

[Original]

PHYTOCHEMICAL ANALYSIS OF THE LEAF OF THE FUJI APPLE TREE

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Abstract

We obtained volatile oil from the leaf of *Malus pumila*, var domestica CV 'Fuji,' by steam distillation, and extracted floral water (hydrosol) with hexane. We also analysed the aroma component of the leaf using the headspace method (HS method). Analysis of the extracts by flame ionization detector-gas chromatography and mass spectrometer-gas chromatography identified 34 components in the oil, of which phytol (34.2%) and (*E, E*)- α -farnesene (9.5%) were yielded as the principal components. In contrast, 47 components were identified from floral water, of which terpinen-4-ol (30.4%) and linalool (21.0%) accounted for 50% or more. Twenty-two compounds were identified as scent components, including *E*-2-hexenal (48.4%), 1-penten-3-ol (12.4%), and 3-pentanone (7.4%).

Farnesene showed the antioxidant action. In order to shut down free radicals in the body and to prevent lifestyle-related diseases, and arteriosclerosis, I think that such fundamental studies become still more important.

Key words: Fuji apple, essential oil, steam distillation, head-space method, phytol

Introduction

Apple trees belong to the family *Rosaceae* and originated in the Caucasus region of southwestern Europe. The fruit of the apple tree has been developed for human consumption since ancient times, and a 2014 output report by the Japanese Ministry of Agriculture, Forestry, and Fisheries noted that apples are now produced in every country of the world. Production is highest by far in China, while Japan is in 16th place. About 50% of apples produced in Japan come from Aomori Prefecture, and the flower of the apple serves as the prefectural flower. Apple harvesting in Aomori extends from August to November; the most popular variety is the Fuji, a late variety harvested in November which has excellent taste and shelf life. According to 2013 data, Japanese apple production has recently tended to decrease, likely due to delays in fruiting due to unseasonable weather and to the aging of apple farmers [1, 2]. The Standard Tables of Food Composition in Japan, Fifth Revised and Enlarged Edition

list the three major nutrients in apples as sugar, protein, and lipid [3]. Apples also contain a range of minerals, such as potassium, which aid in the maintenance of blood pressure; and dietary fiber, such as apple pectin, which aids in the prevention of constipation. Moreover, apples also contain organic acids, such as citric acid and malic acid, which participate in energy metabolism; and vitamins, which act in a manner akin to bodily lubricating oils. The polyphenol in apple is said to act in preventing obesity and to exert positive health benefits in cholesterol control and blood circulatory flow, and to thereby contribute to the prevention of lifestyle-related diseases [4–15]. The research on the components contained in the apple leaf has also been reported a little [16, 17].

We previously investigated volatile oil components of a range of botanicals, such as lavender and rugosa rose, and reported their antimicrobial activity [18, 19]. Here, we investigated the volatile oil components, floral water, and aroma components of the leaf of the apple tree.

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2. Materials and Methods

2.1. Plant materials

A total of 1531 g of leaves of *Malus pumila*, var domestica CV 'Fuji' were collected at Hirakawa, Aomori Prefecture, Japan in October, 2011. The fresh leaves were cut finely and used for steam distillation. Scent component analysis was carried out by the headspace method using 0.37 g of leaves.

2.2. Reagents

1,1-Diphenyl-2-picrylhydrazyl and butylated dihydroxyanisole were purchased from Tokyo Chemical Industry Co., Ltd. (Japan), and Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), respectively. Common reagents of biochemistry grade or highest quality were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries. 96-well multiwell plates were purchased from Corning International KK (CIKK) (Akasaka, Tokyo).

2.3. Hydrodistillation, extraction and the head-space method

Steam distillation was performed over about 4 to 6 hours. *Ca.* 2 L of floral water was obtained. A 0.013-g (0.00087%) amount of volatile oil was obtained from the leaves. Floral water was then extracted four times with hexane, and yielded 0.021 g (0.0014%) of extract. The leaves were subject to GC-MS analysis by the head-space method and aroma components were analyzed.

2.4. Analysis

Volatile oils and floral water-extracts were analyzed by flame ionization detector-gas chromatography (GC-FID). GC-FID analysis was performed 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 ended 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 × φ 0.32 mm × 0.25 μm film thickness: coated with 5%-phenyl-poly [methylsiloxane]) 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 [20]. 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.

Head-space method

GC-MS measurement of the leaves was done using the head-space method. The aroma component of the leaves was analyzed by GC-FID under the same conditions as in the volatile oils analysis. GC-MS analysis was performed on a JEOL Q1000GC-Mk II equipped with a JEOL 12031 headspace auto sampler, consisting of an AQUATIC capillary column (Agilent, 60 m × φ 0.32 mm, 0.25 μm film thickness) with a 1 ml/min helium carrier flow rate (15 psi). Temperature was programmed to deliver a 10 °C /min gradient initiated with a 3.0-min hold at 40 °C, and then to increase to 170 °C at 10 °C /min, then to 200 °C (12.5 min) at 20 °C /min. The increase was terminated with a 2-min hold at 280 °C. Injector and GC-transfer line temperatures were both set at 200 °C.

Separated components were identified by comparing retention times and mass spectra with laboratory data and the NIST library. The accordance of retention times in the GC-MS was confirmed by co-injecting the reagent with an n-alkane (C₇-C₄₀) retention index. The relative amounts of components were calculated from their respective GC-MS peak areas.

2.5 Antioxidant activity test

Antioxidant action of reagents contained in the leaves of the apple tree was investigated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical elimination as assay [20–22]. 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.

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:

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

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

3.1. Component of essential oil

The components of the essential oil identified by analysis of the leaves of the apple tree are shown in Table 1. The major component of the volatile oil of leaves was phytol, at 34.2%, followed by (*E,E*)- α -farnesene, which is also known as a scent component of the skin of the apple fruit, at 9.5%. Volatile oil containing phytol is known to exert an anti-itching effect in the skin and a relaxant effect [23].

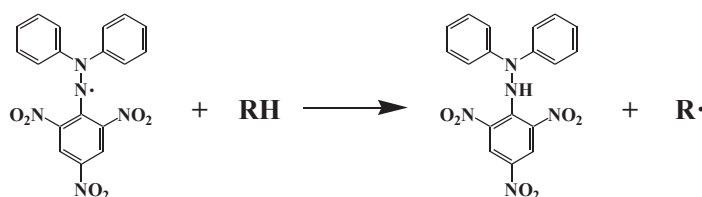


Figure 1. Reaction of DPPH with a test sample (RH).

Table 1. Components of the volatile oil of the apple tree leaves.

No.	RT	RI	compound	rate %	CI	No.	RT	RI	compound	rate %	CI
1	8:37	1099	linalool	0.2	○	19	13:02	1530	calamenene	0.2	-
2	8:41	1104	nonanal	0.1	-	20	13:15	1556	elemol	0.7	-
3	9:37	1179	terpinen-4-ol	0.2	○	21	13:19	1564	nerolidol	0.7	-
4	9:47	1191	α -terpineol	0.1	○	22	13:24	1574	<i>cis</i> -3-hexenyl benzoate	1.8	-
5	9:56	1204	decanal	0.2	-	23	13:27	1580	hexyl benzoate	0.4	-
6	10:50	1289	lavandulyl acetate	1.6	-	24	13:35	1596	caryophyllene oxide	3.3	-
7	11:01	1307	undecanal	0.1	-	25	13:44	1615	cedrol	0.7	-
8	11:33	1363	neryl acetate	0.6	○	26	14:00	1650	τ -cadinol	2.0	-
9	11:44	1382	geranyl acetate	1.0	○	27	14:09	1670	alloaromadendrene oxide	0.1	-
10	11:48	1389	β -damascenone	0.1	-	28	14:17	1687	cadalene	0.2	-
11	11:56	1404	tetrahydrogeranyl acetone	0.2	-	29	14:56	1775	benzyl benzoate	0.2	-
12	11:58	1408	dodecanal	0.2	-	30	15:26	1844	phytone	6.2	-
13	12:09	1428	β -caryophyllene	3.9	○	31	16:08	1945	isophytol	0.9	-
14	12:16	1442	thujopsene	0.6	-	32	16:11	1952	hibaene	1.2	-
15	12:24	1457	β -farnesene	4.6	-	33	16:39	2025	thunbergol	0.3	-
16	12:44	1494	(<i>Z,E</i>)- α -farnesene	1.3	-	34	17:12	2115	phytol	34.2	-
17	12:51	1508	(<i>E,E</i>)- α -farnesene	9.5	-	-	-	-	<i>n</i> -alkane (C ₂₁ -C ₂₈)	2.1	-
18	12:58	1522	γ -cadinene	2.0	-				Total	81.7	

RT: Retention Time,

RI: Retention Index,

CI: Co-injection with authentic sample

3.2. Components of floral water extracts

Analysis of the floral water extract is shown in Table 2. The main components were terpinen-4-ol (30.4%) and linalool (21.0%). Concentration of phytol, which accounted for 30% or more of total volatile oil, was low, at 0.9%. This low concentration in volatile oil suggests that the extract experiment was performed correctly.

3.3. Aroma component of apple tree leaves

Aroma component analysis of the leaves of the apple tree identified 22 compounds (Table 3). (*E*)-2-hexenal concentration was high, at 48.4%. The leaves of the apple tree show a characteristic grassy scent. (*E*)-2-hexenal, called 'green-leaves aldehyde,' is considered to greatly

contribute to this scent. Moreover, minute amounts of (*E,E*)- α -farnesene, which is contained in apple peel, were also identified in the leaves of the apple tree.

3.4. Antioxidative action

Common reagents were selected from leaf components, and their antioxidative actions were investigated (Table 4).

Since farnesene did not dissolve in ethanol but was suspended, its exact absorbance could not be measured and its impact in DPPH radical scavenging was not correctly analyzed. However, the antioxidant action of farnesene could be qualitatively identified by the decolorization of DPPH (Photo. 1).

Table 2. Components of the floral water extracts of apple tree leaves

No.	RT	RI	compound	rate %	CI	No.	RT	RI	compound	rate %	CI
1	6:41	983	1-octen-3-ol	0.2	-	25	10:58	1302	carvacrol	1.0	○
2	6:50	991	6-methyl-5-hepten-2-one	0.1	-	26	11:33	1363	eugenol	0.2	○
3	6:52	993	β -myrcene	0.1	○	27	11:34	1365	neryl acetate	0.8	○
4	7:07	1004	octanol	0.1	-	28	11:45	1384	geranyl acetate	2.1	○
5	7:12	1009	<i>cis</i> -3-hexenyl acetate	0.1	-	29	11:49	1389	β -damascenone	1.2	-
6	7:31	1029	limonene	0.1	○	30	12:05	1421	(<i>E</i>)- β -damascone	0.1	-
7	7:38	1036	2,2,6-trimethyl cyclohexanone	0.1	-	31	12:10	1430	β -caryophyllene	0.1	○
8	7:46	1045	benzenacetaldehyde	0.3	-	32	12:20	1449	coumarin	0.3	-
9	8:14	1075	linalool oxide	4.3	-	33	12:45	1496	(<i>Z,E</i>)- α -farnesene	0.1	-
10	8:38	1099	linalool	21.0	○	34	12:52	1510	(<i>E,E</i>)- α -farnesene	0.5	-
11	8:41	1104	nonanal	0.9	-	35	13:16	1558	elemol	0.9	-
12	8:48	1113	1-octen-3-yl acetate	0.5	-	36	13:20	1566	nerolidol	0.2	-
13	9:09	1141	<i>trans</i> -pinocarveol	0.1	-	37	13:26	1578	<i>cis</i> -3-hexenyl benzoate	1.2	-
14	9:30	1169	lavandulol	1.6	-	38	13:46	1620	cedrol	0.5	-
15	9:37	1179	terpinen-4-ol	30.4	○	39	13:50	1628	cubenol	0.1	-
16	9:43	1187	<i>p</i> -cymen-8-ol	0.5	-	40	14:02	1652	τ -cadinol	1.4	-
17	9:47	1192	α -terpineol	9.0	○	41	14:08	1667	α -eudesmol	0.3	-
18	9:50	1196	methyl salicylate	2.0	-	42	14:10	1672	alloaromadendrene oxide	0.6	-
19	9:57	1206	decanal	0.1	-	43	14:57	1777	benzyl benzoate	0.5	-
20	10:01	1213	<i>cis</i> -verbenone	0.1	-	44	15:27	1847	phytone	0.2	-
21	10:12	1230	nerol	1.1	○	45	16:09	1940	isophytol	tr	-
22	10:29	1256	geraniol	2.6	○	46	16:12	1955	hibaene	0.1	-
23	10:33	1263	carvenone	0.4	-	47	17:14	2146	phytol	0.9	-
24	10:51	1291	lavandulyl acetate	5.4	-				Total	94.4	

tr: trace (< 0.1%)

Table 3. Aroma components of the apple tree leaves.

No.	RT	compound	rate (%)	MS agreement	No.	RT	compound	rate (%)	MS agreement
1	9:20	3-methyl pentanal	1.4	86 %	12	16:14	(<i>E,E</i>)-2,4-hexadienal	0.3	96 %
2	9:34	4,4-dimethyl-2-pentaone	6.1	87 %	13	16:40	3-ethyl-1,5-octadiene	0.1	91 %
3	9:51	1-penten-3-ol	12.4	98 %	14	16:50	2,3-octanedione	0.3	89 %
4	10:22	1-penten-3-one	4.4	97 %	15	17:51	ocimene	0.2	81 %
5	10:28	3-pentanone	7.4	99 %	16	19:08	nonanal	0.5	97 %
6	12:01	5-methyl-1-heptene	0.1	88 %	17	19:26	benzenacetaldehyde	0.1	88 %
7	12:15	<i>cis</i> -2-penten-1-ol	6.2	99 %	18	20:31	<i>n</i> -hexyl butanoate	0.1	86 %
8	12:57	hexanal	5.7	96 %	19	20:36	cucumeraldehyde	0.1	83 %
9	14:10	3-hexen-1-ol	0.2	99 %	20	21:03	decanal	0.2	97 %
10	14:20	isobutyl cyclopentane	5.2	89 %	21	21:24	terpinen-4-ol	0.1	76 %
11	14:33	(<i>E</i>)-2-hexenal	48.4	94 %	22	29:39	(<i>E,E</i>)- α -farnesene	0.8	95 %

RT: retention time

Table 4. Summary of antioxidative activity of reagents

compound	% antioxidant activity			SC ₅₀ /mM
	1.0 mM	10 mM	100 mM	
α -pinene	0	0	5.1	420
β -pinene	0	0	8.3	440
α -terpinene	0	0.6	34.3	180
γ -terpinene	0	22.2	59.1	78
limonene	0	0	3.9	n. c.
<i>p</i> -cymene	0	0	0	n. c.
terpinen-4-ol	0	0	0.7	n. c.
α -terpineol	0	0	0	n. c.
carvacrol	46.7	80.1	86.0	1.1
citronellyl acetate	0	0	13.0	270
β -caryophyllene	0	0	n. d.	n. c.
cedrol	0	0	n. d.	n. c.
coumarin	0	0	0	n. c.
naringenin	11.7	60.4	-	6.5
isophytol	0	0	0	n. c.
butylated hydroxyanisole (positive control)	-	-	-	0.031

SC₅₀: 50% Scavenging Concentration, n.c.: not calculated, n.d.: not detected, -: not tested

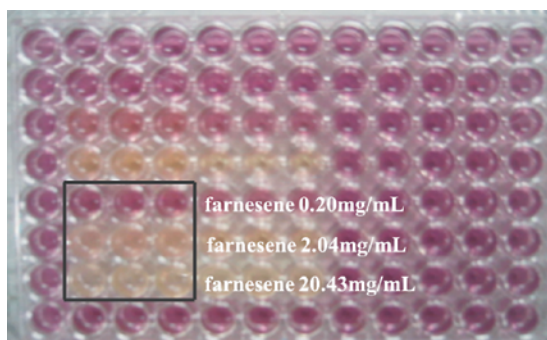


Photo. 1. Antioxidative activity of farnesene

4. Discussion

In this phytochemical study, we obtained the volatile oil from the leaves of *Malus pumila*, 'Fuji apple,' by steam distillation, and extracted floral water with hexane. We also analysed the aroma component of the leaf using the headspace method (HS method). First, results revealed the presence of many substances with antioxidant effects in leaves of this tree, as has also been found in the fruit of the Fuji apple. These findings may point to the possible use of these leaves in cosmetics products.

It is interesting that half of the volatile oil of the leaves of the Fuji apple tree were accounted for by only three or four main components, namely phytol (34.2%), (*E,E*)- α -farnesene (9.5%), phytone (6.2%), and caryophyllene (3.9%). In particular, phytol and phytone are known as precursors of vitamin E and vitamin K, which are known for their antioxidant effect. Moreover, phytol has an emollient effect and has attracted attention for possible use in cosmetics. Investigation of the antioxidative action of farnesene revealed decolorization of DPPH, and its antioxidant activity has now been accepted. It is known that the natural peel of apples is rich in farnesene [22]. Eating the peel of apples may therefore reduce the amount of active oxygen in the human body, protecting cells from oxidative damage and promoting an antiaging effect [23].

Second, terpinen-4-ol (30.4%) and linalool (21.0%) were identified as basic components of floral water extracts. These did not show any antioxidant activity. Finally, (*E*)-2-hexenal, having a characteristic grassy smell, was contained as an aroma component in the apple tree leaf at a concentration as high as 48.4%. Other basic components were penten-3-ol (12.4%) and aldehydes, ketones, and alcohols of C5-C6, as expected.

This study found that the leaves of the Fuji apple tree contain many substances with antioxidant effects, as has already been found with the fruit of this tree. The possible cosmetics use of these compounds warrants consideration.

5. Conclusion

Steam distillation of the volatile oil of the leaves of the 'Fuji' variety of apple tree revealed 34 components, including phytol (34.2%) and farnesene (9.5%). Analysis has shown that phytol, which controls the itchiness of the skin, and farnesene are primarily contained in the peel of the apple fruit as basic components. Forty-seven

alcohol-based components were obtained from floral water, including terpinen-4-ol (30.4%), linalool (21.0%), and α -terpeneol (9.0%). Furthermore, analysis of the scent components of leaves by the headspace method revealed 22 components, including characteristic (*E*)-2-hexenal (48.4%), penten-3-ol (12.4%), and 3-pentenone. To investigate the potential antiaging effects of apple, we also conducted a basic antioxidant assay of the apple tree leaf.

The fruit of the apple tree has been widely studied. We consider that a comprehensive understanding of the apple as a food, and possibly a cosmetic agent, requires basic evaluation of the whole apple tree, including the leaf, stem, root, etc. Our present findings will contribute to understanding of the chemistry of the apple.

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りんご“ふじ”の葉の植物化学的分析

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要 旨

りんご‘ふじ’の葉の水蒸気蒸留を行い製油を得、さらにフローラル・ウォーター（蒸留湯）のヘキサン抽出を行った。精油はGC-MS分析により、主要成分としてフィトール(34.2%)、およびファルネセン(9.5%)等34化合物を同定した。一方、フローラル・ウォーターからテルピネン-4-オール(30.4%)やリナロール(21.0%)をはじめ47種の化合物を同定した。更に、ヘッドスペース法により香気成分として(*E*)-2-ヘキサナール(48.4%)、1-ペンテン-3-オール(12.4%)および3-ペンタノン(7.4%)を含めて22種の化合物を同定した。

ファルネセンには抗酸化作用のあることを確認した。体内のフリーラジカルを遮断し、生活習慣病や動脈硬化を予防するため、このような基礎的研究は重要と考えられる。

キーワード：りんご‘ふじ’、精油、水蒸気蒸留、ヘッドスペース法、抗酸化作用