〔Original〕

Phytochemical analysis of the leaf of the blackcurrant (Aomori Cassis) "*Ribes nigrum* L." and the antioxidant effect of catechins

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Abstract

The volatile oil from the leaf of the blackcurrant (Aomori Cassis) was obtained by hydrodistillation and the floral water (aromatic distilled-water) was extracted with hexane. Analysis of the components by flame ionization detector-gas chromatography and mass spectrometer-gas chromatography identified 39 components in the oil, of which sabinene (32.9%), *cis*-, *trans*-ocimenes (15.3%), and 3-carene (11.6%) were the major. In contrast, principal components of the floral water of black currant were terpinen-4-ol (41.0%), followed by β-caryophyllene (7.8%) and 3-carene (5.9%).

Investigation of the antioxidant effects of five kinds of catechins revealed strong antioxidant activity, suggesting that these might be suitable for development as radical scavengers.

Key words : black currant (Aomori Cassis); essential oil; hydrodistillation; antioxidant activity; radical scavenger

Introduction

The black currant "*Ribes nigrum* L.", also called cassis, is grown across Northern Europe, Russia, the United States and New Zealand, among others. Despite estimations that Russian production is 1.5 times more than that of Poland, Russia has not formerly presented production data, and the largest producer is therefore considered to be Poland. In Japan, Aomori Prefecture accounts for 90 percent of production [1–3]. "Aomori Cassis" is the first geographical indication registration in Japan (registered December 22, 2015), and this name is protected as intellectual property by the geographical indication (GI) protection system, such that the origin of the product can be pinpointed from the name by GI [4, 5].

Cassis belongs to the Rosales currant family, which is a characterized by a refreshing acidity and aroma. This family also includes strawberries and blueberries. Cassis contains many polyphenols, anthocyanins, catechins, vitamins, and minerals, and is used in jam, jelly, sweets, and liqueurs, etc. In general, the polyphenols of anthocyanins and catechins contained in cassis promote blood flow in the peripheral blood vessels, and some reports have described efficacy against eye strain [6–14]. Additional properties include an antibacterial effect, antiviral activity, and pollinosis, and some reports note that these effects are beneficial to health [15–22].

We recently conducted componential analyses of the leaves of the main fruit trees grown in Aomori Prefecture, including apple and Steuben grape, and reviewed their biological activity [23, 24]. We also reported the essential oil components and antimicrobial activity of the rugosa rose, which also grows in Aomori Prefecture [25, 26]. Here, we report our analysis of the leaf of the "Aomori cassis", and then compare its essential oil components with previously reported results for the apple and Steuben grape.

Numerous studies have reported that the polyphenols of anthocyanin and catechin found in black currants have beneficial health effects [6–22]. Based on our marked interest in the biological activity of catechins, and we also the investigated antioxidant actions of these polyphenols.

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2. Materials and Methods

2.1. Plant materials

Steam distillation of 1,123 g of the leaves of black currant in May 2011 yielded 0.75 g (0.067%) of essential oil.

2.2. Reagents

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

2.3. Hydrodistillation and extraction

Steam distillation of black currant leaves was carried out over 4 hours. The yield of essential oil (upper layer) was 0.75 g. Two liters of distillation water (floral water) was obtained. The floral water was then extracted with hexane and 0.035% (0.39 g) of the extract was obtained.

2.4. Analysis

Essential oils and floral water 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 ending upon reaching at 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-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 [27]. 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. Antioxidant activity test

We investigated the antioxidant effect using reagents of the catechins. Antioxidant assay was performed according to a previously reported DPPH radical elimination method [24].

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 radical 50% scavenging effect (SE_{50}) was obtained according to a similar method reported previously. BHA (butylated dihydroxyanisole) was used as positive control.

3. Results and Discussion

3.1. Components of essential oil

Components of the essential oil identified by analysis of the leaf of black currant (Aomori Cassis) "*Ribes nigrum*" are shown in Table 1.

Essential oil of the leaves of black currant are most likely to contain sabinene, at 32.9% of total essential oil components. Other components include 3-carene (11.6%), *cis*- and *trans*-ocimene (9.5% and 6.8%, respectively), and β-caryophyllene (9.1%). In addition, monoterpenes accounted for 80.1% of terpenes.

Table 1. Essential oil components of the leaves of black currant

No.	RT	RI	compound	rate %	CI	No.	RT	RI	compound	rate %	CI
1	5:36	937	α-pinene	1.0	○	22	12:21	1451	aromadendrene	0.1	-
2	6:30	976	**sabinene**	32.9	-	23	12:29	1466	α-caryophyllene	1.7	-
3	6:53	993	β-myrcene	2.1	○	24	12:33	1474	alloaromadendrene	0.4	-
4	7:05	1002	α-phellandrene	0.2	-	25	12:44	1494	2-isopropyl-5-methyl-9-methylene[4.4.0]dec-1-ene	0.1	-
5	7:11	1008	**3-carene**	11.6	-						
6	7:19	1017	α-terpinene	1.7	○	26	12:52	1510	eremophilene	4.2	-
7	7:31	1029	β-phellandrene	3.0	-	27	13:00	1526	γ-cadinene	0.2	-
8	7:42	1041	***cis*-ocimene**	9.5	-	28	13:04	1534	δ-cadinene	0.9	-
9	7:52	1052	*trans*-ocimene	6.8	-	29	13:12	1550	cada-1,4-dine	tr	-
10	8:01	1061	γ-terpinene	3.1	○	30	1317	1560	elemol	0.2	-
11	8:27	1088	α-terpinolene	7.9	-	31	13:23	1572	germacrene B	0.6	-
12	9:38	1180	terpinen-4-ol	0.3	○	32	13:27	1580	denderalasin	tr	-
13	10:30	1258	linalyl acetate	tr	○	33	13:33	1592	spathulenol	tr	-
14	10:51	1290	bornyl acetate	tr	-	34	13:46	1620	cedrol	0.2	-
15	11:22	1344	δ-elemene	0.1	-	35	13:58	1646	γ-eudesmol	tr	-
16	11:35	1367	neryl acetate	tr	○	36	14:03	1657	τ-cadinol	0.3	-
17	11:45	1384	copaene	0.1	-	37	14:09	1670	δ-cadinol	0.4	-
18	11:54	1400	β-elemene	0.2	-	38	14:21	1696	α-bisabolol	tr	-
19	12:05	1421	α-gurjunene	tr	-	39	16:13	1958	hibaene	tr	-
20	12:11	1432	**β-caryophyllene**	9.1	○						
21	12:18	1445	thujopsene	0.1	-				Total	99.0	

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

3.2. Components of floral water extract

Analysis of the floral water extract is shown in Table 2. The main component was terpinen-4-ol (41.2%). Other basic components were β-caryophyllene (7.8%), 3-carene (5.9%), and sabinene (5.3%). Thirty-eight components were identified in floral water.

In view of the structural formulas, the main component of the essential oil of the leaf is sabinene. The main component of the floral water extract was terpinen-4-ol, which is likely secondarily derived from sabinene during steam distillation, as described by Cornwell et al. [28]. The predicted production of terpinen-4-ol in steam distillation is shown in Fig. 1.

To date, we have used steam distillation to report the essential oil and floral water of rugosa rose [25], apple [24], Steuben grape [23] and black currant. All four of these plants are grown in Aomori Prefecture. Components of these four types of plants were identified and their features were observed. Yields of their essential oils, obtained by steam distillation, and of floral water extract are shown in Table 3. The flower and leaves of rugosa rose and the leaves of Steuben grape and apple contained very few essential oil components. Indeed, the leaves of Steuben grape yielded almost no essential oil at all.

Table 2. Components of floral water extracts of the leaves of black currant

No.	RT	RI	compound	rate %	CI	No.	RT	RI	compound	rate %	CI
1	5:30	933	α-thujene	0.2	-	21	11:46	1386	copaene	0.1	-
2	5:38	938	α-pinene	0.4	○	22	11:55	1400	β-elemene	0.2	-
3	6:31	977	**sabinene**	**5.3**	-	23	11:58	1408	jasmone	0.1	-
4	6:54	993	β-myrcene	1.1	○	24	12:12	1434	**β-caryophyllene**	**7.8**	○
5	7:07	1004	α-phellandrene	0.4	-	25	12:22	1453	aromadendrene	0.1	-
6	7:13	1011	**3-carene**	**5.9**	-	26	12:30	1468	α-caryophyllene	1.6	-
7	7:20	1018	α-terpinene	1.7	○	27	12:34	1475	alloaromadendrene	0.5	-
8	7:28	1026	*p*-cymene	0.3	○	28	12:52	1510	eremophilene	2.8	-
9	7:32	1031	β-phellandrene	2.1	-	29	13:01	1528	γ-cadinene	0.3	-
10	7:43	1042	***cis*-ocimene**	**5.3**	-	30	13:04	1534	δ-cadinene	1.0	-
11	7:53	1053	*trans*-ocimene	4.0	-	31	13:18	1562	elemol	0.4	-
12	8:02	1062	γ-terpinene	4.6	○	32	13:24	1574	germacrene B	0.6	-
13	8:28	1089	α-terpinolene	4.9	-	33	13:34	1594	spathulenol	0.3	-
14	8:39	1101	linalool	0.7	○	34	13:41	1609	viridiflorol	0.1	-
15	9:34	1175	ethyl benzoate	0.2	-	35	13:47	1622	cedrol	0.3	-
16	9:39	1181	**terpinen-4-ol**	**41.2**	○	36	13:59	1648	γ-eudesmol	tr	-
17	9:49	1195	α-terpineol	1.5	○	37	14:03	1657	τ-cadinol	0.8	-
18	10:00	1211	*trans*-piperitol	0.1	-	38	14:10	1672	δ-cadinol	1.3	-
19	10:31	1259	geraniol	0.1	○						
20	11:00	1305	carvacrol	0.1	○				Total	98.4	

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

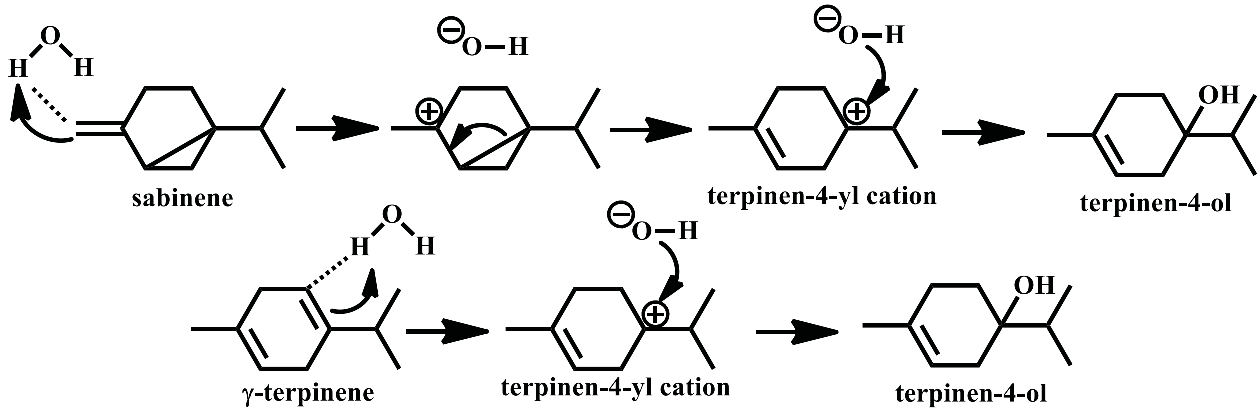

Fig. 1. Predicted production of terpinen-4-ol during steam distillation (Prediction: hydration reaction)

Table 3. Yield of essential oil and floral water extract of four plants grown in Aomori Prefecture

Plant	part	essential oil yield (%)	extraction yield of floral water (%)
Rugosa rose	flower	0.0058	0.054
	leaf	0.062	0.049
Blackcurrant	leaf	0.067	0.035
Grape (Steuben)	leaf	0	0.0075
Apple (Fuji)	leaf	0.00087	0.0014

Essential oil from the flower of rugosa rose contained citronellyl acetate at 12.1% and citronellol at 7.7%. Moreover, the floral water extracts also contained citronellol, at 31.1%, and showed a characteristic scent. The floral water extracts yielded linalool (3.9%), which has a sweet scent and which is also contained in geraniol (4.2%) and lavender. These are typically associated with the scent of rose.

Phytol content was highest in essential oil from the leaves of the apple, at 34.2%. The essential oil also contained (*E, E*)-α-farnesene (9.5%) a scent component found in the epidermis of the apple. Components extracted from the floral water of the apple included terpinen-4-ol at 30.4% and linalool at 21.0%.

τ-Cardinol concentration (8.1%) was highest in the leaves of the Steuben grape τ-Cardinol is reported to have calcium antagonistic activity and has attracted interest in this field.

On the other hand, the main component of the essential oil and floral water from the leaves of the black currant was sabinene.

3.3. Anti-oxidant activity test

The present and previous studies widely suggest that the polyphenols of anthocyanin or catechin contained in the black currant are beneficial for health [6–22].

Since we targeted the analysis of volatile components, we did not isolate polyphenols. However, due to our interest in the biological activity of catechins, we also examined their anti-oxidant effects, with a particular focus on the antioxidant effect of catechins in polyphenols.

The results are summarized in Table 4 and depicted in Photos 1–5. The structure of the catechins used is shown in Fig. 2.

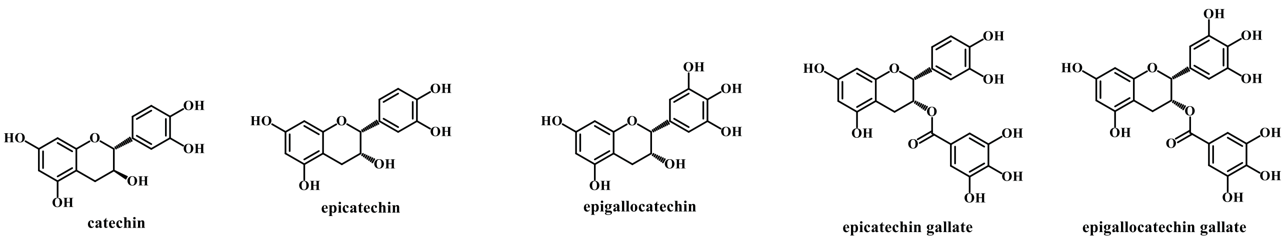

Fig. 2. Structures of catechins

Table 4. Antioxidant effect of catechins.

Radical Scavenging Effect (%)

	0.001mM	0.01mM	0.1mM	SE_{50}(μg/ml)
(+)-catechin	12.4%	60.8%	89.7%	6.6
(−)-epicatechin	58.4%	87.5%	87.1%	16.0
(−)-epigallocatechin	83.6%	85.1%	86.1%	2.1
(−)-epicatechin gallate	78.9%	86.3%	86.6%	1.9
(−)-epigallocatechin gallate	8.6%	47.6%	85.4%	2.4

SE_{50}: 50% Scavenging Effect

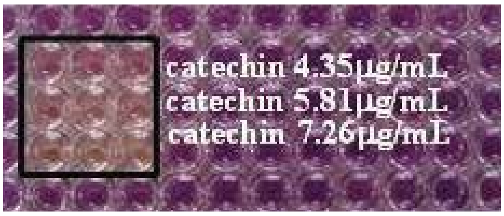

Photo 1. Antioxidant acitivity of catechin

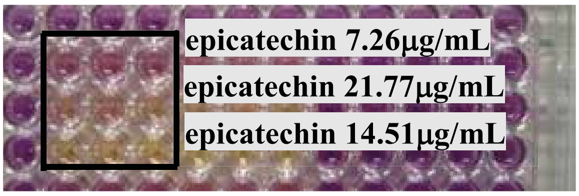

Photo 2. Antioxidant acitivity of epicatechin

Photo 3. Antioxidant acitivity of epigallocatechin

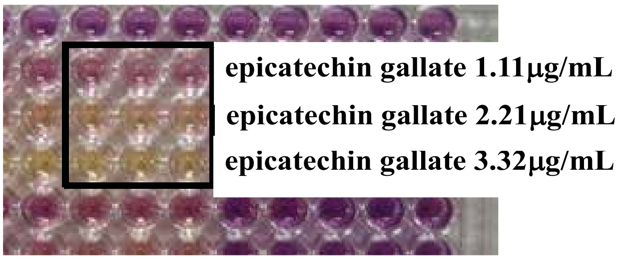

Photo 4. Antioxidant acitivity of epicatechin gallate

Photo 5. Antioxidant acitivity of epigallocatechin gallate

β-Farnessene is contained in the essential oil of the leaf of the apple, as reported previously [24]. As shown in the photos and Table 4, however, five kinds of catechin (catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate) showed high antioxidant activity. In particular, the 50% Scavenging Effect (SE_{50}) of epicatechin gallate was 1.9 μg/ml, indicating very strong antioxidant activity (Table 4). Free radicals damage biological molecules, leading to cell necrosis and apoptosis. This damage is a major cause of certain types of cancer, Alzheimer's disease, Parkinson's disease, and the degenerative conditions of aging [29–33]. Usually, certain enzymes such as superoxide dismutase have the action of inactivating active oxygen. Vitamins, glutathione, cysteine, β-carotene, CoA-SH, and uric acid are also known to have antioxidant properties. An increase in radical production or free radical concentration will result in oxidative damage to various cellular components, such as mitochondria. Consumption of supplements and foods with antioxidant properties as a source of radical scavengers is therefore usually considered important.

4. Conclusion

Aomori Prefecture accounts for 90% of total Japanese production of black currants, as well as 60% of apples and 70% of Steuben grapes.

Comparing the essential oil components of these three kinds of fruit, the highest concentration is generally seen for phytol (34.2%), a diterpene alcohol found in apples. Phytol is also known as a precursor of vitamins E and K.

The Steuben grape yielded only trace amounts of essential oil.

In stark contrast to the apple, sabinene accounted for 32.9% of the essential oil of black currants. On the other hand, terpinen-4-ol was the most common component in the floral water of apples and black currants, and was third-most common in that of Steuben grapes. Overall, strong polar alcohols are the major components of these floral waters.

Oxidative stress is a major cause of not only degenerative diseases but also of aging and lifestyle-related disease. Inactivation of radical species by an antioxidant effect is desirable for both disease prevention and anti-aging.

The accumulation of such basic antioxidant experiments will contribute to future studies in chemistry.

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青森カシス“*Ribes nigrum* L.”の葉の植物化学的分析および カテキン類の抗酸化作用について

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要　　旨

青森カシスの葉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。成分はGC-MS（ガスクロ・マススペクトル）分析を行い、精油から39種類の化合物を同定した。その主成分は、サビネン（32.9%）、*cis/trans*-オシメン（15.3%）および3-カレン（11.6%）であった。それに対し、蒸留湯（フローラル・ウォーター）の主成分はテルピネン-4-オール（41.0%）、β-カリオフィレン（7.8%）、および3-カレン（5.9%）と続き、38種の化合物を同定した。

後半では、5種類のカテキン類の抗酸化作用について調査した。いずれも強力な抗酸化作用を示し、十分なラジカル消去能を有することが確認された。