Table 7. Histopathology of female rats treated with rice bran glycosphingolipid for 13 weeks

		Grade	Control group n = 8	Rice bran glycosphingolipids 1000 mg/kg n = 8
Liver	Microgranuloma	+	3	3
	Hemosiderin deposition, periporta	+	0	1
Spleen			0	0
Lung			0	0
Kidney	Basophilic tubule	+	0	2
	Calcification	+	3	4
Heart	Myocarditis	+	1	2
Muscle			0	0
Tongue			0	0
Cerebrum			0	0
Cerebellum			0	0
Submandibular gland	Vacuolation of striated portion	+	1	1
Submandibular lymph node			0	0
Skin			0	0
Mammary gland			0	0
Esophagus			0	0
Artery			0	0
Thymus			0	0
Trachea	Dilation tracheal gland	+	0	1
Ovary			0	0
Uterus			0	0
Vagina			0	0
Urinary bladder			0	0
Pancreas			0	0
Forestomach			0	0
Glandular stomach			0	0
Duodenum			0	0
Jejunum			0	0
Ileum			0	0
Cecum			0	0
Colon			0	0
Rectum			0	0
Mesentery lymph node			0	0
Pituitary gland	Rathke's pouch	+	2	2
	Pars distalis Cyst	+	1	3
	Pars intermedia		0	0
	Pars nervosa		0	0
Thyroid gland	Follicular cell hypertrophy	+	1	0
Adrenal gland	Accessory adrenocortical tissue	+	0	1
Peripheral nerve			0	0
Eye			0	0
Harderian gland			0	0
Femur			0	0
Sternum			0	0
Spinal cord	Cervical vertebrate		0	0
	Thoracic vertebrate		0	0
	Lumbar vertebrate		0	0

+ : slight

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
杉本直樹，佐藤恭子，山崎　壮，棚元憲一	天然苦味料ジャマイカカッシア抽出物の成分分析	食品衛生学雑誌	44(6)	328-331	2003
Moto, M., Okamura, M., Watanabe, T., Kashida, Y., Mitsumori, K.	Thirteen-week Repeated Dose Toxicity of Rice Bran Glycosphingolipid in Wistar Hanover (GALAS) Rats	The Journal of Toxicological Sciences	29(1)	73- 80	2004
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

ノート

天然苦味料ジャマイカカッシア抽出物の成分分析

(平成15年6月23日受理)

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Analysis of Constituents in Jamaica Quassia Extract, a Natural Bittering Agent

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Jamaica quassia extract, a natural bittering agent, is described as "a substance extracted from bark of Jamaica quassia (*Quassia excelsa* Sw.)" in the List of Existing Food Additives in Japan. The constituents in Jamaica quassia extract product were investigated as a part of an ongoing study to evaluate its quality and safety as a food additive. The main constituents of the extract were identified as quassin and two isomers of neoquassin by using LC/MS. The main constituent, quassin, was isolated and the structure was determined by spectral means. The quantification of their main constituents was performed by HPLC using quassin as a standard, and the concentrations of quassin and total of neoquassin isomers were 21.4% and 55.5%. In addition, it was confirmed that Jamaica quassia extract was different from quassia extract, which is extracted from bark of *Picrasma quassioides* BENN. belonging to the same family as *Q. excelsa*, by comparing their HPLC profiles.

(Received June 23, 2003)

Key words: ジャマイカカッシア抽出物 Jamaica quassia extract; 食品添加物 food additive; 苦味料 bittering agent; クアシン quassin; ネオクアシン neoquassin; ジャマイカカッシア *Quassia excelsa* Sw.; ニガキ *Picrasma quassioides* BENN.

緒　言

天然苦味料ジャマイカカッシア抽出物は，既存添加物名簿収載品目リスト[1]に，その基原・製法・本質として，「ニガキ科ジャマイカカッシア (*Quassia excelsa* Sw.) の幹枝または樹皮より，水で抽出して得られたものである．有効成分はクアシン (quassin (1)) およびネオクアシン (neoquassin (2)) である．」と記載されている (Fig. 1)．基原植物のニガキ科ジャマイカカッシアは，ジャマイカ周辺にのみ自生し，我が国では栽培されていないことから，本抽出物の原料はすべて輸入品と考えられる．また，本抽出物の成分組成についての報告はなく，公的な成分規格もない．著者らは，既存添加物のうち，公的規格や自主規格のない品目，あるいは規格が不十分と考えられる品目を選定し，成分および品質に関する研究を行っている[2),3)]．そこで，その一環として，ジャマイカカッシア抽出物についても主構成成分の分析を行った．また同時に，同科植物ニガキより調製される天然苦味料ニガキ抽出物の成分組成との比較を行い，その差異について検討した．

実験方法

1. 試　料

ジャマイカカッシア抽出物 (Jamaica quassia extract) 製品 (1社1製品：淡褐色粉末) は日本食品添加物協会を通じて入手した．ニガキ科ニガキ (*Picrasma quassioides* BENN.) の細木片は，国立医薬品食品衛生研究所生薬部標本を用いた．

2. 試　薬

カラムクロマトグラフィーには，Silica gel 60N (spherical, neutral), 63～200 μm (関東化学(株)社製, Cat. No. 37565-79) を用いた．TLC には，Silica gel 60 F_{254} (20×20 cm, Merck 社製，Art. 1.05715) を用いた．その他の試薬はすべて市販特級品を用いた．

3. 装　置

各種機器データは，次の機器を用いて測定した．

融点 (mp): Yanaco MP-3 (柳本製作所(株)製) (未補正値)．

高速液体クロマトグラフ：LC-10AVP system ((株)島

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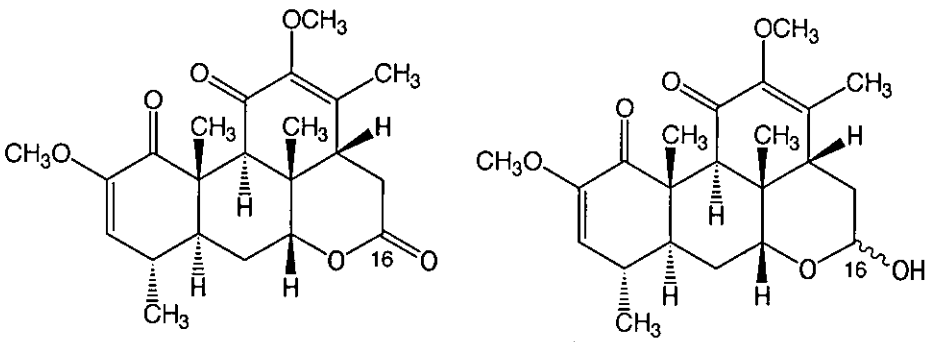

Quassin (1)
$C_{22}H_{28}O_6$
MW: 388

Neoquassin (2)
$C_{22}H_{30}O_6$
MW: 390

Fig. 1. Structures of quassin (1) and neoquassin (2)

津製作所製)

液体クロマトグラフ/質量分析装置: Waters 社製 LC/MS system (LC: 2525 binary high-pressure LC pump, 2767 one-bed injection-collection sample manager; PDA: 2996 photodiode array detector; MS: ZQ-2000 single quadropole mass spectrometer)

核磁気共鳴スペクトル (NMR): JEOL alpha-500 (日本電子(株)製). homonuclear shift correlation spectroscopy (COSY), heteronuclear multiple-quantum coherence (HMQC) および heteronuclear multiple bonds correlation (HMBC), nuclear Overhauser effect (NOE) には磁場勾配システムを用いた. NMR のケミカルシフト値は, TMS (tetramethylsilane) を内部標準とし, δ 値を ppm 単位で表した.

4. HPLC 条件

分析条件: カラム, Atlantis™ dC18 (4.6 mm i.d.×100 mm, 5 μm, Waters 社製); カラム温度, 40℃; 移動相, メタノール-水=40 : 60; 注入量, 5 μL; 流速, 1.0 mL/min; 検出波長, 255 nm

5. LC/MS 条件

LC 条件: カラム, Atlantis™ dC18 (4.6 mm i.d.×100 mm, 5 μm, Waters 社製); カラム温度, 室温; 移動相, メタノール-水=40 : 60 (0 min)→55 : 45 (25 min)→100 : 0 (35 min); 注入量, 10 μL; 流速, 1.0 mL/min; スプリット比, PDA-MS=4 : 1; 検出波長, 255 nm

MS 条件: ソース温度, 120℃; 脱溶媒温度, 350℃; 脱溶媒ガス, 350 L/hr; コーンガス, 60 L/hr; キャピラリー電圧, 3.0 kV; コーン電圧, 45 V (ESI pos.); −25 V (ESI neg.); スキャン範囲, *m/z* 50〜600.

6. ニガキ抽出物の調製

水抽出物: ニガキ細木片 0.5 g に水 50 mL を加えて 90℃ の水浴中で 30 分間加熱し, 温時綿栓ろ過した. ろ液を減圧留去して残さ 27 mg を得た.

メタノール抽出物: ニガキ細木片 0.4 g にメタノール 40 mL を加えて 70℃ の水浴中で 30 分間加熱し, 温時綿栓ろ過した. ろ液を減圧留去して残さ 22 mg を得た.

7. LC/MS 分析試料の調製

ジャマイカカッシア抽出物製品およびニガキ細木片より調製したニガキ抽出物 (水抽出物, メタノール抽出物) をそれぞれメタノールに溶解し, 濃度 5.0 mg/mL に調製したものを LC/MS 分析試料とした.

8. Quassin (1) の単離・精製

ジャマイカカッシア抽出物製品 1 g をシリカゲルカラム (内径: 50 mm; 長さ: 250 mm; 移動相: クロロホルム-メタノール=19 : 1) に付し, 流出液を TLC (展開溶媒: クロロホルム-メタノール=19 : 1; 検出波長: UV 254 nm) でモニターしながら, 約 10 mL ずつ分画した後, quassin (1) 流出部を合わせ, 減圧下, 溶媒を留去して粗 quassin (1) (36 mg) を得た. 得られた粗 quassin (1) を酢酸エチル-メタノールにより再結晶して quassin (1) (34 mg) を得た.

Quassin (1): colorless powder, mp 222〜223℃ (Merck Index: mp 222℃), MS (ESI pos.): *m/z* 389 $[M+H]^+$, 411 $[M+Na]^+$. ^{1}H-NMR ($CDCl_3$) δ: 1.12(3H, d, J=7.0 Hz, H-18), 1.20(3H, s, H-20), 1.56(3H, s, H-19), 1.81(1H, m, H-5), 1.84(1H, m, H-6a), 1.88(3H, s, H-21), 2.09(1H, ddd, J=3.0, 3.3, 14.0 Hz, H-6b), 2.42(1H, dd, J=7.0, 11.6 Hz, H-14), 2.49(1H, m, H-4), 2.60(1H, dd, J=11.9, 18.5 Hz, H-16a), 2.99(1H, s, H-9), 3.00(1H, dd, J=7.0, 18.5 Hz, H-16b), 3.59(3H, s, OMe (C-2)), 3.67(3H, s, OMe (C-12)), 4.30(1H, dd, J=2.1, 3.3 Hz, H-7), 5.31(1H, d, J = 2.5 Hz, H-3). ^{13}C-NMR ($CDCl_3$) δ: 25.8(C-6), 31.1(C-4), 31.6(C-15), 37.0 (C-8), 43.2 (C-5), 45.8 (C-10), 46.2 (C-9), 46.5 (C-14), 54.8 (C-3-OMe), 59.2 (C-12-OMe), 81.8 (C-7), 116.3 (C-3), 137.5(C-13), 147.9(C-2), 148.2(C-12), 169.0(C-16), 190.9 (C-11), 196.1(C-1), 197.7(C-1).

9. HPLC 分析試料の調製および quassin (1), neoquassin (2) の定量

ジャマイカカッシア抽出物製品 50 mg を精密に量りメタノール 10 mL に溶解し, HPLC 分析試料とした. また, 単離・精製した quassin (1) 3.0 mg を精密に量り, メタノール 1.5 mL に溶解し, 1/2 希釈を繰り返し, 2.0,

1.0, 0.5, 0.25 mg/mL に調製した．検出波長 255 nm において，各濃度の quassin (1) のピーク面積より作成した絶対検量線より，ジャマイカカッシア抽出物中の quassin (1) および neoquassin (2) を定量した．

結果および考察

1. Quassin (1) および neoquassin (2) の確認

ジャマイカカッシア抽出物製品およびニガキ細木片より調製したニガキ抽出物（水抽出物，メタノール抽出物）を LC/MS 装置に付した結果を Fig. 2 に示した．ジャマイカカッシア抽出物製品は，検出波長 255 nm において，保持時間 7.9 分（ピーク A），13.3 分（ピーク B），14.2 分（ピーク C）の 3 つのピークが観察された．ピーク A は，ESI-MS (pos.) において，*m/z* 389 $[M+H]^+$, 411 $[M+Na]^+$ を与えたことから本抽出物の有効成分の 1 つとされる quassin (1) であると推定された．そこで，別にジャマイカカッシア抽出物製品をシリカゲルカラムクロマトに付し，ピーク A に由来する成分を単離・精製した．得られた化合物は，融点 222～223℃ を示し，また，NMR 測定値が文献値[5] と一致したことから，ピーク A を quassin (1) と決定した．一方，ピーク B, C は ESI-MS (pos.) において，共に *m/z* 391 $[M+H]^+$, 413 $[M+Na]^+$ を与え，ESI-MS (neg.) においても，共に 389 $[M-H]^-$ を与えた．また，ピーク B, C は，255 nm 付近に極大吸収を持つピーク A とほぼ等しい PDA スペクトルを与えたことから，これらは quassin (1) と同様に分子内に α,β 不飽和カルボニルを含むことが示唆された (Fig. 3)．よって，ピーク B, C は neoquassin (2) の 16 位の立体異性体[4] であると決定した．さらに，検出波長 192～600 nm において本抽出物製品を走査したところ，ピーク A, B, C 以外に特に大きなピークは観察されなかったことから，本抽出物製品が主に quassin (1) および neoquassin (2) により構成されるものと考えられた．

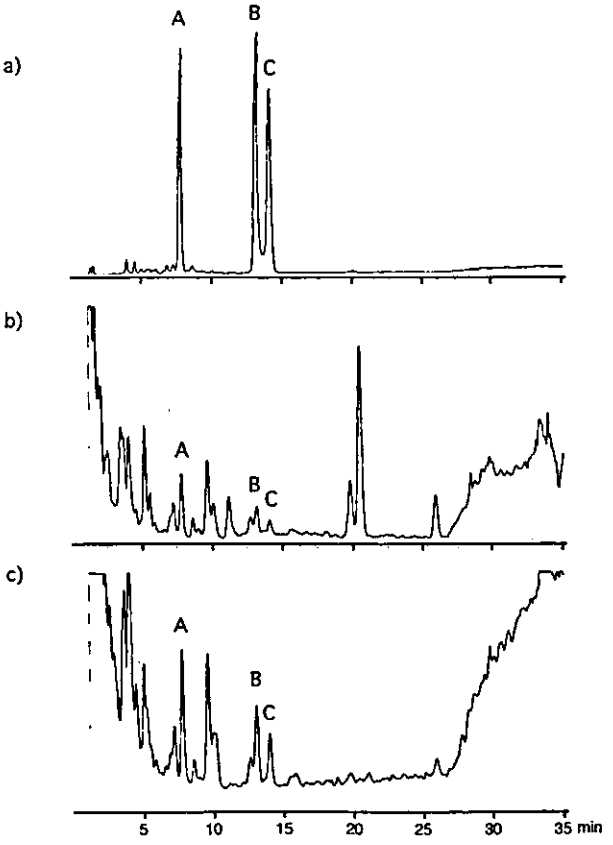

Fig. 2. LC/MS profiles of Jamaica quassia extract product and quassia extract prepared from bark of *P. quassioides*
a) Jamaica quassia extract product, b) Quassia methanol-extract, c) Quassia water-extract, A: *m/z* 389 $[M+H]^+$, 411 $[M+Na]^+$ (quassin (1)); B and C: *m/z* 391 $[M+H]^+$, 413 $[M+Na]^+$, 389 $[M-H]^-$ (neoquassin isomers (2))
LC conditions: column, Atlantis™ dC18 (4.6 mm i.d.×100 mm, 5 μm); column temp., r.t.; solvent, A=water, B=methanol, 0 min (B=40%)→25 min (B=60%)→35 min (B=100%); flow rate, 1.0 mL/min; injection, 10 μL; split rate, PDA–MS=4 : 1; detection, 255 nm

2. ニガキ抽出物の成分

一方，ニガキ抽出物は，既存添加物名簿収載品目リストに，その基原・製法・本質として，「ニガキ科ニガキ (*Picrasma quassioides* Benn.) の幹枝または樹皮より，水で抽出して得られたもの，またはメタノールで抽出したものから得られたものである．有効成分はクアシン (quassin (1)) である．」と記載されている．よって，ジャマイカ

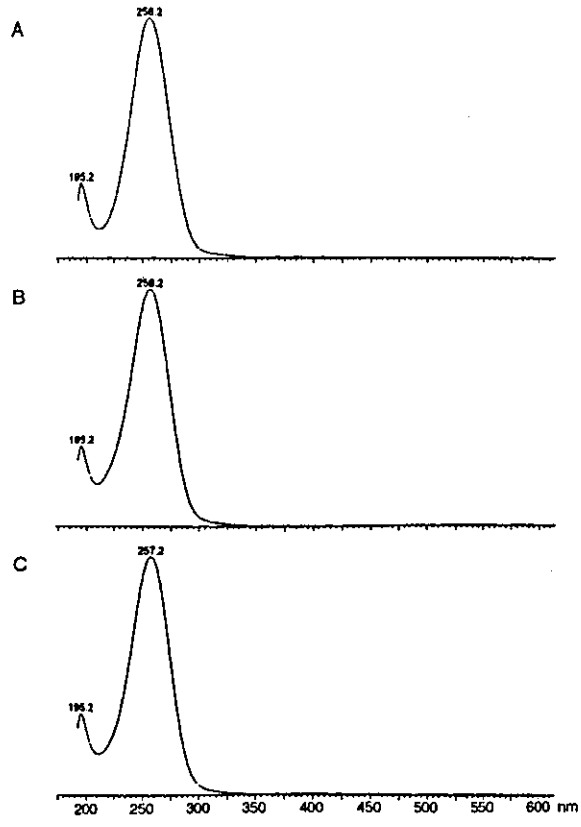

Fig. 3. PDA spectra of peaks A, B and C
A: quassin (1); B and C: neoquassin isomers (2)

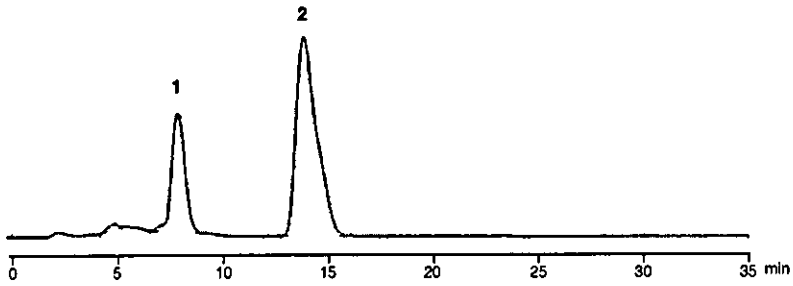

Fig. 4. HPLC profile of Jamaica quassia extract
1: quassin; 2: neoquassin isomers
LC conditions: column, Atlantis™ dC18 (4.6 mm i.d.×100 mm, 5 μm); column temp., 40℃; solvent, methanol–water=40 : 60; flow rate, 1.0 mL/min; injection, 5 μL; detection, 255 nm

カッシア抽出物と同科の植物より調製されるニガキ抽出物は，成分的にほぼ同等である可能性が考えられる．天然苦味料としてのニガキ抽出物は，現在，市場に流通していないとされており入手できなかったが，この点を確認しておく必要がある．そこで，既存添加物名簿収載品目リストに記載の製法に従って，ニガキ抽出物（水抽出物，メタノール抽出物）を調製し，その成分組成について検討した．その結果，今回調製したニガキ抽出物（水抽出物，メタノール抽出物）の成分組成は，ジャマイカカッシア抽出物とは異なり，quassin (1) および neoquassin (2) 以外に多数の成分を含む混合物であることが明らかとなった (Fig. 2).

3. ジャマイカカッシア抽出物中の quassin (1) および neoquassin (2) の定量

ジャマイカカッシア抽出物製品中の quassin (1) および neoquassin (2) を定量した．HPLC において，quassin (1) は保持時間 7.8 分に観察され，また，neoquassin (2) の 2 つの立体異性体は 13.7 分に 1 つのピークとして観察された (Fig. 4)．定量用標品としてジャマイカカッシア抽出物製品よりシリカゲルカラムクロマトグラフィーにより単離・精製した quassin (1) を用い，その濃度と検出波長 255 nm におけるピーク面積より絶対検量線 ($y=7109285x+120080$, $r^2=1.000$) を作成した．なお，quassin (1) と neoquassin (2) は，極大波長 255 nm において同一のモル吸光係数 ($\varepsilon=11650$, Merck index) を持つことから，neoquassin (2) の定量には quassin (1) より作成した検量線を用いた．その結果，今回試料に用いたジャマイカカッシア抽出物製品 5 mg 中には，quassin (1) が 1.07 mg (21.4%), neoquassin (2) が 2.77 mg (55.5%) 含まれることが分かった．

ま と め

現在，既存添加物には，公的規格や自主規格のない品目が数多く残されている．著者らは，将来，既存添加物の規格および品質評価法を設定する目的で，各品目の成分および品質について研究している．その一環として，天然苦味料の 1 つであるジャマイカカッシア抽出物の分析および主構成成分の定量を行った．ジャマイカカッシア抽出物製品の主構成成分 quassin (1) および neoquassin (2) の分析には，LC/MS が有効であった．また，quassin (1) および neoquassin (2) の定量は，本抽出物製品よりシリカゲルカラムクロマトグラフィーによって比較的容易に単離・精製することができる quassin (1) を定量用標品とすることで可能であり，製品中に quassin (1) (21.4%) および neoquassin (2) (55.5%) が含有されることを明らかとした．また同時に，同科植物を基原とするニガキ抽出物を調製して検討したところ，ジャマイカカッシア抽出物製品とその成分組成が異なることを明らかとした．本研究結果は，ジャマイカカッシア抽出物の品質評価および規格設定のための基盤となると考えられる．

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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.

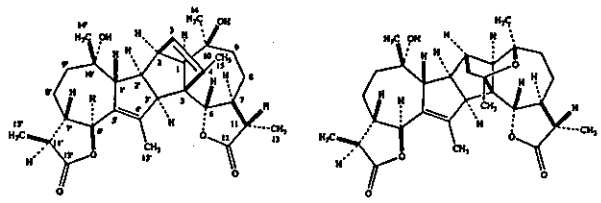
III. Results and Discussion

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).



Absinthin (1) Anabsinthin (2)

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.

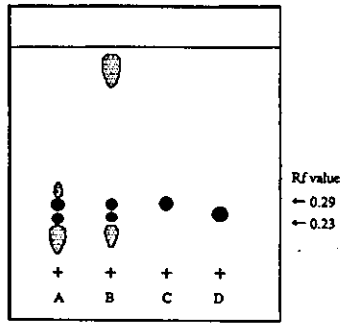


Fig. 2 RP-18 TLC profiles of absinth extract product and 50% ethanol extract from the dry leaves of *A. absinthium*.
A) absinth extract product.
B) 50% EtOH extract prepared from the dry leaves of *A. absinthium*.
C) absinthin (**1**).
D) anabsinthin (**2**).
Solvent: MeOH : water = 3 : 2.
Spots visualized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

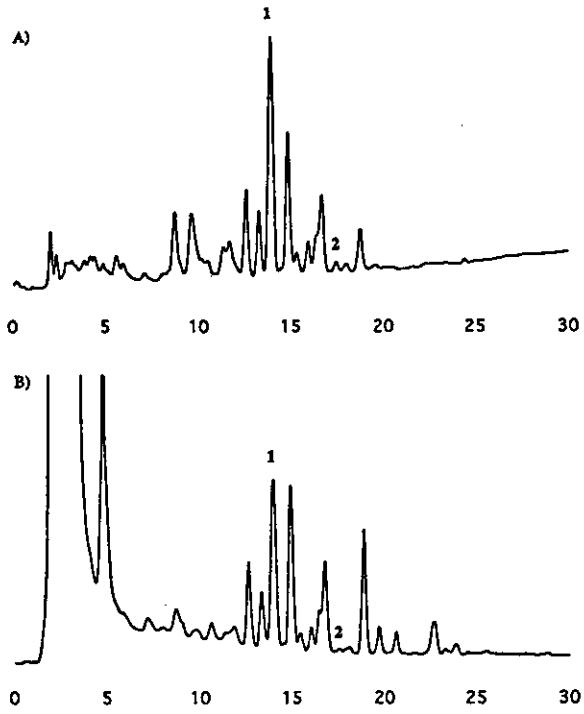


Fig. 3 HPLC profiles of absinth extract product and 50% ethanol extract from the leaves of *A. absinthium*.
A) absinth extract product.
B) 50% EtOH extract prepared from the leaves of *A. absinthium*.
absinthin (1). anabsinthin (2).

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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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

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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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THIRTEEN-WEEK REPEATED DOSE TOXICITY OF RICE BRAN GLYCOSPHINGOLIPID IN WISTAR HANNOVER (GALAS) RATS

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ABSTRACT — Rice bran glycosphingolipid (RBGSL), one of the glycosphingolipids (GSLs), has been widely used as a food additive, a base of cosmetics, and so on. As a part of the safety assessment of RBGSL, a 13-week repeated dose toxicity study was performed in Wistar Hannover (GALAS) rats. Male and female rats were divided into 4 groups consisting of 8 animals and were given 0, 60, 250, and 1000 mg/kg BW of RBGSL orally 5 times weekly for 13 weeks. During the experiment, no deaths were observed in any groups, and there were no remarkable changes in general appearance, body weight, food and water consumption, hematological and serum biochemical parameters, organ weight and histopathological findings between the control and treated groups. On the basis of these data, the no-observed-adverse effect level (NOAEL) of RBGSL in Wistar Hannover rats was considered to be 1000 mg/kg BW/day or more.

KEY WORDS: Food additive, Rat, Repeated dose toxicity, Rice bran glycosphingolipid

INTRODUCTION

Glycosphingolipids (GSLs) are amphipathic sphingolipids consisting of oligosaccharide and ceramide moieties (Fig. 1), and known as typical constituents of various cell membranes in a wide variety of organisms including mammalian species (Hakomori, 1981). GSLs have been used in skin care products because the constitutive ceramide accelerates the skin barrier repair after damage (Leonardi, 2002). Additionally, GSLs were found to be enriched with cholesterol and GPI-anchor proteins to form lipid microdomains on the plasma membrane of vertebrates, and have been suggested to mediate cell-cell interaction, attachment, proliferation and differentiation (Hakomori, 1981; Ito *et al.*, 1995; Spiegel and Merrill, 1996; Yamada, 2002). Furthermore, it has been reported that GSLs were absorbed orally (Schmelz *et al.*, 1994). Therefore, GSLs or their catabolites are expected to be origins of new drugs, and developments have been performed in various fields (Ceccarelli *et al.*, 1976; Vantini *et al.*, 1988; Bradly, 1990).

GSLs have been artificially synthesized or extracted from mammalian tissues such as the bovine brain containing rich GSLs so far (Yamada, 2002). However, it has become difficult to use tissues of animal origin because of the risk of prion-virus-infection of bovine spongiform encephalopathy (BSE) in recent years. Therefore, much attention has been currently focused on GSLs that are derived from tissues of non-animal origin such as rice bran or rice with germs, and the GSLs of plant origin have been widely used as cosmetics, cosmetic foods and food additives. In Japan, rice bran GSL (RBGSL) has been used as a food additive. However, there is little information regarding the safety of GSLs derived from non-animal tissues.

In the present study, as a part of the safety assessment of food additives in the Japanese Ministry of Health, Labor and Welfare, a 13-week repeated dose toxicity study of RBGSL was performed in rats.

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MATERIALS AND METHODS

Animals

Male and female Wistar Hannover (GALAS) rats were obtained from Clea Japan, Inc. (Tokyo, Japan) at 5 weeks of age and acclimated for 1 week prior to commencement of the experiment. The animals were housed 2 or 3 rats per wire-mesh steel cage under conventional conditions (12 hr light/dark cycle, 55 ± 10% humidity and 24 ± 2°C temperature) and given a pellet-type diet (CE-2, Clea Japan Inc., Tokyo, Japan) and water throughout the experimental period.

Chemicals and preparation of the test substance

RBGSL was kindly provided by Okayasu Shoten Co, Ltd. (Saitama, Japan). Sodium carboxymethylcellulose (Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in water at 0.5% (w/v) was used as a vehicle, and dosage volume was set at 10 mL/kg BW. Prior to the 13-week study, a preliminary study using the oral dosage of 1000 to 2000 mg/kg BW was performed, but it was difficult to perform oral administration by gavage because of the strong viscosity of the test substance suspension of 2000 mg/10 mL. Based on the results of a preliminary study (data not shown), the dose levels for the present study were set as 1000, 250, 60, and 0 (vehicle) mg/kg BW. RBGSL was newly suspended in vehicle at each concentration every 1 or 2 weeks and preserved in a dark condition at 4°C until use.

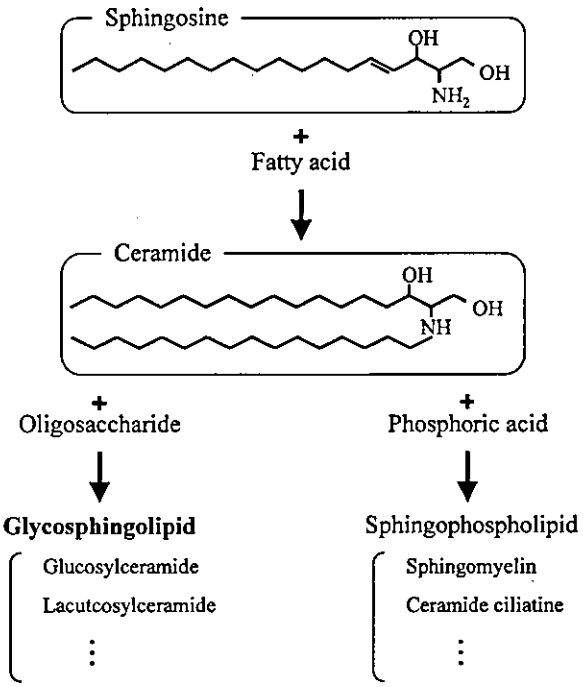

Fig. 1. Basic structure and classification of sphingolipid.

Experimental design

Animals were divided into 4 groups consisting of 8 male and 8 female rats, respectively, and given 0, 60, 250, or 1000 mg/kg BW of RBGSL orally on a daily basis 5 times weekly for up to 13 weeks (Table 1).

Clinical signs and general appearances were observed once a day, and body weights as well as food and water consumption were measured once a week. An autopsy was performed at the end of the experiment. Blood samples were collected from the abdominal aorta under light ether anesthesia after 16 hr starvation.

Hematological parameters, white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HB), hematocrit (HT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC) and platelet (PLT) count were assessed using an automatic multichannel blood cell counter (Sysmex SE-9000, Sysmex Co., Hyogo, Japan). Additionally, the ratios of neutrophil (NEU), eosinophil (EOS), lymphocyte (LYMP) and monocyte (MONO) on blood smears were determined with an automatic blood cell analyzer (MICROX HEG-120A, Tateishi Electric Co., Kyoto, Japan).

Serum biochemical examinations were performed at SRL Co. Ltd. (Tokyo, Japan), and the following parameters were measured: total protein (TP), albumin/globulin ratio (A/G), total cholesterol (TC), blood urea nitrogen (BUN), creatinine (CRE), calcium (Ca), inorganic phosphate (IP), sodium (Na), potassium (K), chloride (Cl), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and albumin (ALB).

At autopsy, weights of brain, heart, lungs, liver, kidneys, spleen, thymus, adrenal glands, pituitary gland, thyroid glands, testes, uterus, and ovaries were measured. In addition, the artery, bone/marrow, coagulation gland, esophagus, epididymidis, large intestine, lymph node, mammary gland, pancreas, peripheral nerve, prostate gland, salivary gland, skeletal muscle, skin, small intestine, spinal cord, stomach, urinary bladder, tongue, trachea and vagina were fixed in 10% neutral buffered formalin. Eyeballs and Harderian glands were fixed with Davidson's solution for 12 hr

and then replaced into 95% ethanol. Testes were fixed with 4% acetic acid / 12% formalin mixture for 24 hr and then were removed into 10% neutral buffered formalin. Bone was decalcified in Plank Rychlo solution before embedding. These tissues were routinely embedded in paraffin, sectioned at 4 μm thick for hematoxylin and eosin staining, and examined by light microscopy. Histopathological examinations were carried out only on the 0 and 1000 mg/kg groups for both sexes.

Statistical analysis

The data obtained from the measurements of body weight, food and water consumption, organ weight, hematology and serum biochemistry were expressed as mean ± S.D.. The significance of the differences between the control and RBGSL groups was determined by Dunnett's test (Dunnett, 1955) after one-way ANOVA. A p-value less than 0.05 was considered statistically significant in both analyses.

RESULTS

Neither deaths nor remarkable changes in general appearance were observed in treated groups during the experimental period. Changes of body weights during the experiment are shown in Fig. 2. There was no suppression of body weight gain in groups treated during the experiment.

As shown in Table 1, there were no remarkable differences in food and water consumption between the control and treated groups in either sex.

The results of hematological and serum biochemical analyses are shown in Table 2 and 3. In hematological examinations, a significant increase in the ratio of EOS was observed in females of the 1000 mg/kg group, although no remarkable difference was observed in the other parameters. All data in male treated groups were similar to the control group. In serum biochemical examinations, a significant decrease in A/G ratio and an increase in BUN level were observed in males of the 1000 and 250 mg/kg groups, respectively. In females, there were no significant differences in any parameters between the control and treated groups.

The relative organ weights are shown in Table 4. There were no significant differences in any organ weights between the control and treated groups in either sex.

The results of histopathological examinations are summarized in Table 5. In males of the 1000 mg/kg group, microgranuloma of the liver, basophilic tubule and/or calcification of the kidney, myocarditis, dilation of tracheal glands, chronic inflammation of the prostate, and Rathke's pouch and/or cyst of the pituitary *pars distalis* were observed. In females of the high dose group, hemosiderin deposition and/or microgranuloma of the liver, basophilic tubule and/or calcification of the kidney, myocarditis, vacuolation of striated portions of the submandibular gland, dilation of tracheal glands, Rathke's pouch and/or cyst of the pituitary *pars distalis*, thyroid follicular cell hypertorophy, and accessory adrenocortical tissue of the adrenal gland were observed. These findings in male and female rats of the 1000 mg/kg group were also observed in the control group of either sex, and there were no significant differences in the incidences of these changes between these two groups.

Table 1. Body weights and food and water consumptions in rats treated with rice bran glycosphingolipid for 13 weeks.

	Dose level (mg/kg)	No. of animals	Final body weight (g)	Food consumption (g/rat/day)	Water consumption (g/rat/day)
Male	0	8	456.1 ± 54.6 [a)]	25.9	36.3
	60	8	443.6 ± 39.7	25.2	33.9
	250	8	466.8 ± 26.3	26.5	33.6
	1000	8	463.4 ± 33.4	26.4	34.2
Female	0	8	262.3 ± 17.1	19.2	32.6
	60	8	255.4 ± 12.5	19.1	25.9
	250	8	252.5 ± 12.1	19.7	40.9
	1000	8	257.8 ± 13.6	19.6	31.5

[a)] : Mean ± S.D.

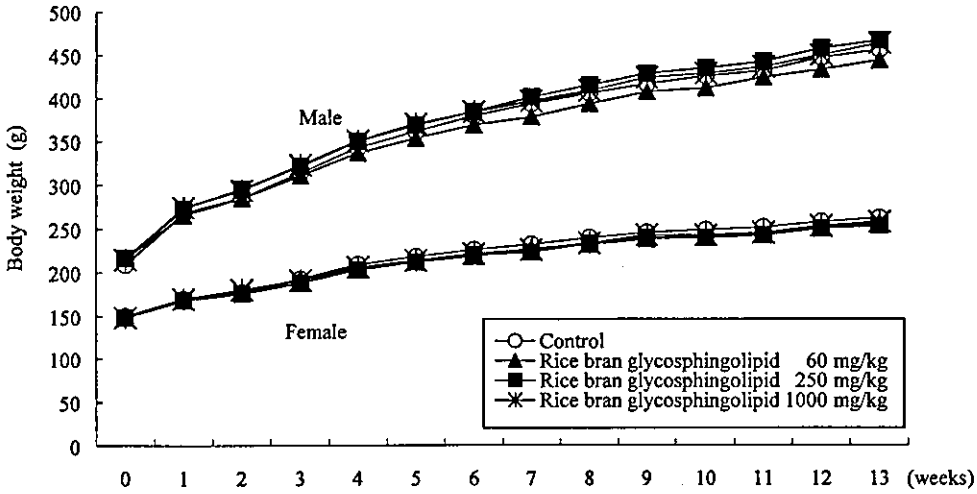

Fig. 2. Growth curves of male and female rats treated with rice bran glycosphingolipid for 13 weeks.

Table 2. Hematological data in rats treated with rice bran glycosphingolipid for 13 weeks.

Dose (mg/kg)		0		60		250		1000	
No. of animals		8		8		8		8	
Male									
RBC	$\times 10^4/mm^3$	712.9 ±	49.2 [a)]	736.6 ±	31.8	755.4 ±	24.0	750.1 ±	49.2
HB	g/dl	13.6 ±	0.7	14.1 ±	0.4	14.0 ±	0.4	14.0 ±	0.7
HT	%	40.1 ±	2.4	41.3 ±	2.3	41.4 ±	1.4	41.1 ±	2.9
MCV	m^3	56.4 ±	1.7	55.9 ±	1.6	54.8 ±	1.7	54.6 ±	0.7
MCH	pg	19.1 ±	0.6	19.0 ±	0.8	18.6 ±	0.5	18.6 ±	0.5
MCHC	%	34.0 ±	0.9	34.3 ±	1.2	34.0 ±	0.9	34.1 ±	1.1
WBC	/ml	2737.5 ±	776.3	3387.5 ±	603.4	3487.5 ±	1172.8	3387.5 ±	1089.5
Differential cell count (%)									
NEU		17.9 ±	7.4	15.4 ±	4.1	16.0 ±	5.0	16.4 ±	4.4
EOS		1.7 ±	1.0	3.0 ±	1.9	1.7 ±	0.6	2.4 ±	1.1
MONO		2.0 ±	1.4	1.0 ±	0.0	2.0 ±	0.9	1.5 ±	0.8
LYMP		79.4 ±	8.9	81.4 ±	6.0	81.9 ±	5.0	81.0 ±	4.7
Female									
RBC	$\times 10^4/mm^3$	659.1 ±	39.0	640.1 ±	27.0	655.3 ±	22.7	638.9 ±	22.0
HB	g/dl	13.6 ±	0.6	13.3 ±	0.6	13.6 ±	0.5	13.4 ±	0.7
HT	%	39.1 ±	2.4	38.0 ±	2.1	38.8 ±	1.6	38.0 ±	2.1
MCV	m^3	59.4 ±	2.9	59.5 ±	1.9	59.1 ±	1.5	59.5 ±	2.1
MCH	pg	20.5 ±	0.9	20.8 ±	0.5	20.8 ±	0.5	21.0 ±	0.8
MCHC	%	34.6 ±	1.2	34.9 ±	0.8	35.0 ±	0.8	35.4 ±	0.9
WBC	/ml	1900.0 ±	1028.2	1862.5 ±	855.1	1425.0 ±	525.8	2512.5 ±	693.7
Differential cell count (%)									
NEU		17.8 ±	6.0	15.4 ±	2.7	16.1 ±	5.0	13.9 ±	4.4
EOS		1.3 ±	0.5	1.5 ±	0.6	2.3 ±	0.6	4.0 ±	1.4 **
MONO		1.9 ±	1.5	1.2 ±	0.4	1.3 ±	0.5	2.2 ±	1.3
LYMP		79.4 ±	6.6	83.1 ±	2.3	82.0 ±	4.1	83.8 ±	4.8

[a)] : Mean ± S.D.

**: Significantly different from control at $p<0.01$.

DISCUSSION

GSLs are ubiquitous membrane components essential in all eukaryotic cells, and consist of both sugar moiety such as monosaccharide, disaccharide or oligosaccharide and ceramide moiety such as cerebroside, globoside or ganglioside (Hakomori, 1981, 1990). Due to variations in these sugar chains, it is known that GSLs have many physiological functions such as coordination of cell surface by oligosaccharide, receptors for bacterial toxins and viruses, regulation of the differentiation of nerve cells, control of intercellular signal transductions, and regulation of the function of cell membranes (Hakomori, 1990; Yamada, 2002). Therefore, it is clear that GSLs in cell membranes have very important functions against many diseases such as cancer, nerve disease, virus infection and so on, in spite of the very small quantity of the constituents (Arita *et al.*, 1989; Kitagawa *et al.*, 1989; Schengrund, 1990; Spiegel and Merrill, 1996; Yamada, 2002). On the other hand, there is little information regarding the safety of GSLs including RBGSLs, although they are capable of being incorporated into cellular membranes when exogenously added. In the present study, as a part of a comprehensive safety evaluation of food additives in the Japanese Ministry of Health, Labor and Welfare, a 13-week repeated dose toxicity study of the RBGSL was performed in rats.

Table 3. Data of serum biochemistry in rats treated with rice bran glycosphingolipid for 13 weeks.

Dose (mg/kg)		0	60	250	1000
No. of animals		8	8	8	8
Male					
TP	g/dl	5.9 ± 0.5 [a]	6.1 ± 0.2	6.3 ± 0.2	6.0 ± 0.4
A/G		1.8 ± 0.2	1.8 ± 0.1	1.7 ± 0.1	1.6 ± 0.2 *
TC	mg/dl	50.8 ± 7.0	50.6 ± 4.5	51.6 ± 6.2	50.6 ± 7.9
BUN	mg/dl	23.9 ± 1.7	23.3 ± 2.6	26.6 ± 0.8 *	24.0 ± 2.2
CRE	mg/dl	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Ca	mg/dl	9.6 ± 0.4	9.6 ± 0.2	9.7 ± 0.2	9.6 ± 0.5
IP	mg/dl	4.8 ± 0.5	4.7 ± 0.5	4.5 ± 0.4	4.7 ± 0.6
Na	mEQ/l	147.9 ± 2.2	146.0 ± 1.5	146.3 ± 0.7	147.1 ± 1.6
K	mEQ/l	3.2 ± 0.3	3.2 ± 0.2	3.4 ± 0.2	3.2 ± 0.2
Cl	mEQ/l	107.0 ± 3.5	105.4 ± 1.8	105.9 ± 2.0	106.3 ± 2.7
AST	IU/l	74.5 ± 6.8	73.5 ± 8.9	67.4 ± 5.7	73.9 ± 13.9
ALT	IU/l	50.6 ± 10.9	44.8 ± 5.2	43.6 ± 6.0	44.6 ± 11.1
ALP	IU/l	396.8 ± 150.5	387.1 ± 93.6	409.4 ± 145.0	379.1 ± 149.3
ALB	g/dl	3.8 ± 0.4	3.9 ± 0.1	3.9 ± 0.2	3.7 ± 0.3
Female					
TP	g/dl	6.2 ± 0.4	6.1 ± 0.4	6.2 ± 0.4	6.3 ± 0.5
A/G		2.2 ± 0.3	2.1 ± 0.4	1.9 ± 0.2	2.3 ± 0.4
TC	mg/dl	53.5 ± 9.7	49.0 ± 9.2	50.8 ± 7.6	53.1 ± 8.3
BUN	mg/dl	22.5 ± 2.6	23.3 ± 1.9	23.1 ± 2.7	24.2 ± 3.0
CRE	mg/dl	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
Ca	mg/dl	9.4 ± 0.7	9.3 ± 0.5	9.4 ± 0.3	9.7 ± 0.6
IP	mg/dl	4.4 ± 1.3	4.5 ± 0.7	4.7 ± 0.7	4.7 ± 0.7
Na	mEQ/l	147.9 ± 1.7	147.9 ± 1.4	148.5 ± 1.2	146.5 ± 1.6
K	mEQ/l	3.0 ± 0.5	2.9 ± 0.3	2.8 ± 0.2	3.0 ± 0.2
Cl	mEQ/l	108.4 ± 2.5	109.5 ± 2.4	109.3 ± 1.8	108.0 ± 3.9
AST	IU/l	69.1 ± 18.9	66.0 ± 18.1	64.5 ± 13.7	67.0 ± 15.8
ALT	IU/l	44.5 ± 14.8	44.1 ± 17.1	43.9 ± 13.1	44.1 ± 15.8
ALP	IU/l	224.3 ± 124.0	179.5 ± 58.9	199.5 ± 62.8	231.6 ± 106.7
ALB	g/dl	4.3 ± 0.4	4.1 ± 0.4	4.1 ± 0.3	4.4 ± 0.4

[a]: Mean ± S.D.

* : Significantly different from control at $p<0.05$.

In hematological examinations of the present study, the ratio of EOS in the differential blood count was significantly increased in male rats of the 1000 mg/kg group. The high level of EOS ratio was observed only in one rat of this group, and no change was observed in the value of WBC in this group. Therefore, this change is considered to be of no toxicological significance. In serum biochemical examinations, BUN level was significantly increased in males of the 250 mg/kg group and the A/G ratio was reduced in females of the 1000 mg/kg group. As these alterations were very small in their fluctuation range and there were no changes in other associated parameters, these changes were considered to be incidental. In histopathological examinations, slight changes were observed in some tissues of both sexes of the 1000 mg/kg group. However, since these changes were also observed in control groups and their incidence in the 1000 mg/kg group were not significantly different from the control group, they were regarded as spontaneous lesions.

Prior to the present study, there were some concerns about the onset of toxicities due to the accumulation of GSLs and/or their metabolites because of the oral administration of a large amount of RBSGL to rats. However, no toxic changes were observed in RBSGL-treated rats. Until now, some conclusions have been reported about the digestion, uptake and distribution of GSLs: GSLs, without any cleavage in the stomach, were hydrolyzed by intestinal enzymes and microflora in the small intestine and colon of rats and mice, exchanged to ceramide and/or sphingoid-base

Table 4. Relative organ weights (g/100 g BW) in rats treated with rice bran glycosphingolipid for 13 weeks.

Dose (mg/kg)	0	60	250	1000
No. of animals	8	8	8	8
Male				
Body weight (g)	456.1 ± 54.6 [a)]	443.6 ± 39.7	466.8 ± 26.3	463.4 ± 33.4
Brain	0.46 ± 0.05	0.47 ± 0.04	0.44 ± 0.03	0.46 ± 0.04
Heart	0.24 ± 0.02	0.25 ± 0.02	0.25 ± 0.01	0.25 ± 0.02
Lungs	0.29 ± 0.02	0.29 ± 0.01	0.28 ± 0.01	0.29 ± 0.02
Liver	3.20 ± 0.12	3.29 ± 0.12	3.30 ± 0.12	3.14 ± 0.23
Kidneys	0.61 ± 0.04	0.61 ± 0.06	0.60 ± 0.03	0.60 ± 0.05
Spleen	0.16 ± 0.02	0.17 ± 0.02	0.17 ± 0.02	0.15 ± 0.02
Thymus	0.08 ± 0.01	0.09 ± 0.02	0.08 ± 0.02	0.09 ± 0.02
Adrenal glands	0.002 ± 0.000	0.002 ± 0.001	0.002 ± 0.000	0.002 ± 0.001
Pituitary gland	0.014 ± 0.002	0.015 ± 0.003	0.016 ± 0.002	0.016 ± 0.003
Thyroid glands	0.005 ± 0.001	0.006 ± 0.003	0.006 ± 0.001	0.005 ± 0.001
Testes	0.79 ± 0.11	0.81 ± 0.07	0.78 ± 0.05	0.79 ± 0.09
Female				
Body weight (g)	262.3 ± 17.1	255.4 ± 12.5	252.5 ± 12.1	257.8 ± 13.6
Brain	0.71 ± 0.05	0.76 ± 0.03	0.75 ± 0.03	0.72 ± 0.03
Heart	0.29 ± 0.02	0.30 ± 0.02	0.41 ± 0.29	0.30 ± 0.01
Lungs	0.38 ± 0.04	0.38 ± 0.03	0.39 ± 0.02	0.39 ± 0.02
Liver	3.37 ± 0.25	3.50 ± 0.18	3.57 ± 0.19	3.56 ± 0.23
Kidneys	0.65 ± 0.06	0.66 ± 0.04	0.68 ± 0.05	0.68 ± 0.06
Spleen	0.21 ± 0.01	0.21 ± 0.02	0.19 ± 0.02	0.29 ± 0.21
Thymus	0.12 ± 0.03	0.13 ± 0.03	0.11 ± 0.01	0.13 ± 0.02
Adrenal glands	0.006 ± 0.001	0.006 ± 0.001	0.006 ± 0.000	0.006 ± 0.001
Pituitary gland	0.032 ± 0.004	0.036 ± 0.004	0.035 ± 0.003	0.031 ± 0.004
Thyroid glands	0.008 ± 0.002	0.007 ± 0.001	0.008 ± 0.001	0.006 ± 0.003
Uterus	0.332 ± 0.092	0.283 ± 0.091	0.259 ± 0.080	0.291 ± 0.166
Ovaries	0.038 ± 0.010	0.044 ± 0.010	0.037 ± 0.003	0.039 ± 0.005

[a)]: Mean ± S.D.

(including sphingosine) throughout the intestine, including the colon, and then absorbed by the intestinal epithelial cells (Nilsson, 1968, 1969; Schmelz *et al.*, 1994; Vesper *et al.*, 1999). In addition, when sphingoid-base-labeled sphingolipids including GSLs were fed to rats, a small amount of the radiolabeled sphingoid-base was found in the lymph, blood and liver, which implied that some components of dietary sphingolipids were transported through the mucosa and appeared in systemic circulation (Nilsson, 1968; Schmelz *et al.*, 1994; Vesper *et al.*, 1999). The data of these references may suggest that RBGSL administered orally is probably absorbed and distributed in various tissues and organs of rats. Therefore, it could be considered that the RBGSL absorbed from the intestine in rats did not result in any toxicity under the treatment condition of the present study.

In conclusion, there were no toxic changes related to the administration of RBGSL in rats given 1000 mg/kg BW, and the NOAEL (no-observed-adverse effect level) of the RBGSL in Wistar Hannover rats was estimated to be 1000 mg/kg/day or more. This report is the first investigation concerning the subchronic oral toxicity of RBGSL in rats, and studies on the chronic toxicity and carcinogenicity of RBGSL have not been performed yet. Further studies such as chronic toxicity and carcinogenicity are needed for safe use of this substance in consumers.

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Table 5. Histopathological findings in rats treated with rice bran glycosphingolipid for 13 weeks.

Organ	Pathological findings	Grade	Control 0 mg/kg n = 8	Rice bran glycosphingolipids 1000 mg/kg n = 8
Male				
Liver	Microgranuloma	+	3 [a)]	3
Kidney	Basophilic tubule	+	1	2
	Calcification	+	0	1
Heart	Myocarditis	+	4	2
Submandibular gland	Vacuolation of striated portion	+	2	0
Trachea	Dilation, tracheal gland	+	0	1
Prostate	Chronic inflammation	+	2	3
Pituitary gland	Rathke's pouch	+	0	1
	Pars distalis, cyst	+	1	1
Female				
Liver	Microgranuloma	+	3	3
	Hemosiderin deposition, periportal	+	0	1
Kidney	Basophilic tubule	+	0	2
	Calcification	+	3	4
Heart	Myocarditis	+	1	2
Submandibular gland	Vacuolation of striated portion	+	1	1
Trachea	Dilation tracheal gland	+	0	1
Pituitary gland	Rathke's pouch	+	2	2
	Pars distalis, cyst	+	1	3
Thyroid gland	Follicular cell hypertrophy	+	1	0
Adrenal gland	Accessory adrenocortical tissue	+	0	1

+: slight

[a)]: Number of animals showing pathological findings.

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