic acid	0.6	-
3	9:20	1091	*p*-cymenene	0.1	-	20	13:22	1338	menthoglycol	0.1	-
4	10:23	1148	*neo*-isopulegol	0.6	-	21	14:08	1392	cedr-9-ene	0.3	-
5	10:34	1158	isopulegol	0.1	-	22	14:30	1419	α-cedrene	1.4	-
6	10:38	1162	sabina ketone	0.1	-	23	14:36	1427	β-cedrene	0.3	-
7	10:45	1169	borneol	0.1	○	24	14:45	1438	thujopsene	10.3	-
8	10:56	1177	terpinen-4-ol	14.8	○	25	15:22	1486	β-chamigrene	0.8	-
9	11:04	1187	*p*-cymen-8-ol	3.3	-	26	15:29	1495	β-eudesmene	0.1	-
10	11:10	1192	α-terpineol	3.1	○	27	15:43	1514	cuparene	4.8	-
11	11:21	1203	*o*-cumenol	0.2	-	28	15:55	1530	δ-cadinene	0.3	-
12	11:25	1208	*p*-menth-2-en-7-ol	0.5	-	29	16:47	1601	cedrol	0.9	-
13	11:30	1213	*cis*-verbenone	0.1	-	30	16:55	1611	*epi*-cedrol	16.1	-
14	11:47	1231	*m*-cumenol	6.4	-	31	17:12	1638	δ-cadinol	0.9	-
15	12:12	1258	piperitone	tr	-	32	17:49	1691	α-bisabolol	1.3	-
16	12:15	1261	carvenone	tr	-						
17	12:17	1263	myrtanol	tr					Total	75.5	

tr: trace (< 0.1%)

Hinokitiol showed very strong activity (+++) against the Gram-negative bacteria *E. coli* and enterohemorrhagic *E. coli* (EHEC) and the Gram-positive bacterium methicillin-resistant *Staphylococcus aureus* (MRSA). It also showed strong activity (++) against a Gram-negative bacterium, *P. aeruginosa*, and a fungus, *C. albicans*.

Cedrol showed strong (++) activity against EHEC and MRSA and (+) activity against *E. coli* and *C. albicans*. However, it showed no activity against *P. aeruginosa*.

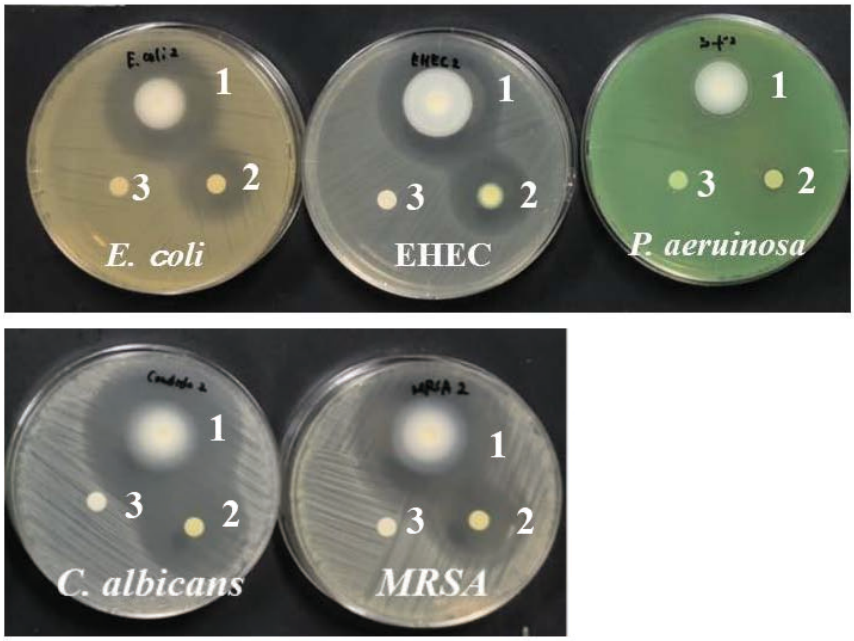
We therefore repeated these antimicrobial tests using the essential oil and distilled water extract obtained from lavendar (*Lavandula angustifolia*), another plant from Aomori prefecture. The results are shown in Table 4.

We previously showed that the essential oil of lavender contains 29% linalool, 14% linalyl acetate and 15% terpinen-4-ol [14]. Antimicrobial activity was exhibited even on 50% dilution of this oil, suggesting that the antimicrobial activity might be ascribable to

Table 3. Result of antimicrobial activity tests

	E. coli	EHEC	*P. aeruginosa*	*C. albicans*	MRSA
1: hinokitiol	+++	+++	++	++	+++
2: cedrol	+	++	−	+	++
3: negative control	−	−	−	−	−

Classification criteria of inhibition circles: (±) <10 mm, (+) < 20 mm, (++) < 30 mm, 30 mm ≤ (+++)



1: hinokitiol, 2: cedrol, 3: negative control

Photo 1. Results of antimicrobial assay.

Table 4. MIC values in antimicrobial activity

Plants of Aomori Prefecture	MIC(%)			
	S. aureus	MRSA	*E. coli*	*S. sonnei*
Essential oil of *Lavandula angustifolia*	50	50	40	40
Distilled water of *L. Angustifolia*	n.a.	n.a.	n.a.	n.a.
Essential oil of hiba (wood chips)	12.5	12.5.	6.3	5.0
Distilled water of hiba (wood chips)	n.a.	n.a.	n.a.	n.a.

n.a.: not active. The MIC value was expressed as % of undiluted solution as 100% (*v/v*).
For reference, we plated on lavender essential oil and distilled water extract.

these components.

The essential oil of hiba wood chips was also found to have antimicrobial activity, even when diluted to 12.5% for Gram-positive bacteria and 6.3% for Gram-negative bacteria.

Although we have previously conducted antibacterial activity tests of isoprenoids such as terpinen-4-ol and linalool [15], we have not reported assay of the essential oil of lavendar itself. Mixtures of these isoprenoids are also interesting as they tend to show antibacterial tendencies.

When we compared the wood chip components examined in this study with those of the leaves of Hiba that we reported last year [9], we found that the unique scent of Hiba is mainly attibutable to components of the essential oil of the chips, including *epi*-cedrol, thujopsene and others. In contrast, the leaves of Hiba have similar components to the leaves of the Aomori prefectural fruits we previously evaluated, many of which showed a fresh fragrance [6–8]. This finding was somewhat surprising. In addition, the distilled water of many of these fruits also contain hydrophilic compounds.

4. Conclusion

We conducted steam distillation of wood chips of the Aomori hiba tree to obtain essential oil and distilled water extract. Analysis of the oil identified 25 components, of which the main component was thujopsene (35.6%). In addition, GC-MS analysis of distilled water extract identified 32 components, of which the main component was *epi*-cedrol (16.1%).

When antimicrobial activity was evaluated using a newly obtained essential oil sample, activity was seen against Gram-positive bacteria (*S. aureus*, MRSA) and Gram-negative bacteria (*E. coli*, *S. sonnei*).

We have been studying the components of fruits commonly grown in Aomori (Fuji apples, Steuben, Cassis) and "Aomori hiba" for 5 years. We hope that these results will contribute to understanding of the special products of Aomori Prefecture, and will aid in regional development.

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青森ヒバ（*Thujopsis dolabrata* SIEB. *et* ZUCC. var. *hondai* MAKINO）木材チップの植物化学的成分のプロファイルと抗菌活性

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要　旨

私たちは、青森ヒバの木材チップの水蒸気蒸留を行い、精油を得た。さらに蒸留湯はヘキサン抽出を行った。精油から25種類の化合物を同定したが、主要成分はthujopsene（36.5%）、次いで*epi*-cedrol（15.9%）と続いた。更に、蒸留湯抽出物から32種類の成分を同定した。主成分は*epi*-cedrolが多く16.1%、続いてterpinen-4-olが14.8%、thujopseneが10.3%であった。

更に、私たちは精油と2、3の成分について抗菌活性試験を行った。Hinokitiolは、使用したすべての菌（大腸菌、腸管出血性大腸菌、緑膿菌、MRSA、および真菌のカンジダアルビカンス）で強い活性がみられた。cedrolでも緑膿菌以外は活性を示した。ヒバチップの精油では、グラム陽性菌（黄色ブドウ球菌、MRSA）およびグラム陰性菌（大腸菌、赤痢菌）に活性を示した。