〔Original〕

Phytochemical Profile and Antioxidant Activity of the Leaf of Aomori Hiba (*Thujopsis dolabrata* SIEB. *et* ZUCC. var. *hondai* MAKINO)

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Abstract

We obtained the essential oil from the leaves of Aomori hiba by steam distillation and extracted the distilled water with hexane. Of the 22 ingredients identified, the major essential oil components were sabinene (35.7%), γ-terpinene (13.5%), α-pinene (7.7%), and β-myrcene (7.7%), From distilled water, the content of terpinen-4-ol was very high, at 71.1%, followed by *p*-cymene (4.8%), terpineol (4.6%) and γ-terpinene (2.5%).

We investigated the antioxidant effect of the essential oil of Aomori hiba leaf using the DPPH method. Results showed a relatively high antioxidant effect (SC_{50}: 11%　concentration, *v/v*). Furthermore, SC_{50} for γ-terpinene and α-terpinene, which are also essential oil components of the leaves, were 78 mM and 180 mM, respectively, also showing high antioxidant effects.

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

1. Introduction

"Aomori hiba *(Thujopsis dolabrata* Sieb. *et* Zucc. var. *hondai* Makino*),*" regarded together with "Kiso Hinoki" in Nagano Prefecture and "Akita Sugi" in Akita Prefecture as one of Japan's three major beautiful forest trees, is a coniferous tree of the *Thujopsis* genus of the *cypressaceae* family. The Japanese name is "Hinoki Asunaro", or more commonly "Aomori hiba", given that about 80% are clustered in Aomori Prefecture, which has selected it as the official "prefecture tree". "Aomori hiba" is characterized by its excellent durability as a residential building wood, as well as insecticide (termite etc.), deodorant and deodorizing, and relaxant effects [1–4].

We have studied the essential oils and distilled water components of the leaves of apple, steuben and cassis, three fruits for which Aomori Prefecture has the highest production in Japan. Among these, apples show high concentrations of farnesene, which shows antioxidant activity. Sabinene concentrations are remarkably high in Aomori cassis. Although essential oil could not be obtained from the leaves of steuben, concentrations of terpinen-4-ol and linalool in three types of distilled water were high. We have reported that these compounds show antibacterial activity [5–8].

Here, we examined the essential oil and distilled water components of Aomori hiba and their biological activity in a non-fruit plant of Aomori Prefecture.

2. Materials and Methods

2.1. Plant materials

A total of 2132 g of leaves of Aomori hiba collected in March and April were used. The fresh leaves were cut finely and used for steam distillation.

2.2. Reagents

1,1-Diphenyl-2-picrylhydrazyl and butylated dihydroxyanisole were purchased from Tokyo Chemical

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Industry Co., Ltd. (Japan) and Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), respectively. Biochemical grade or highest quality reagents, including α-terpinene, γ-terpinene, and terpinen-4-ol, were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries. 96-well multiwell plates were purchased from Corning International KK (Akasaka, Tokyo).

2.3. Steam distillation and extraction of leaves

Steam distillation was performed over about 4 to 6 hours. Approximately 2 L of distilled water was obtained. A 17.4 g (0.81%) amount of volatile oils was obtained from the leaves. Distilled water was then extracted four times with hexane, and yielded 3.3 g (0.15%) of extract.

2.4. Analysis

Volatile oils and distilled water extracts 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 [9]. 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 antioxidant activity

Antioxidant action of reagents contained in the leaves of Aomori hiba was investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical elimination as assay [9–11]. DPPH shows purple absorption at 517 nm (violet) of visible portions. When DPPH is reduced, it fades and absorption is lost, as shown Figure 1.

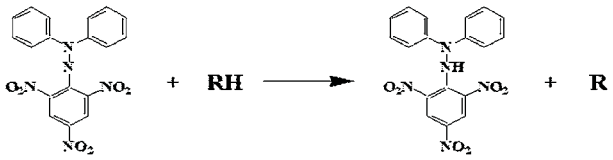

Fig. 1. Reaction of DPPH with a test sample (RH).

To the 96-well plates were added 120 μl portions of 0.1 M acetate buffer (pH 5.5) and the ethanol solution of the sample, followed by 60 μl of a 0.5 mM DPPH ethanol solution. As blank, ethanol was added instead of the sample. After stirring, the mixture was allowed to stand for 30 minutes, and absorbance at 550 nm was measured with a Multiskan JX microplate reader (Thermo Labsystems, MA, U.S.A). Using this absorbance, antioxidant activity (%) of the sample was calculated using the following formula:

$$\% \text{ Antioxidant activity} = 100 \times (A_{blank} - A_{sample})/A_{blank}$$

The regression line obtained with this formula was used to plot the radical 50% scavenging concentration (SC_{50}). BHA (butylated dihydroxyanisole) was used as positive control.

3. Results and Discussion

3.1. Essential oil components

Analysis of the essential oil of Aomori hiba leaves by GC-MS identified 22 components, as shown in Table 1.

Essential oil of Aomori hiba leaves contains as much as 35.7% sabinene, followed by 13.5% of γ-terpinene. Overall, more than 68% of the essential oils were composed of C10 monoterpene. The essential oil obtained from the leaves had a refreshing scent, in contrast to the calming fragrance of the so-called hiba tree.

3.2. Distilled water-extracted components

Components identified from distilled water extract are shown in Table 2. The distilled water extract contained terpinen-4-ol as domonant component (71% of total), in contract to only 4.5% in essential oil.

Table 1. Components of essential oil obtained from leaves of Aomori hiba

No.	RT	RI	compound	rate %	CI	No.	RT	RI	compound	rate %	CI
1	5:29	932	α-thujene	5.2	–	13	9:42	1185	*p*-cymen-8-ol	0.2	–
2	5:37	938	α-pinene	7.7	○	14	9:49	1195	α-terpineol	0.1	○
3	6:31	976	sabinene	35.7	–	15	11:29	1356	α-terpinyl acetate	0.4	–
4	6:54	993	β-myrcene	7.7	○	16	12:11	1432	β-caryophyllene	0.2	○
5	7:13	1011	3-carene	1.9	–	17	12:30	1468	α-caryophyllene	0.1	–
6	7:20	1018	α-terpinene	6.7	○	18	13:01	1528	γ-cadinene	tr	–
7	7:28	1026	*p*-cymene	5.1	○	19	13:04	1534	δ-cadinene	0.1	–
8	7:32	1030	limonene	4.8	○	20	13:17	1560	elemol	0.1	–
9	8:02	1062	γ-terpinene	13.5	○	21	13:59	1548	γ-eudesmol	0.1	–
10	8:28	1089	α-terpinolene	2.9	–	22	14:10	1672	α-eudesmol	0.2	–
11	8:39	1101	sabinene hydrate	0.1	–						
12	9.39	1181	terpinen-4-ol	4.5	○				Total	97.4	

RT: Retention Time RI: Retention Index CI: Co-injection with authentic sample tr: trace (< 0.1%)

Table 2. Ingredients contained in distilled water extract of Aomori hiba leaves

No.	RT	RI	compound	rate %	CI	No.	RT	RI	compound	rate %	CI
1	5:35	936	α-pinene	1.8	○	15	10:40	1273	*cis*-verbenyl acetate	0.3	–
2	6:29	975	sabinene	1.4	–	16	10:50	1289	bornyl acetate	0.2	–
3	6:52	992	β-myrcene	1.5	○	17	10:53	1294	myrtenyl acetate	0.2	–
4	7:05	1002	α-phellandrene	0.2	–	18	10:58	1302	terpinen-4-yl acetate	0.2	–
5	7:11	1008	3-carene	0.5	–	19	11:27	1353	α-terpinyl acetate	0.6	–
6	7:18	1016	α-terpinene	0.7	○	20	11:33	1363	α-limonene diepoxide	0.1	–
7	7:26	1024	*p*-cymene	4.8	○	21	12:10	1430	β-caryophyllene	0.2	○
8	7:30	1028	limonene	1.7	○	22	12:29	1466	α-caryophyllene	0.1	–
9	8:00	1060	γ-terpinene	2.6	○	23	12:59	1524	γ-cadinene	tr	–
10	8:27	1088	α-terpinolene	0.9	–	24	13:03	1532	δ-cadinene	0.1	–
11	9:37	1179	terpinen-4-ol	71.1	○	25	13:16	1558	elemol	0.8	–
12	9:43	1187	*p*-cymen-8-ol	0.9	–	26	13:58	1646	γ-eudesmol	0.4	–
13	9:47	1192	α-terpineol	4.6	○	27	14:08	1667	α-eudesmol	0.5	–
14	10:22	1245	isothymol methyl ether	0.1	–				Total	96.5	

Therefore, compared with the essential oil of leaves of Aomori hiba, distillation water showed a greater than 65% concentration of terpinen-4-ol, owing to its easy dissolution in distilled water due to its high hydrophilicity. We consider that the ingredients sabinene and γ-terpinene were then generated in the process of steam distillation. Similar results were seen in distilled water of the leaves of Aomori Cassis [7].

3.3. Assay of antioxidant activity

The antioxidant effect (radical scavenging ability) was examined using the essential oil of leaves of Aomori hiba in the range of 2.5% (*v*/*v*) to 20% (*v*/*v*). The results are shown in Fig 2. The plate used in this study is shown in Photo 1.

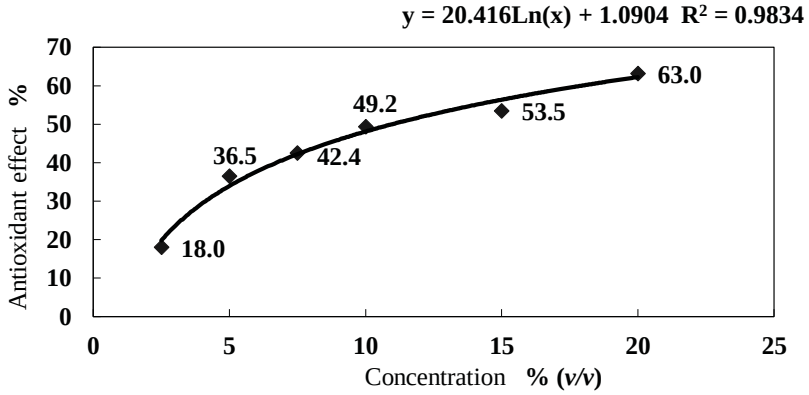

Fig. 2. Antioxidant effect (radical scavenging effect) of essential oil of the leaf of Aomori hiba

Results showed that concentration giving the 50% radical scavenging effect (SC_{50}) was 11.0% (*v/v*).

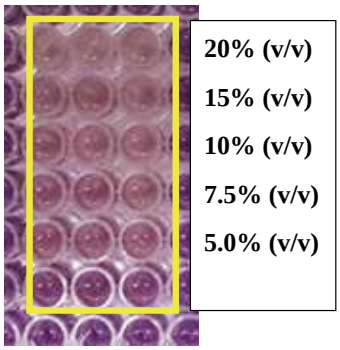

Photo 1. Antioxidant effect of essential oil of the leaf of Aomori Hiba (in yellow frame)

Antioxidant assay using reagents (α-terpinene, γ-terpinene and terpinen-4-ol) was also performed. The antioxidant effect of γ-terpinene in the range of 0.010 M to 0.15 M is shown in Fig. 3, while that of α-terpinene in the range of 0.025 M to 0.20 M is shown in in Fig. 4.

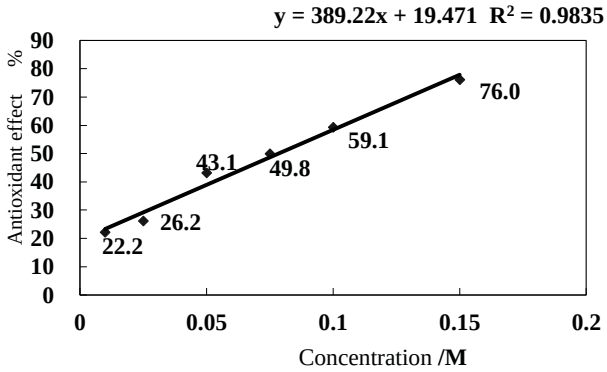

Fig. 3. Antioxidant effect of γ-terpinene

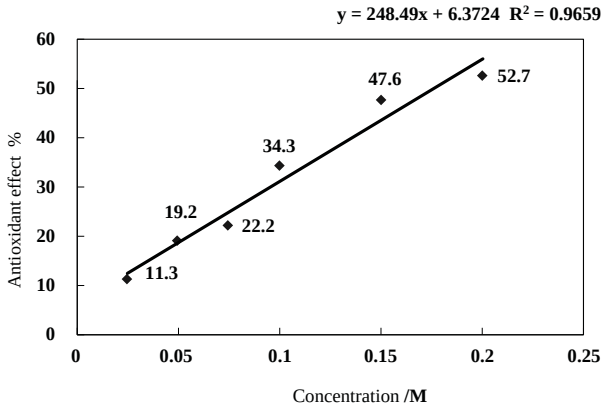

Fig. 4. Antioxidant effect of α-terpinene

Results showed that γ-terpinene had an antioxidant effect of 76.0% at 0.15 M and SC_{50} was calculated as 78 mM (10.6 mg/ml). Further, α-terpinene showed an antioxidant effect of 52.7% at 0.20 M and SC_{50} was calculated as 0.18 M (23.9 mg/ml). Photos of the plate immediately after measurement are shown in Photos 2 and 3.

Terpinen-4-ol showed no activity at all (data not shown).

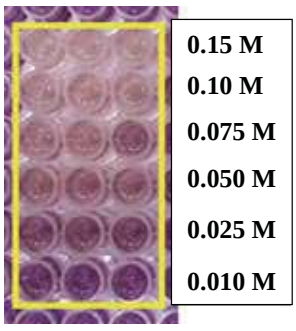

Photo 2. Antioxidative effect (in yellow frame) of γ-terpinene (**left**).

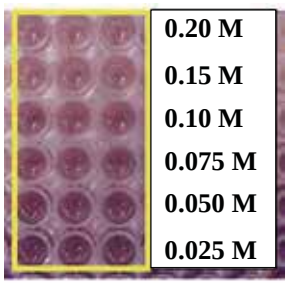

Photo 3. Antioxidative effect (in yellow frame) of α-terpinene (**right**)

The antioxidant effect of γ-terpinene was more than twice as high as that of α-terpinene, suggesting a difference in antioxidant effect depending on the position of the double bond.

As shown in Table 1, γ-terpinene and α-terpinene content in the essential oil was about 13.5% and 6.7%, respectively. These have been confirmed to have a relatively high antioxidant effect, and to be one factor in the antioxidant effect of the essential oil of leaves of hiba.

Recently, a number of conditions have been found to induce the accumulation of proteins with abnormal functions. These include Alzheimer's disease, Parkinson's disease, and ALS (amyotrophic lateral sclerosis). [14–16].

The combination of these proteins with some reactive oxygen species [ROS] can lead to the onset of disease [17].

Radical stress produced by this ROS damages blood vessels and cells, leading to a range of lifestyle diseases and aging.

Therefore, the accumulation of fundamental research to examine the ability to remove radicals by using antioxidant substances is considered to be useful in the future for prevention of such diseases and prevention of aging.

4. Conclusion

We previously reported that sabinen is contained in the leaves of Aomori Cassis. Similarly, however, the essential oil of "Aomori Hiba" also contains sabinen (35.7%) [7]. The fragrance peculiar to Aomori hiba, which is said to have a relaxant effect [3] was not found in the essential oil of the leaves. Furthermore, terpinen-4-ol (71.1%) was the main component in the distilled water of Aomori hiba leaves, as has also been the case for apples, steuben, and Aomori cassis [5–7].

In addition, the essential oil of the leaves of Aomori hiba showed relatively high antioxidant effect. This essential oil of these leaves contained γ-terpinene and α-terpinene, and these single components (reagents) also showed an antioxidant effect. We consider that the antioxidant effects of these agents will act synergistically.

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青森ヒバ（*Thujopsis dolabrata* SIEB. *et* ZUCC. var. *hondai* MAKINO）の葉の植物化学的プロファイルと抗酸化活性について

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要　　旨

私たちは、青森ヒバの葉の精油を得るために水蒸気蒸留を行った。さらに蒸留湯はヘキサン抽出を行った。精油から22種類の化合物を同定したが、主要成分はsabinene（35.7%）、γ-terpinene（13.5%）、α-pinene（7.7%）、およびβ-myrcene（7.7%）であった。蒸留湯にはterpinen-4-olが非常に多く71.1%含有していた。更に*p*-cymene（4.8%）、terpineol（4.6%）、およびγ-terpinene（2.5%）と続き、27種類の化合物を同定した。

更に、私たちは精油と2、3の成分についてDPPH法により抗酸化試験を行った。その結果、ヒバの葉の精油には強力な抗酸化作用（SC_{50}：50%ラジカル消去能が11%）があることが分かった。