Available online at www.sciencedirect.com
SCIENCE DIRECT
Phytochemistry 67 (2006) 1143–1150
PHYTOCHEMISTRY
www.elsevier.com/locate/phytochem

Immunosuppressive diacetylenes, ceramides and cerebrosides from *Hydrocotyle leucocephala*

Freddy Ramos [a], Yoshihisa Takaishi [a,*], Kazuyoshi Kawazoe [a], Coralia Osorio [b], Carmenza Duque [b], Ricardo Acuña [c], Yoshinori Fujimoto [d], Mitsunobu Sato [e], Masato Okamoto [e], Tetsuya Oshikawa [e], Sharif Uddin Ahmed [e]

[a] *Pharmacognosy Laboratory, Graduate School of Pharmaceutical Sciences, University of Tokushima, Shomashi 1-78, Tokushima 770-8505, Japan*
[b] *Departamento de Química, Universidad Nacional de Colombia, AA 14490, Bogota, Colombia*
[c] *Centro Nacional de Investigaciones del Café, Planalto Chinchina, Colombia*
[d] *Department of Chemistry and Material Science, Tokyo Institute of Technology, Meguro, Tokyo 152-8551, Japan*
[e] *School of Dentistry, University of Tokushima, Kuramoto-cho, Tokushima 770-8504, Japan*

Received 8 October 2005; received in revised form 18 February 2006
Available online 2 May 2006

Abstract

Three C-17 diacetylenic compounds (**1**–**3**), one monoterpenoid (**4**), seven ceramides (leucoceramides A–G, **5a**–**g**), six cerebrosides (leucocerebrosides A–F, **6a**–**f**) and nine known compounds were isolated from the methanolic extract of *Hydrocotyle leucocephala*. Their structures were established by spectroscopic methods. The isolated compounds **1**–**3**, **5a**–**g**, **6a**–**f** and **7** were shown to be active in the lipopolysaccharide (LPS) induced cytokine production assay for IL-10, IL-12, and TNF-α.

Keywords: *Hydrocotyle leucocephala*; Apiaceae; C-17 diacetylenes; 4-hydroxy-4-(1-hydroxy-1-methylethyl)-cyclohex-2-en-1-one; Leucoceramides; Leucocerebrosides; Cytokine production assay

1. Introduction

Hydrocotyle leucocephala Cham. & Schlecht. (Apiaceae), commonly known as ''orejitas'', ''sombrillita'', or Brazilian pennyworth, is an aquatic plant originally from Brazil, and widespread worldwide due to its use as an ornamental aquarium plant. The plant is used as a diuretic, antihelminthic and antidiarrheal in Colombia, and is also used as a bioremediator to remove inorganic nutrients from fresh water in recirculating aquaculture systems in Korea (Ma et al., 2003). Previous chemical studies of members of the genus *Hydrocotyle* have resulted in isolation of quercetin-3-*O*-galactoside from *H. umbellata* L. (Adams et al., 1998), *trans*-β-farnesene, thymol methyl ether and α-terpinenes from *H. sibthorpioides* Lam. and *H. maritima* Honda (Asakawa et al., 1982), monogalactosylmonoacylglycerols and other known compounds from *H. ramiflora* Maxim. (Kwon et al., 1998), oleane and ursane type glycosides from *H. ranunculoides* Blume (Della Greca et al., 1994a,b), *H. sibthorpoides* (Matsushita et al., 2004), and *Centella asiatica* Urb. (Matsuda et al., 2001), as well as fourteen C-15 diacetylene compounds from *H. sibthorpoides* (Shulte et al., 1973).

As part of our study of medicinal plants collected in Colombia, we report the isolation, identification, and biological evaluation of three new diacetylenes, a monoterpenoid, a mixture of seven leucoceramides (**5a**–**g**), a mixture of six leucocerebrosides (**6a**–**f**), along with nine other known compounds (**7**–**15**) obtained from the methanolic extract of *H. leucocephala*. The mixtures were identified through chemical degradation analyses. The purified compounds

* Corresponding author. Tel.: +81 886337275; fax: +81 886339501.
E-mail address: takaishi@ph.tokushima-u.ac.jp (Y. Takaishi).

0031-9422/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2006.03.004

were evaluated for their potential immunosuppressive activity, by testing in a LPS-induced cytokine induction assay.

2. Results and discussion

The methanolic extract of *Hydrocotyle leucocephala* was partitioned between EtOAc, BuOH, and H_2O. The EtOAc layer was fractionated by column chromatography and HPLC to give three new C-17 diacetylenic compounds (**1**–**3**), one monoterpenoid (**4**), mixtures of seven ceramides (**5a**–**g**) and six galactocerebrosides (**6a**–**f**) as well as nine known compounds (**7**–**15**).

Compound **1** was obtained as yellow oil. It showed IR absorptions for hydroxyl groups (broad, 3374 cm^{-1}), acetylenic carbons (2235 and 2150 cm^{-1}), and double bonds (1646 cm^{-1}). The UV spectra showed the absorption maxima at 287 (3.00), 269 (3.07), 253 (3.11) nm. These spectroscopic data are characteristic for acetylenic compounds (Shim et al., 1987). The ^1H NMR spectrum (Table 1) showed signals for a vinyl group at δ_H 5.94 (*ddd*, 1H, $J = 17.0$, 10.2, 5.2 Hz), 5.48 (*d*, 1H, $J = 17.0$ Hz), and 5.27 (*d*, 1H, $J = 10.2$ Hz), four hydroxymethines at δ_H 4.96 (*d*, 1H, $J = 5.2$ Hz), 4.55 (*d*, 1H, $J = 6.5$ Hz), 4.27 (*dt*, 1H, $J = 8.7$, 4.0 Hz), and 3.74 (*dd*, 1H, $J = 6.5$, 4.0 Hz), and a carbon chain with a terminal methyl. The ^{13}C NMR spectrum (Table 1) and DEPT experiments exhibited resonances for a vinyl group (δ_C 135.9 and 117.8), four hydroxymethines (δ_C 63.7, 64.6 × 2 and 75.9), four signals assignable to acetylenic carbons (δ_C 78.7, 78.2, 70.9, 70.3), in addition to an aliphatic chain of six methylenes and a terminal methyl. These data suggest a molecular formula of $C_{17}H_{26}O_4$ in agreement with the adduct ion LRFABMS at *m/z* 299 $[M+Na-H_2O]^+$. The foregoing data indicated that **1** is a C-17 diacetylenic compound with a vinyl group and four hydroxyls in its structure. Several C-17 diacetylenic derivatives have been isolated from *Panax ginseng* roots, (Shim et al., 1987; Hirakura et al., 1992) and the ^{13}C and ^1H NMR spectroscopic data of **1** were very similar to those of ginsenoynes I–K by Hirakura et al. (1992). However, some differences were detected, in particular, the presence of four hydroxyl groups never before reported for acetylenic compounds. ^1H–^1H correlations between the resonance at δ_H 4.96 with the vinyl protons at δ_H 5.94, 5.48 and 5.27, and HMBC correlations for this same proton with the vinyl carbons (δ_C 135.9, 117.8) and one acetylenic carbon (δ_C 78.7), permitted the assignment of a hydroxyl at position C-3. In the same way, the COSY spectrum showed correlations between the signals at δ_H 4.55, 3.74 and 4.27, and the HMBC spectrum showed correlations for the hydroxymethine resonance at δ_H 4.55 with the acetylenic carbon at δ_C 78.2 leading to assignments of the hydroxyls at positions C-8, C-9 and C-10 as well as that of the acetylenic carbons. Additionally, the hydroxymethine resonance at δ_H 4.27 showed HMBC correlations with the methylene signal at δ_C 34.8. These correlations allowed us to establish that compound **1** was 1-heptadecen-4,6-diyne-3,8,9,10-tetraol. The stereochemistry for compound **1** at the triol system (C-8, C-9 and C-10) was estimated as α, α, β by the method proposed by Higashibayashi et al. (2003), where the value of the coupling constant for a triol system was characteristic for each diasteromer measured in CD_3OD, D_2O, DMSO or d_6-pyridine. From that, the coupling constant

Table 1
^1H- and ^{13}C NMR spectroscopic data for compounds **1**–**3** ($CDCl_3$)

	1		**2**		**3**	
	δ_H[a]	δ_C[b]	δ_H[a]	δ_C[b]	δ_H[a]	δ_C[b]
1	5.48, *d*, 1H, (17.0)	117.8	5.50, *d*, 1H, (17)	117.8	5.48, *d*, 1H, (17.0)	117.5
	5.27, *d*, 1H, (10.2)	–	5.28, *d*, 1H, (10.0)	–	5.29, *d*, 1H, (10.2)	–
2	5.94, *ddd*, 1H, (17.0, 10.2, 5.2)	135.9	5.94, *ddd*, 1H, (17.0, 10.0, 5.2)	135.9	5.95, *ddd*, 1H, (17.0, 10.2, 5.2)	135.6
3	4.96, *d*, 1H, (5.2)	63.7	4.95, *d*, 1H, *d*, (5.2)	63.7	4.95, *d*, 1H, (5.2)	63.5
4	–	78.7	–	78.8	–	78.6
5	–	70.3	–	71.2	–	70.0
6	–	70.9	–	70.2	–	70.6
7	–	78.2	–	74.9	–	76.1
8	4.55, *d*, 1H, (6.5)	64.6	5.47, 1H, *d*, (6.6)	65.7	4.78, *d*, 1H, (7.3)	62.7
9	3.74, *dd*, 1H, (6.5, 4.0)	75.9	3.91, *dd*, 1H, (6.6, 3.1)	74.4	5.15, *dd*,1H, (7.3, 2.9)	75.8
10	4.27, *dt*, 1H, (8.7, 4.0)	64.6	4.10, *ddd*, 1H, (8.5, 4.4, 3.1)	63.9	4.29, *ddd*, 1H, (8.3, 5.5, 2.9)	60.8
11	1.79, *m*, 2H	34.8	1.86, *m*, 2H	35.0	1.72, *m*, 2H	34.6
12	1.45, *brm*, 1H	26.7	1.52, *m*, 1H	26.8	1.55, *m*, 1H	26.4
	1.39, *brm*, 1H	–	1.42, *m*, 1H	–	1.41, *m*, 1H	–
13	1.29, *m*, 2H	29.4	1.29, *m*, 2H	29.4	1.29, *m*, 2H	29.0
14	1.29, *m*, 2H	29.3	1.29, *m*, 2H	29.3	1.29, *m*, 2H	28.9
15	1.29, *m*, 2H	32.0	1.29, *m*, 2H	32.0	1.29, *m*, 2H	31.7
16	1.29, *m*, 2H	22.9	1.29, *m*, 2H	22.9	1.29, *m*, 2H	22.6
17	0.88, *brt*, 3H, (7.0)	14.4	0.89, *brt*, 3H, (7.0)	14.4	0.89, *brt*, 3H, (7.0)	14.0
COO	–	–	–	169.4	–	170.3
CH_3	–	–	2.14, 3H, *s*	21.0	2.19, 3H, *s*	20.7

[a] δ_H (400 MHz) in ppm, (*J* in Hz).
[b] δ_C (100 MHz) in ppm.

for a 8α,9α,10α system in methanol should have a coupling constant value $J_{8,9} = J_{,9,10,} = 4.0$ Hz, for 8α, 9β, 10α, $J_{8,9} = J_{,9,10,} = 7.0$ Hz; for 8α, 9α, 10β $J_{8,9} = 8.0$ Hz, $J_{,9,10,} = 2.5$; and for 8α, 9β,10β $J_{8,9} = 2.5$ Hz; $J_{,9,10,} = 8.0$ Hz. These values are reported in the supporting information of the cited reference, and C-10 was adopted as the terminal extreme to apply the model. The obtained values for compound **1** coupling constant in CD_3OD at positions 8,9,10 were $J_{8,9} = 8.8$ Hz, $J_{,9,10,} = 2.0$ in agreement with an α,α,β system. From this analysis the structure of compound **1** was established as is shown in Fig. 1.

Compound **2**, isolated as a pale yellow oil, showed very similar UV data to that of **1** and the IR spectrum had the same absorption bands, with the exception of an absorption for a carbonyl group at 1749 cm^{-1}. The ^1H- and ^{13}C NMR spectroscopic data for **2** were very similar to those obtained for **1** (Table 1), except for the presence of an acetyl group with methyl signals at δ_H 2.14 (*s*, 3H), δ_C 21.0 and a carbonyl at δ_C 169.4. This information, and the LRFABMS data, suggested that the molecular formula of **2** is $C_{19}H_{28}O_5$ (*m/z* 341 $[M+Na-H_2O]^+$). From the COSY spectrum, the hydroxymethines at δ_H 5.47 (*d*, 1H, $J = 6.6$ Hz), 3.91 (*dd*, 1H, $J = 6.6$, 3.1 Hz) and 4.10 (*ddd*, 1H, $J = 8.5$, 4.4, 3.1 Hz) were assigned at the C-8, C-9 and C-10 positions, respectively. The chemical shift for the hydroxymethine at δ_H 5.47, and its correlations in the HMBC spectrum with the hydroxymethine resonance at C-10 at δ_C 63.9 and the carbonyl at δ_C 169.4 allowed us to establish that compound **2** is 1-heptadecen-4,6-diyne-8-acetyl-3,9,10-triol. From comparison of the coupling constants for compounds **1** and **2** in the triol system (see Table 1), an 8α,9α,10β stereochemistry was adopted.

Compound **3** was assigned a molecular formula $C_{19}H_{28}O_5$ from the LRFABMS data, and gave UV and IR data closely related to **2**. This suggested that compound **3** is a C-17 diacetylenic compound with a vinyl terminal, three hydroxyl groups, and an acetyl group in its structure. However, from these data a different position of the acetyl group in **3** was detected according to the chemical shifts observed by ^1H and ^{13}C NMR spectroscopy for hydroxymethines at C-8, C-9 and C-10 (Table 1). The COSY spectrum allowed the assignment of the hydroxymethine signals at δ_H 4.78 (*d*, 1H, $J = 7.3$ Hz), 5.15 (*dd*, 1H, $J = 7.3$, 2.9 Hz), and 4.29 (*ddd*, 1H, $J = 8.3$, 5.5, 2.9 Hz) to the C-8, C-9 and C-10 positions, respectively. In the HMBC spectrum of **3**, correlations between the hydroxymethine signal at δ_H 5.15 (*dd*, 1H, $J = 7.3$, 2.9 Hz), assigned to H-9 from the COSY spectrum and the carbonyl signal at δ_C 170.3 were detected. Accordingly, **3** was identified as 1-heptadecen-4,6-diyn-9-acetyl-3,8,10-triol. From the

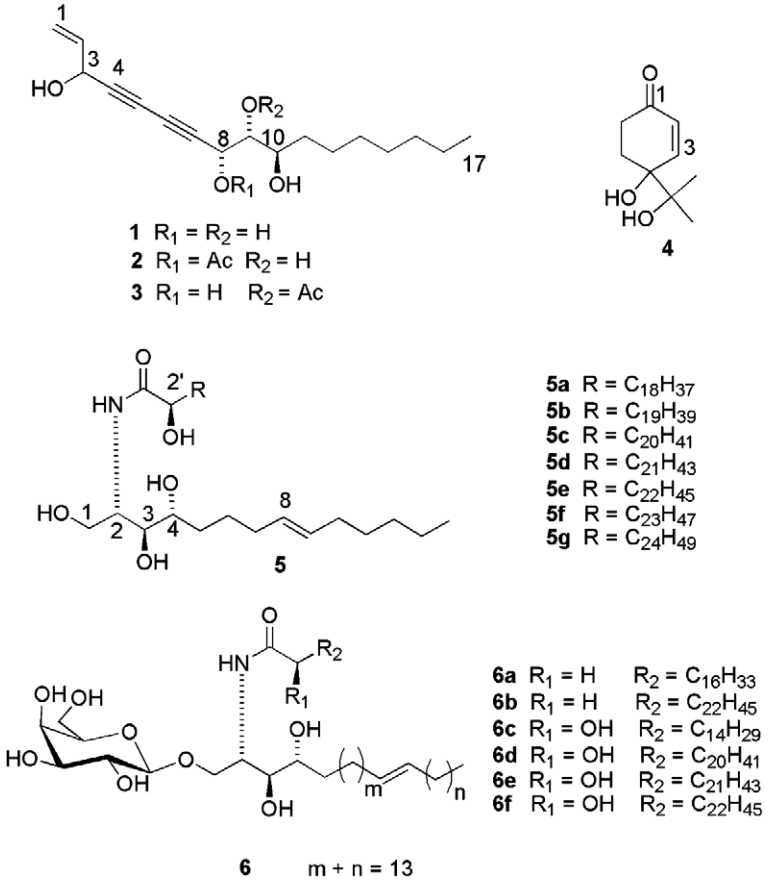

Fig. 1. Structure of the new compounds isolated from *H. leucephala.*

comparison of the coupling constants for compounds **1**, **2** and **3** in the triol system (see Table 1), an 8α,9α,10β stereochemistry was adopted.

Compounds **1**–**3** have the same stereochemistry at C-9 and C-10 to that of panaxytriol, a related compound isolated form *Ginseng radix rubra*, but without an hydroxyl at C-8. The stereochemistry of C-9 and C-10 for panaxitryol results from the opening of the epoxide ring of its precursor panaxydol (Kobayashi et al., 1997). From that, the new acetylenic compounds probably derive from the 1-heptadecen-4,6-diyne-9,10-epoxy-3,8,-diol, previously isolated from *Panax quinquefolium* (Fujimoto et al., 1991). However, this compound could not be detected in the extract.

Compound **4** has a molecular formula of $C_9H_{14}O_3$ according to the HRFABMS data. The IR spectrum gave absorption bands for OH at 3405 cm^{-1} and an α,β-unsaturated ketone with an absorption at 1683 cm^{-1}. The UV spectrum gave an absorption for an α,β-unsaturated cyclic ketone at 215 nm. The ^1H and ^{13}C NMR spectroscopic data for this compound confirmed the presence of a *cis* α,β-unsaturated ketone with signals at δ_H 7.06 (*d*, 1H, $J = 10.3$ Hz), δ_C 148.3; 6.06 (*d*, 1H, $J = 10.3$ Hz), δ_C 130.3, and a carbonyl at δ_C 199.6. The NMR spectroscopic data also showed the presence of a methylene group with resonances at δ_H 2.79 (*ddd*, 1H, $J = 16.8$, 10.6, 10.6 Hz) and 2.46 (*dt*, 1H, $J = 16.8$, 4.2 Hz) and δ_C 33.7; a second methylene with resonances at δ_H 2.12 (*ddd*, 1H, $J = 14.8$, 10.6, 4.2 Hz), 2.10 (*ddd*, 1H, $J = 14.8$, 10.6, 4.2 Hz) and δ_C 29.7; two quaternary carbons, each one bonded to oxygen at δ_C 74.4 and 73.1, and two methyls at δ_H 1.33 (*s*, 3H) δ_C 24.1 and δ_H 1.23 (*s*, 3H) and δ_C 25.0. From the HMBC spectrum, a cyclohexenone substructure was identified for the correlations between the methylene protons at δ_H 2.79 and 2.46 and the carbon resonances for the methylene, the carbonyl and the quaternary carbon at δ_C 29.7, 199.6 and 73.1, respectively. The methyl signals at δ_H 1.33 (*s*, 3H) and δ_H 1.23 (*s*, 3H) were shown to be geminal and correlated with the quaternary carbons at δ_C 74.4 and 73.1. These correlations allowed us to identify compound **4** as 4-hydroxy-4-(1-hydroxy-1-methylethyl)-cyclohex-2-en-1-one.

From fraction E-14.5, was obtained a white amorphous solid of a mixture of compounds **5a**–**g** by crystallization in MeOH. Analysis of the ^1H NMR spectroscopic data (Table 2) showed characteristic signals for ceramides at δ_H 8.57 (*d*, $J = 8.7$ Hz) for the amide protons; resonances at δ_H 5.10 (m, 1H) for the methines bonded to nitrogen, and signals for a hydroxymethylene at δ_H 4.49 (*dd*, 1H, $J = 10.3$, 4.2 Hz) and 4.42 (*dd*, 1 H, $J = 10.3$, 4.1 Hz). Additionally, signals for three hydroxymethines at δ_H

Table 2
^1H- and ^{13}C NMR spectroscopic data for compounds **5a**–**g** and **6a**–**f** (d_5-pyridine)

	5a–**g**			**6a**–**f**	
	δ_H[a]	δ_C[b]		δ_H[a]	δ_C[b]
1	4.49, *dd*, 1H, (10.3, 4.2)	62.0	1	4.70, *dd*, 1H, (10.6, 6.6)	70.5
	4.42, *dd*, 1H, (10.3, 4.1)	–		4.51, *d*, 1H, (10.6, 4.3)	–
2	5.10, *m*, 1H	52.9	2	5.27, *m*, 1H	51.8
3	4.33, *dd*, 1H, (7.9, 4.7)	76.8	3	4.28, *d*, 1H, (4.7)	75.9
4	4.28, *brt*, 1H, (7.9)	72.8	4	4.57, brs, 1H	75.2
5	2.26, *m*, 2H	33.8	5	2.25, *m*, 2H	33.9
6	1.70, *m*, 2H	26.7	6	1.68, *m*, 2H	26.7
7	2.21, *m*, 2H,	33.3	7–18[c]	2.21, *m*, 2H	33.4
8	5.52, *ddd*, 1H, (16.7, 6.0, 5.6)	130.8	7–18[c]	5.52, *ddd*, 1H, (16.7, 6.0, 5.6)	130.9
9	5.48, *ddd*, 1H, (16.7, 6.0, 5.6)	130.7	7–18[c]	5.49, *ddd*, 1H, (16.7, 6.0, 5.6)	130.7
10	2.00, *m*, 2H	33.0	7–18[c]	1.98, *m*, 2H	33.0
11	1.25, *s*	25.8	7–18[c]	1.24, *s*	30.0
12	1.25, *s*	32.1	19	1.24, *s*	32.2
13	1.25, *s*	22.9	20	1.24, *s*	22.9
14	1.25, *s*	14.3	21	1.24, *s*	14.3
1′	–	175.3	1′	–	175.8
2′	4.61, *brs*, 1H	72.4	2′	4.57, *brt*, 1H, (4.0)	72.5
3′	2.02, *m*, 2H	35.7	3′	2.02, *m*, 2H	35.6
4′	1.73, *m*, 2H	25.8	4′	1.73, *m*, 2H	25.7
1″	–	–	1″	4.90, *d*, 1H, (7.9)	105.6
2″	–	–	2″	4.00, *dd*, 1H, (7.9, 8.2)	75.2
3″	–	–	3″	4.18, *d*, (8.2)	78.6
4″	–	–	4″	4.19, *brs*, 1H	71.5
5″	–	–	5″	3.86, *brs*, 1H	78.5
6″	–	–	6″	4.48, *d*, 1H, (12)	62.8
	–	–		4.34, *dd*, (12, 5.4)	–
N–H	8.57, *d*, (8.7)	–	N–H	8.56, *d*, (9.2)	–

[a] δ_H (400 MHz) in ppm, (J in Hz).
[b] δ_C (100 MHz) in ppm.
[c] Position of the double bond could not be determined.

4.33 (*dd*, 1H, $J = 7.9$, 4.7 Hz), δ_H 4.28 (*brt*, 1H, $J = 7.9$ Hz), δ_H 4.61 (*brs*, 1H), a double bond at δ_H 5.52 (*ddd*, 1H, $J = 16.7$, 6.0, 5.6 Hz), 5.48 (*ddd*, 1H, $J = 16.7$, 6.0, 5.6 Hz) and two aliphatic chains with resonances at δ_H 2.26, 2.02, 1.73, 1.70 and 1.25 were observed. The ^{13}C NMR spectrum (Table 2) showed the resonances for a hydroxymethylene at δ_C 62.0, three hydroxymethines at δ_C 76.8, 72.8, 72.4, a methine bonded to nitrogen at δ_C 52.9, the olefinic carbons at 130.8 and 130.7, a carbonyl at δ_C 175.3, and resonances for two aliphatic chains. The methine at δ_H 5.10 exhibited correlations in the COSY NMR spectrum with the amide proton (δ_H 8.57), the hydroxymethylene (δ_H 4.49, 4.42) and the hydroxymethine at δ_H 4.33. The same methine proton (δ_H 5.10) showed correlations in the HMBC spectrum with the hydroxymethylene (δ_C 62.0), the carbonyl (δ_C 175.3), and the hydroxymethines at δ_C 76.8 and 72.8. The hydroxymethine at δ_H 4.61 was correlated in the HMBC spectrum with the carbonyl at δ_C 175.3. Therefore, compounds **5a**–**g** are suggested to have a 2-hydroxy fatty acid moieties with a normal chain and a 1,3,4-trihydroxy substitution in the sphingosine moiety (Yamada et al., 1998). In order to determine the length of each chain, compounds **5a**–**g** were methanolyzed with methanolic hydrochloric acid, obtaining a mixture of fatty acid methyl esters and the sphingosine moieties, and each fraction was analyzed by GC-MS. The mixture of methyl esters revealed the presence of 2-hydroxyeicosanoic acid, 2-hydroxyeneicosanoic acid, 2-hydroxydocosanoic acid, 2-hydroxytricosanoic acid, 2-hydroxytetracosanoic acid, 2-hydroxypentacosanoic acid, and 2-hydroxyhexacosanoic acid methyl esters in ratios 1:2:25:16:43:5:3, respectively. The sphingosine fraction was analyzed by GC-MS as a TMSi derivative. In the gas chromatogram for this fraction was observed three peaks with ions at *m/z* 331 $[M+TMSi-H]^+$, 405 $[M+2TMSi]^+$, and 479$[M+3TMSi+H]^+$. These ions correspond to adducts of a sphingosine moiety with a molecular formula $C_{14}H_{29}NO_3$ bearing one, two and three TMSi derivative groups, respectively. From these data, and the NMR spectroscopic correlations, the presence of 2-amino-1,3,4-trihydroxy-8-tetradecene was found as the only sphingosine. The position of the double bond was proposed on the basis of the strong ion at *m/z* 417 $[M+(TMSi)_3\text{-}H\text{-}57]^+$, observed in the mass spectrum of the tri-TMSi derivative, probably due to allylic fragmentation at position C-10. The double bond *trans* geometry was established by the coupling constant values and the chemical shift of the methylene carbon adjacent to the olefinic carbon at δ_C 33.0 and 33.4 (Yamada et al., 1998). Chemical shifts in the ^1H and ^{13}C NMR spectra and the optical rotation value for the mixture of compounds **5a**–**g** were shown to be nearly identical with those reported for the ceramide *N*-(2′*R*-hydroxynonacosanoyl)-D-*erythro*-2,3,4-trihydroxy-2-amino-octadecane (Lourenço et al., 1996). As a result of the comparison, the stereochemistry is presumed to be 2*S*, 3*S*, 4*R* for the ceramide moiety and 2′*R* for the fatty acid moiety. From the above analysis, the structures of the ceramide mixture **5a**–**g** were determined as shown in Fig. 1.

From fraction E-14.11, compounds **6a**–**f** were obtained as white crystals by sequential passages over Sephadex LH-20 and CC. Analysis of the NMR spectra (^1H and ^{13}C, Table 2) showed characteristic signals for cerebrosides (Loukaci et al., 2000) at δ_H 8.56 (d, $J = 9.2$ Hz) for the amide proton; a galactose moiety, a 2-hydroxy fatty acid with a normal chain, and a 1,3,4-trihydroxy substituted sphingosine, the same as the sphingosine moiety of compounds **5a**–**g**. Positive FAB MS showed a series of ions at *m/z* 754–908. To identify the length of each chain (fatty acid and sphingosine), compounds **6a**–**f** were treated with methanolic hydrochloric acid, to give a mixture of fatty acid methyl esters and the sphingosine moieties with methyl galactoside; both fractions were analyzed by GC-MS in the same way as compounds **5a**–**g**. The mixture of methyl esters revealed the presence of octadecanoic acid, tetracosanoic acid, 2-hydroxyhexadecanoic acid, 2-hydroxydocosanoic acid, 2-hydroxytricosanoic acid, and 2-hydroxytetracosanoic acid methyl esters in ratios 3:2:2:4:2:3, respectively. The sphingosine fraction was analyzed by GC-MS as a TMSi derivative. In this fraction the presence of 2-amino-1,3,4-trihydroxyeneicosene was found as the only sphingosine due to the only one peak present at R_t 4.00 min and the adduct ion at *m/z* 429, corresponding to $[M+TMSi-H]^+$. Stereochemistry was adopted as 2*S*, 3*S*, 4*R* and 2′*R* by comparison of the chemical shifts reported for homologous galactocerebrosides (Loukaci et al., 2000). The *trans* geometry of the double bond was established by the coupling constant values and the chemical shift of the methylene carbon adjacent to the olefinic carbon at δ_C 33.0 and 33.4 (Yamada et al., 1998). The position of the double bond could not be assigned because of the small amount of sphingosine obtained. Accordingly, the structures of the cerebrosides mixture **6a**–**f** were determined as shown in Fig. 1.

Additionally, nine known compounds were identified by comparison of their spectroscopic data with literature values as follows: (−)-hinokinin **7** (da Silva et al., 2005), spinasterol **8** (Kojima et al., 1990), loliolide **9** (Okada et al., 1994), dehidrovomifoliol **10** (Kato et al., 1977), 3-hydroxy-5α,6α-epoxy-7-megastigmen-9-one **11** (Duan et al., 2002), kaempferol-3-*O*-α-L-arabinopyranoside **12** (Väsange et al., 1997), quercetin-3-*O*-α-L-arabinopyranoside **13** (Almeida et al., 1998), quercetin-3-*O*-β-D-galactopyranoside **14** (Arisawa et al., 1984), and kaempferol-3-*O*-β-D-galactopyranoside **15** (Scharbert et al., 2004).

The effects of the test compounds in LPS-induced cytokine production are shown in Table 3, with data expressed as ratios to cytokine production induced by LPS. Compound **1** inhibited LPS-induced IL-10 production but not production of TNF-α and IL-12. Compounds **2**, **3** and **7** inhibited production of all cytokines tested. Although LPS-induced IL-10 was inhibited by compounds **4**, **5a**–**g**, **6a**–**f**, **12**–**15**, these compounds potentiated rather than inhibited the production of IL-12 induced by LPS. No significant effect of these compounds on TNF-α production

Table 3
Inhibitory effects of compounds **1**–**7**, **9**, **12**–**15** and prednisolone on cytokine production

Treatment	Cytokine production ratio[a]		
	TNF-α	IL12	IL10
LPS alone	1	1	1
1	0.79	0.98	ND[b]
2	0.72	0.57	ND[b]
3	0.4	ND[b]	ND[b]
4	0.91	2.56	0.34
5a–**g**	1	2.02	0.34
6a–**f**	1.13	2.92	0.23
7	0.36	0.44	0.37
9	1.05	3.44	0.35
12	0.89	2.48	0.35
13	0.99	2.73	0.37
14	1.06	2.86	0.36
15	0.88	3.01	0.42
Prednisolone	0.6	0.2	0.41

[a] Cytokine production ratios were expressed as ratios to cytokine production induced by LPS. For protocols used, see Section 3. Concentrations used: test compounds 10 μg/ml; prednisolone, 0.3 μg/ml.
[b] ND, not detected.

was observed. Inhibition of the cytokine production rate for TNF-α and IL-12 was markedly more potent for the acetylenic compounds **2** and **3** compared with **1**, and suggests some importance of the acetyl group in the activity of these compounds against Th1 cells. For compounds **5a**–**g** and **6a**–**f**, inhibition of Th2 cells was increased by the presence of the galactose moiety, according to the data for IL-10. For the TNF-α and IL-12 data, no structural relationships could be observed for these compounds. Compound **7**, like other related γ-valerolactone type lignans showed activity as a regulator of TNF-α production (Scharbert et al., 2004). Activity of this type of lignans is dose-dependent.(Yeşilada et al., 2001). These findings indicated that compounds **2**, **3**, and **7** are inhibitors against the whole immune response, while compound **1** is a strong Th2 inhibitor, and compounds **4**, **5a**–**g**, **6a**–**f**, **8** and **12**–**15** act as Th1 inducers as well as Th2 inhibitors. This suggests that these compounds, from *H. leucocephala* may be effective therapeutic agents for Th2-type diseases.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured with a JASCO DIP-370 digital polarimeter. UV spectra were recorded on a UV2100 UV–vis recording spectrometer (Shimadzu). IR spectra were recorded on a JASCO Fourier transform infrared spectrometer (FT/IR-420). NMR spectra (400 MHz for ^{1}H NMR, 100 MHz for ^{13}C, both use TMS as internal standard) were measured on a Bruker AVANCE 400 spectrometer. EIMS were obtained on a JEOL JMSDX-303 and FABMS run on a SX-102A instrument. The solid supports for column chromatography and gel permeation chromatography (GPC) were: silica gel 60N (Spherical Neutrial, Kanto Kagaku), Toyopearl HW-40 (Tosoh Bioscience), and Sephadex LH-20 (Pharmacia). The packed columns used were: Shodex H-2001, 2002 for GPC ($CHCl_3$); Shodex Asahipack GS310-2G for GPC (MeOH); Mightysil Si60 (250 × 20 mm; Merck) for HPLC normal phase and Mightysil RP-18 (250 × 20 mm; Merck) for HPLC-ODS. For HPLC JASCO equipment was used with detectors UV at 256 nm and RI. For GC-MS an HP-5870 series instrument was used, coupled to a mass analyzer Automass JMS-AM150 (JEOL). A DB-1 (30 m × 0.25 mm × 25 μm film thickness) column was utilized. The injector temperature was maintained at 250 °C. The column oven was programmed from 50 to 300 °C, at 30°/min. The carrier gas flow (He) was at 1.0 mL/min. The ratios of compounds were determined by area peaks in the chromatogram. All other solvents used for this work were analytical grade.

3.2. Plant material

Aerial parts of *Hydrocotyle leucocephala* Cham. & Schlecht. were collected in October 2002 from Manizales City in the central part of Colombia. A voucher specimen was identified by Dr. Julio Betancourt at the Instituto de Ciencias Naturales at Universidad Nacional de Colombia and registered as COL495157 specimen number 15. The plant material was dried in air.

3.3. Extraction and isolation

The dried aerial parts from *Hydrocotyle leucocephala* (662 g) were crushed and extracted three times with MeOH at 60 °C. Then, the methanolic extract was concentrated in vacuo to give a residue (102.6 g), which was partitioned between EtOAc, BuOH, and H_2O. The EtOAc layer was concentrated to obtain a residue (21 g), which was fractionated by silica gel CC eluted with different solvents of increasing polarity (hexane – EtOAc, EtOAc – MeOH) to obtain 15 fractions (E1–E15).

Fraction E7 (0.9 g) was further fractionated by silica gel CC with a discontinuous gradient of $CHCl_3$–MeOH (100:0–90:10) as eluent to give six fractions (E7.1–E7.6). In turn, fraction E7.1 (606 mg) was purified over silica gel column with a discontinuous gradient of $CHCl_3$–MeOH (100:0–98:2) to obtain 10 fractions (E7.1.1–E7.1.10), from that, fractions E7.1.1–E7.1.3 (84 mg) were combined and then separated by GPC ($CHCl_3$) to afford **7** (43 mg) and **8** (4 mg). Fraction E7.1.6 was subjected to GPC ($CHCl_3$) to yield **2** (3 mg) and **3** (2 mg). Fraction E9 (2.1 g) was fractionated by silica gel CC with a discontinuous gradient of $CHCl_3$–MeOH (100:0–90:10) as eluent to give 14 fractions (E9.1–E9.14). Compound **1** (12 mg) was isolated by GPC ($CHCl_3$) from E9.12.

The mixture of E11–E13 (2.2 g) was fractionated by silica gel CC with a discontinuous gradient of $CHCl_3$–MeOH (100:0–90:10) as eluent to give nine fractions (E11.1–E11.9). From these, E11.2 (510 mg) was fractionated by GPC using Toyopearl HW-40 with $CHCl_3$–MeOH (1:1)

as eluent to give six fractions (E11.2.1–E11.2.6). E11.2.5 (80 mg) was selected for further fractionation by GPC ($CHCl_3$), and led to seven fractions (E11.2.5.1–E11.2.5.7). From that, Fraction E11.5.7 corresponds to **9** (7 mg). Fraction E11.2.4 (100 mg) was isolated by GPC ($CHCl_3$) to produce eight additional fractions (E11.2.4.1–E11.2.4.8). From these, fractions E11.2.4.4 and E11.2.5.4 (18 mg) were recombined and fractionated by HPLC on a silica gel column with $CHCl_3$–MeOH 95:5 to obtain **10** (2 mg) and **11** (3 mg). From E11.9 was obtained **4** (5 mg) by two successive GPC purifications with $CHCl_3$ as solvent.

Fractions E14 and E15 (5.1 g) were recombined and then fractionated by silica gel CC with a discontinuous gradient of $CHCl_3$–MeOH (90:10–7:3) to give 16 further fractions (E14.1–E14.16). From that, fractions E14.11–E14.12 (1.6 g) were recombined and then fractionated by Sephadex LH-20 with MeOH as eluent to give **12** (18 mg) and **13** (12 mg). Fractions E14.14 and E14.15 (1.3 g), were combined and then fractionated by Sephadex LH-20 with MeOH as eluent to give **14** (378 mg) and **15** (90 mg). From E14.11.5 was obtained the mixture of ceramides **5a**–**g** (7 mg) by crystallization in MeOH. Finally silica gel CC of E14.11.6–E14.11.7 using a discontinuous gradient of $CHCl_3$–MeOH (95:5–7:3) led to purification of **6a**–**f** (5 mg).

3.4. Methanolysis of *5a–g* and *6a–f*

Analysis of compounds present in fractions **5** (6 mg) and **6** (2 mg) was made after methanolysis with 5% HCl in MeOH (3 mL) at 70 °C for 18 h (Yamada et al., 2001). The reaction mixture was extracted with hexane and concentrated in vacuo to obtain the mixtures of fatty acid methyl esters for analysis by GC-MS. The methanol layer was concentrated in vacuo and redissolved in MeOH four times in order to remove the acidic residues and obtain the sphingosine moiety and methyl galactoside. The sphingosine moiety was analyzed by GC-MS as trimethylsilyl (TMSi) ethers using *N*-trimethylsilylimidazole (merck)/pyridine (1:1) for 1 h at 70 °C for the derivatization process. Then, the TMSi ethers of sphingosine were analyzed using the same chromatographic method as for the fatty acid methyl esters. Ratios between compounds were established according to their area peaks by GC.

3.5. 1-Heptadecen-4,6-diyn-3,8α,9α,10β- tetraol (*1*)

Pale yellow oil, $[\alpha]_D^{25}$ + 5.5 (MeOH; *c* 1.08); UV λ_{max}^{MeOH}nm (logε) 287 (3.00), 269 (3.07), 253 (3.11); IR ν_{max}^{KBr} cm^{-1}: 3374, 2360, 2340, 2235, 2150; for ^1H and ^{13}C NMR spectra, see Table 1; EIMS 70 eV, *m/z* (rel. int): 238 (3), 203 (7), 195 (1), 165 (3), 149 (5), 134 (34), 119 (56), 118 (100); FABMS *m/z* 299 $[M+Na-H_2O]^+$, 277 $[M+H-H_2O]^+$.

3.6. 1-Heptadecen-4,6-diyn-8α-acetyl-3,9α,10β-triol (*2*)

Pale yellow oil, $[\alpha]_D^{25}$ − 6.0 (MeOH; *c* 0.9); UV λ_{max}^{MeOH} nm (log ε) 284 (3.99), 269 (4.08), 254 (4.08); IR ν_{max}^{KBr} cm^{-1}: 2359, 2340, 2239, 2145, 1749; for ^1H and ^{13}C NMR spectra, see Table 1; EIMS 70 eV, *m/z* (rel. int): 354 (1), 319 (1), 309(1), 259(7), 207 (10), 152 (32) 150 (52), 136(57), 134 (95); FABMS *m/z* 341 $[M+Na-H_2O]^+$, 281 $[(M+Na)-H_2O-CH_3COOH]^+$.

3.7. 1-Heptadecen-4,6-diyn-9α-acetyl-3,8α,10β-triol (*3*)

Pale yellow oil, $[\alpha]_D^{25}$ + 24.0 (MeOH;*c*0.2); UV λ_{max}^{MeOH}nm (log ε) 284 (2.87), 269 (2.99), 256 (3.04); IR ν_{max}^{KBr} cm^{-1}: 2359, 2341, 2239, 2147, 1749; for ^1H and ^{13}C NMR spectra, see Table 1; EIMS 70 eV, *m/z* (rel. int): 353 (1), 309 (1), 259 (9), 207 (4), 203 (5), 152 (14), 150 (9), 136 (10), 134 (41), 122 (85), 118 (100); FABMS *m/z* 337 $[M+H]^+$.

3.8. 4-Hydroxy-4-(1-hydroxy-1-methylethyl)-cyclohex-2-en-1-one (*4*)

Colorless oil, $[\alpha]_D^{25}$ + 5.8 (MeOH; *c* 0.8); UV λ_{max}^{MeOH} nm (log ε) 215 (3.99); IR ν_{max}^{KBr} cm^{-1}: 3405, 2362, 2338, 1683; ^1H NMR ($CDCl_3$, 400 MHz, TMS) δ 7.06 (1H, *d*, *J* = 10.3 Hz, H-3), 6.06 (1H, *d*, *J* = 10.3 Hz, H-2), 2.79 (1H, *ddd*, *J* = 16.8, 10.6, 10.6 Hz, H-6a), 2.46 (1H, *dt*, *J* = 16.8, 4.2 Hz, H-6b), 2.12 (1H, *ddd*, *J* = 14.8, 10.6, 4.2 Hz, H-5a), 2.10 (1H, *ddd*, *J* = 14.8, 10.6, 4.2 Hz, H-5b); ^{13}C NMR ($CDCl_3$, 100 MHz, TMSi) δ 199.6 (C-1), 148.3 (C-3), 130.3 (C-2), 74.4 (C-7), 73.1 (C-4), 29.7 (C-5), 33.7 (C-6), 24.1 (C-8), 25.0 (C-9). EIMS 70 eV, *m/z* (rel. int.): 166 (33), 151 (22), 137 (9), 126 (100), 123 (28), 108 (46), 81 (59), 79 (64), 59 (93); HRFABMS *m/z* $[M+Na]^+$ 193.0864 (calcd. for $C_9H_{14}O_3Na$, 193.0841).

3.9. Leucoceramides A–G (*5a–g*)

Amorphous solid, $[\alpha]_D^{25}$ + 11.8 (Pyridine; *c* 0.35); IR ν_{max}^{KBr} cm^{-1}: 3330, 2917, 1622; for ^1H (d_5-pyridine, 400 MHz) and ^{13}C NMR (d_5-pyridine, 100 MHz) spectra, see Table 2; 2-hydroxyfatty acid methyl esters EIMS 70 eV, *m/z* (rel. int.): C-20 342 $[M]^+$; C-21 356 $[M]^+$; C-22 370 $[M]^+$; C-23 384 $[M]^+$; C-24 398 $[M]^+$; C-25 412 $[M]^+$; C-26 426 $[M]^+$. Sphingosine EIMS 70 eV, *m/z* (rel. int): 331(100) $[M+TMSi-H]^+$, 417 (13). FABMS positive *m/z* **5a** $C_{34}H_{67}NO_5$ 570, 610; **5b** $C_{35}H_{69}NO_5$, 584, 624; **5c** $C_{36}H_{71}NO_5$, 598, 638; **5d** $C_{37}H_{73}NO_5$, 612, 652; **5e** $C_{38}H_{75}NO_5$, 626, 666; **5f** $C_{39}H_{77}NO_5$, 640, 680; **5g** $C_{40}H_{79}NO_5$, 654, 694; corresponding to $[M+H]^+$ and $[M+Na+H_2O]^+$, respectively.

3.10. Leucocerebrosides A–F (*6a–f*)

Amorphous solid, $[\alpha]_D^{25}$ − 1.3 (Pyridine; *c* 0.28); IR ν_{max}^{KBr} cm^{-1}: 3378, 2920, 1630; for ^1H (d_5-pyridine, 400 MHz) and ^{13}C (d_5-pyridine, 100 MHz) spectra, see Table 2; fatty acid methyl esters EIMS 70 eV, *m/z* (rel. int): C-18 298 $[M]^+$; C-24 370 $[M]^+$. 2-hydroxyfatty acid methyl esters EIMS 70 eV, *m/z* (rel. int): C-16 286 $[M]^+$; C-22 370 $[M]^+$; C-23 384 $[M]^+$; C-24 398 $[M]^+$. Sphingosine EIMS 70 eV,

m/z (rel. int): 429 (100) $[M+TMSi]^+$. FAB-MS *m/z* **6a** $C_{45}H_{87}NO_9$ *m/z* 768 $[M-H_2O+H]^+$; **6b** $C_{51}H_{99}NO_{10}$ *m/z* 852 $[M-H_2O+H]^+$; **6c** $C_{43}H_{83}NO_{10}$ *m/z* 754 $[M-H_2O-H]^+$, not detected $[M+Na]^+$; **6d** $C_{49}H_{95}NO_{10}$*m/z* not detected $[M-H_2O-H]^+$, 880 $[M+Na]^+$; **6e** $C_{50}H_{97}NO_{10}$ *m/z* 852 $[M-H_2O-H]^+$, not detected $[M+Na]^+$; **6f** $C_{51}H_{99}NO_{10}$ *m/z* 866 $[M-H_2O-H]^+$, 908 $[M+Na]^+$.

3.11. Bioassays for cytokine activity

The inhibition of cytokine production was measured for human peripheral blood mononuclear (PBMC)s cells (1×10^6/ml) which were isolated from heparinized venous blood by Ficoll–Hypaque gradient density centrifugation according to standard procedures (Boyum, 1968). After isolation, the cells were treated with 100 ng/ml of lipopolysaccharide (LPS) derived from *Escherichia coli* 055:B5 (Sigma, St Louis, MO) in the absence or presence of the test compounds (10 μg/ml) for 48 h at 37 °C. Prednisolone (0.3 μg/ml) (Sigma) was used as reference sample for inhibition of LPS-induced cytokine production. Then, cytokines in the supernatants from these cultures were analyzed by commercial ELISA kits. ELISA kits for human tumor necrosis factor TNF-α, IL-10 and IL-12 were purchased from BioSurce International, Inc. (Camarillo, CA).

Acknowledgements

This study was supported by a Grant-in-Aid by the Ministry of Education, Science Sports, Culture and Technology of Japan, The Ministry of Environment Housing and Territorial Development of Colombia and the National Coffee Growers of Colombia (Contract No. 21/2002).

References

Adams, A.A., Norris, J.A., Mabry, T.J., 1998. A flavonoid from *Hydrocotyle umbellata* L. (Umbelliferae, Hydrodotyleae). Rev. Latinoamer. Quim. 20, 67–68.

Almeida, A.P., Miranda, M.M.F.S., Simoni, I.C., Wigg, M.D., Lagrota, M.H.C., Costa, S.S., 1998. Flavonol monoglycosides isolated from the antiviral fractions of *Persea americana* (Lauraceae) leaf infusion. Phytotherapy Res. 12, 562–567.

Arisawa, M., Hamabe, M., Sawai, M., Hayashi, T., Kiuzu, H., Tomimori, T., Yoshizaki, M., Naokata, M., 1984. Constituents of *Liquidamber formosa* (Hamamelidaceae). Shoyakugaku Zasshi 38, 216–220.

Asakawa, Y., Matzuda, R., Takemoto, T., 1982. Mono- and sesquiterpenoids from *Hydrocotyle* and *Centella* species. Phytochemistry 21, 2590–2592.

Boyum, A., 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand. J. Clin. Lab. Invest. Supplementum 97, 77–89.

Della Greca, M., Florentino, A., Monaco, P., Previtera, L., 1994a. Polyoxigenated pleane triterpenes from *Hydrocotyle ranunculoides*. Phytochemistry 35, 201–204.

Della Greca, M., Florentino, A., Monaco, P., Previtera, L., 1994b. Oleane glycosides from *Hydrocotyle ranunculoides*. Phytochemistry 36, 1479–1483.

Duan, H., Takaishi, Y., Momota, H., Ohmoto, Y., Taki, T., 2002. Immunosuppressive constituents from *Saussurea medusa*. Phytochemistry 59, 85–90.

Fujimoto, Y., Satho, M., Takeuchi, N., Kirisawa, M., 1991. Cytotoxic acetylenes from *Panax quinquefolium*. Chem. Pharm. Bull. 39, 521–523.

Higashibayashi, S., Czechtisky, W., Kobayashi, Y., Kishi, Y., 2003. Universal NMR databases for contiguous polyols. J. Am. Chem. Soc. 125, 14379–14393.

Hirakura, K., Morita, M., Nakajima, M., Ikeya, Y., Mitsuhashi, H., 1992. Three acetylenic compounds from roots of *Panax ginseng*. Phytochemistry 31, 899–903.

Kato, T., Tsunakawa, M., Sasaki, N., Aizawa, H., Fujita, K., Kitahara, Y., Takahashi, N., 1977. Growth and germination inhibitors in rice husks. Phytochemistry 16, 45–48.

Kobayashi, M., Mahmud, T., Umezome, T., Wangs, W., Murakami, N., Kitakawa, I., 1997. The absolute stereochemistry of the polyacetylenic constituents of *Ginseng radix rubra*. Tetrahedron 53, 15691–15700.

Kojima, H., Sato, N., Hatano, A., Ogura, H., 1990. Sterol glucosides from *Prunella vulgaris*. Phytochemistry 29, 2351–2355.

Kwon, H.Ch., Zee, O.P., Lee, K.R., 1998. Two new monogalactosylacylglycerols from *Hydrocotyle ramiflora*. Planta Med. 64, 477–479.

Loukaci, A., Bultel-Ponce, V., Longeon, A., Guyot, M.J., 2000. New lipids from the tunicate *Cystodytes* cf. *dellechiajei*, as PLA2 inhibitors. J. Nat. Prod. 63, 799–802.

Lourenço, A., Lobo, A.M., Rodriguez, B., Jimeno, M.L., 1996. Ceramides from the fungus *Phellinus pini*. Phytochemistry 43, 17–620.

Ma, J.S., Oh, S.J., Jo, J.Y., 2003. Inorganic nutrient removal efficiency of aquatic plants from recirculating aquaculture system. Han'guk Yangsik Hakhoechi 16, 171–178.

Matsuda, H., Morikawa, T., Ueda, H., Yoshikawa, M., 2001. Medicinal foodstuffs. XXVII. Saponin constituents of Gotu Kola (2): Structures of new ursane- and oleanane- type triterpene oligoglycosides, Centellasaponins B,C,D, from *Centella asiatica* cultivated in Sri Lanka. Chem. Pharm. Bull. 49, 1368–1371.

Matsushita, A., Sasaki, Y., Warashima, T., Miyase, T., Noguchi, H., Van der Velde, D., 2004. Hydrocotylosides I–VII, new oleane saponins from *Hydrocotyle sibthorpioides*. J. Nat. Prod. 67, 384–388.

Okada, N., Shirata, K., Niwano, M., Koshino, H., Uramoto, M., 1994. Immunosuppressive activity of a monoterpene from *Eucommia ulmoides*. Phytochemistry 37, 281–282.

Scharbert, S., Holzmann, N., Hofmann, T., 2004. Identification of the astringent taste compounds in black tea infusions by combining instrumental analysis and human bioresponse. J. Agric. Food. Chem. 52, 3498–3508.

Shim, S.C., Chang, S.K., Hur, C.W., Kim, C.K., 1987. A polyacetylenic compound from *Panax ginseng* roots. Phytochemistry 26, 2849–2850.

Shulte, K.E., Rucker, G., Abdel-Bary, E., 1973. Polyacetylene aus *Hydrocotyle asiatica* L. Arch Pharmaz. 306, 197–209.

da Silva, R., de Souza, G.H.B., da Silva, A.A., de Souza, V.A., Royo, V.A., e Silva, M.L.A., Donate, P.M., de Matos Araujo, A.L.S., Carvalho, J.C.T., Bastos, J.K., 2005. Synthesis and biological activity evaluation of lignan lactones derived from (−)-cubebin. Bioorg. Med. Chem. Lett. 15, 1033–1037.

Väsange, M., Liu, B., Welch, C.J., Rolfsen, W., Bohlin, L., 1997. The flavonoid constituents of two *Polypodium* species (Calagula) and their effect on the elastase release in human neutrophils. Planta Med. 63, 511–577.

Yamada, K., Hara, E., Miyamoto, T., Higuchi, R., Isobe, R., Honda, S., 1998. Isolation and structure of biologically active glycosphingolipids from sea cucumber *Cucumaria echinata*. Eur. J. Org. Chem. 2, 371–378.

Yamada, K., Matsubara, R., Kaneko, M., Miyamoto, R., Higuchi, R., 2001. Constituents of Holothuroidea. 10. Isolation and structure of a biologically active ganglioside molecular species from the sea cucumber *Holothuria leucospilota*. Chem. Pharm. Bull. 49, 447–452.

Yeşilada, E., Taninaka, H., Takaishi, Y., Honda, G., Sezik, E., Momota, H., Ohmoto, Y., Taki, T., 2001. In vitro inhibitory effects of *Daphne oleoides* ssp. *oleoides* on inflammatory cytokines and activity-guided isolation of active constituents. Cytokine 13, 359–364.