付表
IET No. :
検体名 : エレミ樹脂
機関名 : 神奈川県衛生研究所
動物 : マウス/ddY /雄/8週齢/経口投与

mg/kg/day	SCHEDULE	MOUSE	MNPCE : %	PCE/(PCE+NCE):%	BW:g	CODE
0	×2, 24hr	E0-1	0.15	61.4	36.0	16187
		E0-2	0.10	66.2	38.6	16164
Vehicle		E0-3	0.15	62.4	38.2	16119
(olive oil)		E0-4	0.25	59.8	40.3	16109
		E0-5	0.05	55.2	42.7	16140
		Mean	0.14	61.0	39.2	
		Std	0.07	4.0	2.5	
		Min	0.05	55.2	36.03	
		Max	0.25	66.2	42.7	
		Total No.	14			
500	×2, 24hr	EL-1	0.30	56.4	36.5	16108
		EL-2	0.15	54.8	37.4	16163
		EL-3	0.05	65.2	39.5	16130
		EL-4	0.25	61.4	39.1	16181
		EL-5	0.00	62.2	40.0	16116
		Mean	0.15	60.0	38.5	S^K
		Std	0.13	4.3	1.5	
		Min	0	54.8	36.48	
		Max	0.30	65.2	40.0	判定
		Total No.	15			−
1000	×2, 24hr	EM-1	0.25	63.6	35.8	16106
		EM-2	0.15	53.6	37.1	16149
		EM-3	0.10	53.4	37.4	16125
		EM-4	0.10	63.8	37.7	16165
		EM-5	0.20	54.2	41.5	16194
		Mean	0.16	57.7	37.9	S^K
		Std	0.07	5.5	2.1	
		Min	0.1	53.4	35.83	
		Max	0.25	63.8	38.7	判定
		Total No.	16			−
2000	×2, 24hr	EH-1	0.15	61.4	36.6	16114
		EH-2	0.25	63.8	38.4	16178
		EH-3	0.05	57.2	37.8	16167
		EH-4	0.10	61.4	38.6	16182
		EH-5	0.25	61.2	40.2	16121
		Mean	0.16	61.0	38.3	S^K
		Std	0.09	2.4	1.3	
		Min	0.05	57.2	36.58	
		Max	0.25	63.8	40.2	判定
		Total No.	16			−
MMC 2	×1, 24hr	PC-1	3.10	56.0	35.7	16150
(i.p.)		PC-2	2.85	49.8	35.7	16173
		PC-3	2.70	56.8	37.3	16110
		PC-4	4.35	61.8	40.0	16133
		PC-5	3.40	59.8	39.1	16146
		Mean	3.28	56.8	37.6	χ^2
		Std	0.65	4.6	2.0	
		Min	2.70	49.8	35.7	
		Max	4.35	61.8	40.0	判定
		Total No.	328			++

B.W. : Body weight at the 1st day of dosing.
MNPCE : Frequency of micronucleated polychromatic erythrocytes.
PCE/(PCE+NCE) : Ratio of polychromatic erythrocytes to total erythrocytes.
S^K :Kastenbaum-Bowmanの数表による検定
χ^2 : カイ二乗検定による検定 ($p<0.01$)
MMC : Mitomycin C

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yashiro, T., Sugimoto, N., Sato, K., Yamazaki, T., Tanamoto, K.	Analysis of Absinthin in Absinth Extract Bittering Agent	Japanese Journal of Food Chemistry	11	86- 90	2004

Regular article

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Analysis of Absinthin in Absinth Extract Bittering Agent

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Abstract

The constituents of absinth extract product, a natural bitter flavoring, were investigated as a part of an ongoing study to evaluate its quality and safety as a food additive. Two constituents, namely absinthin and anabsinthin were isolated. The concentration of absinthin, the main bitter constituent, was 2.0% in the absinth extract product. It was also confirmed that the origin of the product was the aerial part of *Artemisia absinthium* L. (Compositae), as determined by comparing TLC and HPLC profiles of the product and 50% ethanol extract prepared from the aerial part of *A. absinthium*.

Key words: natural bittering agent; absinth extract; *Artemisia absinthium* L.; absinthin; anabsinthin

I. Introduction

Artemisia absinthium L. (Japanese name: Nigayomogi), a perennial plant belonging to Compositae family distributed throughout Europe and Siberia, is known as wormwood, and used for anthelmintic, antimalarial, gastric and tonic effects. The aqueous or ethanol extract of *A. absinthium* is called absinth extract or wormwood extract (Japanese name: nigayomogi extract) and is used as a natural bittering agent for alcoholic or non-alcoholic beverages, because of its bitter taste and fragrance. The List of Existing Food Additives in Japan[1] stipulates that absinth extract is a substance composed mainly of absinthin (1)[2] from the whole plant of *A. absinthium*.

To date, the constituents of absinth extract as a food additive have not been fully clarified. In this paper, the main bitter constituent and other dimeric sesquiterpenes in absinth extract are investigated, to contribute to an ongoing comprehensive safety evaluation of food additives by the Japanese Ministry of Health, Labor and Welfare.

II. Materials and Methods

1. Sample and chemicals

Absinth extract product prepared as a 50% EtOH solution, the commercially available product in the Japanese market, was supplied by San-Ei Gen F.F.I. Co. Ltd. for safety evaluation. The dry leaves of *A. absinthium* was purchased from an internet shop specializing in herbal tea, e-tisanes (the web site: www.rakuten.co.jp/e-tisanes/). All chemicals were of reagent grade, and were used without further purification. Silica gel 60 F_{254} (20 cm x 20 cm, Art. 1.05715) (Merck Co., Ltd.) and RP-18WF_{254s} (10 cm x 10 cm, Art.13124) (Merck) were used for TLC. Silica gel 60 F_{254} (20 cm x 20 cm, Art. 1.05744) (Merck) was used for preparative TLC. Silica gel 60 (70-230 mesh Art. 1.07734 (Merck)) and ODS (200-350 mesh, Chromatorex ODS (Fuji Silica Chemical Ltd.)) were used for open column chromatography.

2. Spectroscopic analysis

NMR spectra were recorded on a JNM-ECA (600 MHz and/or 800 MHz) (JEOL Co. Ltd.) with chloroform-*d* as the solvent. Spectra were referenced internally to tetramethylsilane (TMS) in ^{1}H-NMR and to the solvent in ^{13}C-NMR. Assignments of the proton and carbon signals of all isolated compounds were confirmed by pulse field gradient (PFG) heteronuclear multiple quantum coherence (HMQC) and PFG heteronuclear multiple bond connectivity (HMBC) experiments. Fast atom bombardment mass spectrometry (FAB-MS) spectra were performed using a JMS-700 (JEOL) mass spectrometer in the positive and negative modes.

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3. Isolation of compounds 1 and 2 from absinth extract product

Absinth extract product (20 mL) was dissolved in water, and the solution was partitioned with chloroform ($CHCl_3$), affording a $CHCl_3$-soluble part (1.29 g). The $CHCl_3$-soluble part was fractionated subsequently on a Silica gel column by eluting successively with $CHCl_3$ - acetone (each 200 mL of 19 : 1, 8 : 2 and 5 : 5, 100 mL of 0 : 10) and $CHCl_3$ - MeOH (each 100 mL of 5 : 5 and 0 : 10) with monitoring by TLC. The eluates were concentrated *in vacuo*, affording eleven fractions (Fr. 1~11). Then, half of Fr. 5 was fractionated on an ODS column with MeOH - water (3 : 2) with monitoring by TLC, affording crude compounds **1** (36 mg) and **2** (23 mg). The crude compound **1** was developed on preparative TLC with toluene - $CHCl_3$ – ethylacetate (AcOEt) (3 : 5 : 12) to give compound **1** (15 mg) as colorless needles. The crude compound **2** was re-crystallized with hexane-acetone to give colorless needles of compound **2** (5 mg). ^{1}H- and ^{13}C-NMR data of compounds **1** and **2** are shown in Table 1.

4. Preparation of sample solutions of TLC and HPLC analyses

Absinth extract product (10 μL) was diluted with 50% ethanol (EtOH) (990 μL) and the solution was used as the sample solution of the product. The dry leaves of *A. absinthium* (5.0 g) were extracted with 50% ethanol (EtOH) (100 mL) for 3 days, and then 10 mL of the solution was filtered and the filtrate was evaporated *in vacuo*. The remaining residue was dissolved in 2.0 mL of 50% EtOH and the solution was filtered through a Millex 0.45 μm filter (Millipore Co.). The filtrate was used as the sample solution of 50% EtOH extract from *A. absinthium*.

5. TLC and HPLC analyses

RP-18 TLC was developed with MeOH-water (3:2) as a solvent system. The spots were detected after spraying with 10% sulfuric acid (H_2SO_4) in MeOH and gentle heating. The HPLC system (Waters Co. Ltd.) consisted of an Alliance 2965 LC system with a 2996 Photodiode Array detector (PDA). HPLC conditions were as follows: column, Atlantis^{TM} dC_{18} (2.1 x 150 mm, 3 μm) (Waters); flow rate, 0.2 mL/min; mobile phase, 50% MeOH (0 min)→90% MeOH (30min); injection volume, 10 μL. The on-line PDA detector monitored between 191 and 600 nm. The quantity of compound **1** was determined by using an absolute calibration curve to peak area at UV 210 nm of compound **1** isolated from absinth extract product.

III. Results and Discussion

1. Structures of compounds 1 and 2

The absinth extract product was fractionated *via* silica gel and ODS column chromatography, and finally purified by preparative TLC, affording compounds **1** and **2** mainly. The structures of compounds **1** and **2** were identified on the basis of their spectral data.

Compound **1** was obtained as colorless needles. The molecular formula was determined as $C_{30}H_{40}O_6$ (MW = 496), so the FAB-MS data of compound **1** indicated molecular related ion peaks at *m/z* 519 $[M+Na]^+$, 497 $[M+H]^+$ and 479 $[M-H_2O+H]^+$ along with the reverse Diels-Alder product ion peak at *m/z* 249 $[M/2+H]^+$. The ^{13}C-NMR spectrum showed two carboxyl groups (-COO) (δ 178.50, 178.88) and four olefinic signals (δ 122.16, 134.94, 147.72, 148.44). The results suggested that compound **1** consisted of a Diels-Alder adduct of two guaianolide-type sesquiterpenes such as artabsin[3] ($C_{15}H_{20}O_3$, MW = 248). The assignments of the proton signals of compound **1** were based on ^{1}H-^{1}H COSY. The ^{1}H- and ^{13}C-NMR data of compound **1** were compared to previously reported data[2, 3, 4] for constituents of *A. absinthium*. From this comparison, it was concluded that compound **1** was absinthin[2], the main bitter constituent of absinth extract (Fig. 1).

Compound **2** was also obtained as colorless needles. The protonated molecular ion peak at *m/z* 497 $[M+H]^+$ was only observed by FAB-MS, suggesting that compound **2** has the same molecular formula as absinthin (**1**). Its ^{13}C-NMR spectrum showed two carboxyl groups (-COO) (δ 178.58, 179.08) and two olefinic signals (δ 132.35, 148.43). By comparison of ^{13}C-NMR data between absinthin (**1**) and compound **2**, it was predicted that the olefinic carbons (δ 122.16, 147.72) at C-3 and C-4 of absinthin (**1**) were replaced with a methylene carbon (δ 34.69) at C-3 and a methine carbon (δ 88.35) bearing an oxygen atom at C-4. Furthermore, the ^{1}H and ^{13}C-NMR data of **2** were identical to these of anabsinthin, which is known as a cyclized derivative of absinthin (**1**) in acidic medium[4] (Fig. 1).

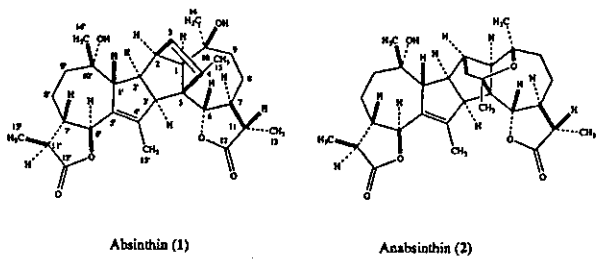

Fig.1 Structures of absinthin (**1**) and anabsinthin (**2**)

2. TLC and HPLC analysis of absinth extract

50% EtOH extract prepared from the dry leaves of *A. absinthium*, and the TLC and HPLC profiles of 50% EtOH extract were compared with these of absinth extract product.

In Fig. 2, the RP-18 TLC profiles of the absinth extract product, 50% EtOH extract of *A. absinthium*, and the isolated dimeric guaianolides absinthin (**1**) and anabsinthin (**2**) are illustrated. The TLC profiles of absinth extract product and 50% EtOH extracts

were very similar. Several spots were observed on the TLC, and two brown spots of absinthin (**1**) and anabsinthin (**2**) were observed at Rf 0.29 and 0.23, respectively, with the tailing spot after spraying with H_2SO_4 and gentle heating. The spot of absinthin (**1**) was the most intense one.

Fig. 3 shows the HPLC profiles of absinth extract product and the 50% EtOH extract prepared from the leaves of *A. absinthium*. The peak patterns between 7.0 and 20.0 min of absinth extract product and 50% EtOH extract were very similar, though 50% EtOH extract showed a large peak before 7.0 min. Therefore, on the results of TLC and HPLC analysis, it was confirmed that the origin of the commercial product was *A. absinthium*. Peak 1 at 14.0 min and peak 2 at 17.5 min were derived from absinthin (**1**) and anabsinthin (**2**), respectively, as proven by injections of isolated absinthin (**1**) and anabsinthin (**2**). Anabsinthin (**2**) was observed as a very small peak on the HPLC, though anabsinthin (**2**) was observed clearly on the TLC. The reason is that the absorption of anabsinthin (**2**) at UV 210 nm is less than that of absinthin (**1**). Other peaks were also observed on the HPLC, and these peaks were thought to be sesquiterpenes. However, we could not identify the structures because they were decomposed in the process of separating these peaks by preparative HPLC.

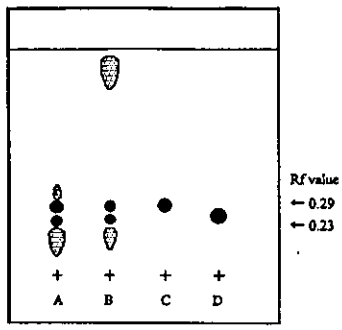

Fig. 2 RP-18 TLC profiles of absinth extract product and 50% ethanol extract from the dry leaves of *A. absinthium*.
A) absinth extract product.
B) 50% EtOH extract prepared from the dry leaves of *A. absinthium*.
C) absinthin (**1**).
D) anabsinthin (**2**).
Solvent: MeOH : water = 3 : 2.
Spots visualized with $H_2SO_4/\triangle$.

Table 1. NMR (δ , $CDCl_3$) signal assignment of absinthin (**1**) and anabsinthin (**2**)

	Absinthin (**1**)			Anabsinthin (**2**)		
position	carbon	proton		carbon	proton	
1	71.50	2.16	br s	62.99	2.35	br s
2	45.75	2.83	br s	41.26	2.19	br s
3	122.16	5.54	s	34.69	1.48, 1.67	
4	147.72	-		88.35	-	
5	64.25	-		62.13	-	
6	82.73	4.71	d, J = 10.1 Hz	82.33	4.14	d, J = 10.3 Hz
7	46.60	1.81		49.20	1.77	
8	27.59	1.86		25.61	1.52, 1.76	
9	43.81	1.88		39.21	1.45, 1.79	
10	74.21	-		77.85	-	
11	42.39	2.23		42.50	2.23	
12	178.50	-		179.08	-	
13	13.19	1.23	d, J = 6.9 Hz	12.10	1.21	d, J = 7.2 Hz
14	29.44	1.17	s	27.08	1.26	s
15	13.81	1.76	s	16.99	1.19	s
1'	57.16	2.26	br s	56.69	2.53	br s
2'	46.82	2.79	m	43.20	2.70	br d, J = 9.6 Hz
3'	58.93	3.18	d, J = 8.2 Hz	52.49	3.47	d, J = 10.2 Hz
4'	134.94	-		132.35	-	
5'	148.44	-		148.43	-	
6'	81.43	4.57	d, J = 11.0 Hz	81.02	4.66	d, J = 11.0 Hz
7'	49.45	1.69		49.45	1.85	
8'	23.68	1.47		23.57	1.53, 1.79	
9'	42.56	1.65		43.88	1.57, 1.87	
10'	72.02	-		74.36	-	
11'	42.14	2.18		42.71	2.24	
12'	178.88	-		178.58	-	
13'	12.29	1.19	d, J = 6.9 Hz	12.38	1.22	d, J = 6.9 Hz
14'	32.37	1.29	s	29.43	1.27	s
15'	18.41	1.92	s	16.66	1.94	s

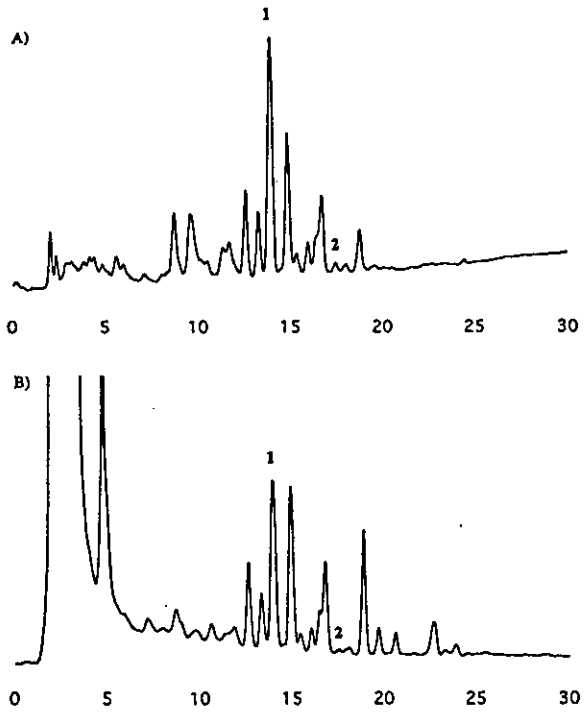

Fig. 3 HPLC profiles of absinth extract product and 50% ethanol extract from the leaves of *A. absinthium*.
A) absinth extract product.
B) 50% EtOH extract prepared from the leaves of *A. absinthium*.
absinthin (**1**). anabsinthin (**2**).

In order to quantify absinthin (**1**), a calibration curve (correlation coefficient r = 0.999) for the peak area against the quantity injected was prepared for absinthin (**1**) within the range of 0.125-0.5 mg/ml, with a retention time for absinthin (**1**) of 14.0 min. The concentration of absinthin (**1**) in absinth extract product was found to be 2.0%.

The sample used in this report, had already been tested in a 13-week repeated dose toxicity study in rat by another group[5], and they concluded that the NOAEL (no-observed-adverse-effect-level) of the extract product in Wistar Hannover rats was estimated to be 2% (equivalent to 1.27 g/kg/day in males and 2.06 g/kg/day in females) or more. It is very important for the safety evaluation of food additives that the analysis of the constituents and their toxicity are carried out using the same sample, since the contents of the constituents in natural food additives may differ depending on the extraction method, processing method, and collection season of the origin plant.

IV. Conclusion

Absinth extract is used as a natural bitter flavoring in Japan. This report is the first investigation of the constituents of commercial absinth extract product. Based on TLC and HPLC analysis, we confirmed that the origin of the absinth extract product is *A. absinthium*, as stipulated in the List of Existing Food Additives in Japan. Two constituents, absinthin and anabsinthin, were isolated from absinth extract product. The content of absinthin, the main bitter constituent, was 2.0% in the extract product.

V. Acknowledgments

The authors are grateful to San-Ei Gen F.F.I. Co., Ltd. for supplying absinth (wormwood) extract. This work was supported by a Grant-in-Aid for Reseach on Food sanitation from the Ministry of Health, Labor and Welfare.

VI. References

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2) Beauhaire, J., Fourrey, J. L., Vuilhorgne, M.: Dimeric sesquiterpene lactones : structure of absinthin, Tetrahedron Lett., 21, 3191-3194 (1980).
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論　　文

天然苦味料ニガヨモギ抽出物中の主成分アブシンチンの分析

(2004年5月6日受付)
(2004年7月27日受理)

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国立医薬品食品衛生研究所

キーワード：天然苦味料, ニガヨモギ抽出物, ニガヨモギ(*Artemisia absinthium* L.), アブシンチン, アナブシンチン

概　要

天然苦味料ニガヨモギ抽出物は、既存添加物収載品目リストにその基原・製法・本質として、「キク科ニガヨモギ(*Artemisia absinthium* L.)の全草より、水又は室温時エタノールで抽出したものである。主成分はセスキテルペン(アブシンチン(absinthin)等)である。」と記載されているが、天然苦味料としての本抽出物の成分組成について十分に検討された例はない。そこで、ニガヨモギ抽出物中の主成分アブシンチンの有無を確認するとともにその分析法について検討した。ニガヨモギ抽出物製品をシリカゲルおよびODSオープンカラムクロマトグラフィーに付し、分画を繰り返し、化合物1および2を得た。NMRおよびFABMSによる解析の結果、化合物1がニガヨモギの主成分とされるアブシンチン、化合物2がセスキテルペン二量体の1つであるアナブシンチンと同定した。ニガヨモギ抽出物製品より単離・精製したアブシンチンを用いて本抽出物製品中のアブシンチンをHPLCにより定量した結果、製品中に2.0%含まれることを明らかとした。また、基原植物とされるニガヨモギ(*A. absinthium*)の地上部を50% EtOHで抽出し、その抽出物とニガヨモギ抽出物製品をTLCおよびHPLCによって比較した結果、ほぼ等しいパターンを示したことから、本抽出物製品が、既存添加物収載品目リストの記載の通り、ニガヨモギを基原植物としていることが確認できた。

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