Pergamon

PII: S0031-9422(97)00127-1

Phytochemistry, Vol. 46, No. 3, pp. 521–524, 1997

Printed in Great Britain
0031-9422/97 $17.00+0.00

CYTOTOXIC FLAVONOIDS FROM *VITEX AGNUS-CASTUS*

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(*Received in revised form* 3 *January* 1997)

Key Word Index—*Vitex agnus-castus*; Verbenaceae; root bark; cytotoxic flavonoids; acylated luteolin *C* and *O*-glucosides.

Abstract—Four new flavonoids, luteolin 6-*C*-(4″-methyl-6″-*O*-*trans*-caffeoylglucoside), luteolin 6-*C*-(6″-*O*-*trans*-caffeoylglucoside), luteolin 6-*C*-(2″-*O*-*trans*-caffeoylglucoside), and luteolin 7-*O*-(6″-*p*-benzoylglucoside), together with four known ones 5, 4′-dihydroxy-3,6,7,3′-tetramethoxyflavone, luteolin, artemetin and isorhamnetin, were isolated from the root bark of *Vitex agnus-castus*. The structures were elucidated by spectroscopic means. © 1997 Elsevier Science Ltd

INTRODUCTION

Vitex agnus-castus (Verbenaceae), is a shrub which is widely distributed in the Middle East and southern Europe. Some iridoids and flavonoids have been isolated from the leaves or fruits [1]. During our study of the cytotoxic principles from this plant, four new flavonoids, luteolin 6-*C*-(4″-methyl-6″-*O*-*trans*-caffeoylglucoside) (**1**), luteolin 6-*C*-(6″-*O*-*trans*-caffeoylglucoside) (**2**), luteolin 6-*C*-(2″-*O*-*trans*-caffeoylglucoside) (**3**), and luteolin 7-*O*-(6″-*p*-benzoylglucoside) (**4**) together with four known ones, 4′,5-dihydroxy-3,3′,6,7-tetramethoxyflavone (**5**) [2], luteolin (**6**) [3], artemetin (**7**) [3–5] and isorhamnetin (**8**) [3] were isolated and applied to cytotoxic bioassay.

RESULT AND DISCUSSION

Luteolin 6-*C*-(4″-methyl-6″-*O*-*trans*-caffeoylglucoside) (**1**) was obtained as a pale yellow powder with a molecular formula of $C_{31}H_{28}O_{15}$ from $[M+H-CH_2+Na]^+$ at *m/z* 634 and $[M+H-CH_2]^+$ at *m/z* 611 in the FABMS. The NMR spectra showed the presence of a flavone skeleton with one methoxyl (δ_H: 3.35 *s*, δ_C: 49.7 *q*), one caffeoyl (δ_H: 6.26 *d* H-8‴, 7.53 *d* H-7‴, 6.88 *dd* H-6‴, 6.73 *d* H-5‴ and 6.99 *d* H-2‴) and one *C*-glucosyl group (δ_H: 4.95 *d* J = 9.9 Hz, H-1″, δ_C: 75.4 *d* C-1″). The flavone skeleton was elucidated as 5,7,3′,4′-tetrahydroxyflavone from the UV spectrum with various shift reagents. The *C*-glucose was assigned to C-6 from the cross peak between C5–OH-5, C10–OH-5, C6–OH-5, C6–H-1″, C7–H-1″ and C5–H-1″ in the HMBC spectrum measured in DMSO-d_6. The methoxyl group was assigned to C-4″ from the cross peak between C-4″–O<u>CH</u>$_3$ in the HMBC spectrum of **1**. The caffeoyl group was assigned to C-6″ from the cross peak between C-9‴–H-6″ in the HMBC spectrum and the *trans*-geometry was elucidated from the coupling constants between H-7‴ and H-8‴ (J = 15.9 Hz).

Luteolin 6-*C*-(6″-*O*-*trans*-caffeoylglucoside) (**2**) was obtained as a pale yellow powder with a molecular formula of $C_{30}H_{26}O_{15}$ from $[M+H+Na]^+$ at *m/z* 634 and $[M+H]^+$ ion at *m/z* 611 in the FABMS. The ^1H and ^{13}C NMR spectra were quite similar to those of **1** except for the absence of the methoxyl signal.

Luteolin 6-*C*-(2″-*O*-*trans*-caffeoylglucoside) (**3**) was obtained as a pale yellow powder with the same molecular formula of $C_{30}H_{26}O_{15}$ as **2** from the $[M+Na]^+$ ion at *m/z* 633 in the FABMS. The NMR spectra were similar to those of **2**, but the chemical shifts of C-2″ and C-5″ were shifted downfield in comparison with those of **2**, and those of C-1″, C-3″, and C-6″ were shifted upfield. Also, the chemical shifts of H-1″, H-2″ and H-3″ in **3** were shifted downfield in comparison with those of **2**, but those of H-5″ and H-6″ were shifted upfield. These results suggested that the caffeoyl group at C-6″ in **2** is at C-2″ in **3**.

Luteolin 7-*O*-(6″-*p*-benzoylglucoside) (**4**) was obtained as a pale yellow powder with a molecular formula of $C_{28}H_{24}O_{13}$ from the 591$[M+Na]^+$ ion at *m/z* 591 in the FAB mass spectrum. The NMR spectra showed the presence of one *p*-benzoyl group (δ_H: 7.84, *d*, H-2‴ and H-6‴; 6.65, *d*, H-3‴ and -5‴), one *O*-β-glucosyl (δ_H: 5.13, *d*, J = 7.3 Hz, H-1″; δ_C: 101.0, *d*, C-1″), and a luteolin skeleton as shown in Tables 1 and 2. The *O*-glucosyl group was confirmed to be

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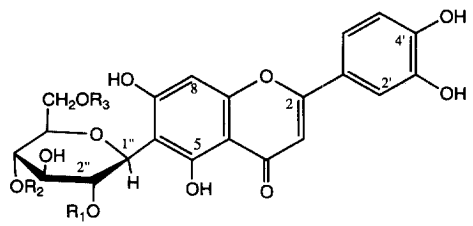
Table 1. ^{1}H NMR data of flavonoids from *Vitex agnus-castus* (400 MH_z, δ, CD_3OD)

	1	2	3	4
3	6.42 *s*	6.51 *s*	6.41 *s*	6.59 *s*
6				6.54 *d* (2.2)
8	6.39 *s**	6.47 *s**	6.38 *s**	6.72 *d* (2.2)
2′	7.27 *br s*	7.33 *br s*	7.26 *br s*	7.35 *br s*
5′	6.73 *d* (8.8)	6.88 *d* (8.9)	6.83 *d* (7.8)	6.88 *d* (8.9)
6′	7.26 *br d* (8.8)	7.34 *br d* (8.9)	7.25 *br d* (7.8)	7.34 *br d* (8.9)
OMe	3.35 *s*			
1″	4.95 *d* (9.9)	4.94 *d* (9.9)	5.12 *d* (10.0)	5.13 *d* (7.3)
2″	4.25 *t* (9.0)	4.23 *t* (8.9)	5.67 *br*	3.55
3″	3.56 *m*	3.53 *m*	3.77 *t* (9.0)	3.55
4″	3.56 *m*	3.53 *m*	3.63 *t* (9.4)	3.44 *t* (9.1)
5″	3.70 *m*	3.68 *m*	3.53 *m*	3.90 *dt* (9.8, 2.2)
6″	4.56 *d* (10.5)	4.54 *d* (10.2)	3.95 *d* (10.5)	4.74 *dd* (12.0, 2.2)
	4.40 *dd* (12.0, 5.4)	4.37 *dd* (12.1, 5.5)	3.82 *dd* (12.1, 5.3)	4.31 *dd* (11.9, 7.8)
2‴	6.99 *d* (1.7)	7.02 *d* (1.9)	6.90 *d* (1.4)	7.84 *d* (8.8)
3‴				6.65 *d* (8.8)
5‴	6.73 *d* (8.2)	6.75 *d* (8.2)	6.69 *d* (8.2)	6.65 *d* (8.8)
6‴	6.88 *dd* (8.2, 1.7)	6.92 *dd* (8.2, 2.0)	6.80 *dd* (8.3, 1.7)	7.84 *d* (8.8)
7‴	7.53 *d* (15.9)	7.56 *d* (15.9)	7.36 *d* (15.9)	
8‴	6.26 *d* (15.9)	6.28 *d* (15.9)	6.05 *d* (15.9)	

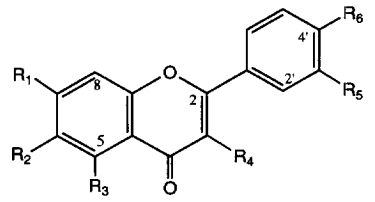
* Signals reduce when immersed in the solvent (CD_3OD) for long periods.

connected to C-7 from the UV spectrum with the absence of a shift of Band II in the presence of sodium acetate. The *p*-benzoyl group was established to be connected to C-6″ from the chemical shifts of C-6″ (δ_C: 65.2, *t*) and H-6″ (δ: 4.74, *dd*; 4.31, *dd*).

Compounds **5**–**8** were identified as 4′,5-dihydroxy-3,3′,6,7-tetramethoxy-flavone, luteolin, artemetin and isorhamnetin, respectively, by comparing their physical and spectral data with those reported in literature, and from chemical evidence [2–5]. The IC_{50} values ($\mu g\ ml^{-1}$) of **1**–**7** showing cytotoxic activity against P388 lymphocytic leukemia cells were 7.6 in **1**, 14 in **2**, 56 in **3**, 70 in **4**, 0.1 in **5**, 0.31 in **6** and 8.1 in **7**.



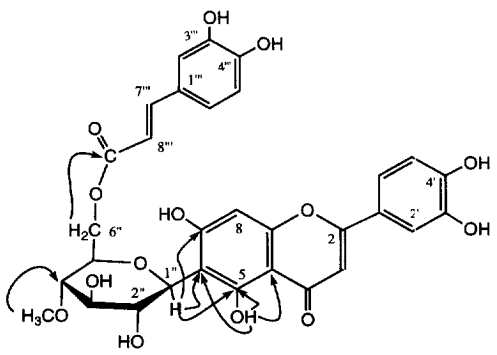
1. R_1=H, R_2=CH_3, R_3=*trans* caffeoyl
2. R_1=R_2=H, R_3= *trans* caffeoyl
3. R_1=*trans* caffeoyl, R_2=R_3=H



4. R_1=O-Glc(6″-*p*-hydroxybenzoyl), R_2=R_4=H, R_3=R_5=R_6=OH
5. R_1=R_2=R_4=R_5=OCH_3, R_3=R_6=OH
6. R_1=R_3=R_5=R_6=OH, R_2=R_4=H
7. R_1=R_2=R_4=R_5=R_6=OCH_3, R_3=OH
8. R_1=R_3=R_4=R_6=OH, R_5=OCH_3, R_2=H

EXPERIMENTAL

General. Mps were uncorr. ^{1}H and ^{13}C NMR: Bruker AM 400 and 500 MHz at 303K. NOESY experiments were made with mixing time of 0.6 sec and precessed on a Bruker data station with an Aspect 3000 computer. Silica gel CC was carried out on Merck Kieselgel 60 (70–230 mesh) at amounts equivalent to 100 × the sample amount. MPLC was performed with a column (22 mm i.d. × 300 mm) packed with 40 μm silica gel or 20 μm ODS. HPLC was



: HMBC correlations of compound **1** measured in DMSO

Table 2. ^{13}C NMR data of flavonoids from *Vitex agnus-castus* (100 MHz, δ, CD_3OD)

	1	2	3	4
2	166.1 *s*	166.3 *s*	166.2 *s*	166.9 *s*
3	103.8 *d*	104.0 *d*	103.9 *d*	103.2 *d*
4	183.9 *s*	184.0 *s*	183.8 *s*	184.1 *s*
5	162.1 *s*	162.2 *s*	161.4 *s*†	158.9 *s*
6	108.8 *s*	108.9 *s*	107.9 *s*	101.2 *d*
7	165.3 *s*	164.9 *s*	164.8 *s*	164.6 *s*
8	95.5 *d*†	95.3 *d*†	95.3 *d*†	96.1 *d*
9	158.7 *s*	158.8 *s*	158.8 *s*	163.1 *s*
10	105.1 *s*	105.3 *s*	105.0 *s*	107.1 *s*
1′	123.4 *s*	123.6 *s*	123.5 *s*	122.0 *s*
2′	114.1 *d*	114.2 *d*	114.2 *d*	114.4 *d*
3′	147.0 *s**	147.0 *s*	146.9 *s**	147.1 *s*
4′	151.1 *s*	151.0 *s*	151.0 *s*	151.3 *s*
5′	116.8 *d*	116.8 *d*	116.8 *d*	116.8 *d*
6′	120.4 *d*	120.4 *d*	120.4 *d*	120.6 *d*
OMe	49.7 *q*			
1″	75.4 *d*	75.5 *d*	73.3 *d*	101.0 *d*
2″	72.5 *d*	72.6 *d*	73.9 *d*	74.6 *d*
3″	79.9 *d*	80.0 *d*	78.0 *d*	77.8 *d*
4″	71.9 *d*	71.9 *d*	71.8 *d*	72.2 *d*
5″	79.9 *d*	80.0 *d*	82.8 *d*	75.7 *d*
6″	65.1 *t*	65.0 *t*	62.8 *t*	65.2 *t*
1‴	127.7 *s*	127.8 *s*	127.7 *s*	123.5 *s*
2‴	115.1 *d*	115.2 *d*	115.1 *d*	132.9 *d*
3‴	146.7 *s**	146.8 *s*	146.7 *s**	116.2 *d*
4‴	149.6 *s*	149.6 *s*	149.5 *s*	163.6 *s*
5‴	116.5 *d*	116.5 *d*	116.5 *d*	116.2 *d*
6‴	123.1 *d*	123.1 *d*	122.9 *d*	132.9 *d*
7‴	147.2 *d*	147.2 *d*	147.0 *d*	168.1 *s*
8‴	114.9 *d*	115.0 *d*	114.9 *d*	
9‴	169.3 *s*	169.3 *s*	168.3 *s*	

* Signals may be interchangable in each column.

† Signals reduce when immersed in the solvent (CD_3OD) for long periods.

performed with a Hibar RT RP-18 column (20 mm i.d. × 250 mm) packed with 7 μm ODS. The NMR coupling constants (J) are given in Hz.

Plant material. The fruits of *Vitex agnus-castus* L. (Verbenaceae) were collected at Israel in 1995. The plant was identified by Professor Dan Palevitch (Action Director, The Northern Research Center, Agricultural Research Organization, Israel). A voucher specimen has been deposited at the Department of Natural Medicines, Tokyo University of Pharmacy and Life Science.

Extraction and isolation. The fruits of *V. agnus-castus* L. (2 kg) were powdered and extracted × 3 with MeOH (3 l). The concd extract (207 g) was partitioned between *n*-hexane and H_2O, between $CHCl_3$ and H_2O, as well as *n*-BuOH and H_2O, successively. The *n*-BuOH soluble fr. (47 g) was subjected to HP-20 CC using H_2O, 40% MeOH, 60% MeOH, 80% MeOH and MeOH as eluting solvent systems to give five frs (Fr. A–E). Fr. D (16.9 g) was further chromatographed on a Sephadex LH-20 column, and then a silica gel column eluted with $CHCl_3$–MeOH (1:1) and $CHCl_3$–MeOH–H_2O (8.9:1:0.1–6.7:3:0.3) successively and finally purified by means of ODS MPLC and HPLC with MeOH–H_2O or CH_3CN–H_2O as eluants to give **1** (46 mg), **2** (13 mg), **3** (32 mg), **4** (2.5 mg), **6** (11 mg), and **8** (1.7 mg). Also, the *n*-hexane extract (55 g) was subjected to silica gel CC and eluted with *n*-hexane–EtOAc (10:0–0:10), followed with EtOAc–MeOH (1:1). The EtOAc–MeOH (1:1) eluate was further isolated with ODS MPLC and finally purified with ODS HPLC using 80% MeOH to give **5** (2.4 mg) and **7** (25 mg).

Luteolin 6-C-(4″-methyl-6″-O-trans-caffeoylglucoside) (**1**). Yellow powder, mp 224–226° (from MeOH), $[\alpha]_D$ −44.1° (MeOH; *c* 0.29), UV λ_{max}^{MeOH}nm: 245, 272 (sh), 295 (sh), 336; NaOMe: 269, 389; $AlCl_3$: 273, 302 (sh), 371 (sh), 425; $AlCl_3$–HCl: 281, 297 (sh), 340, 386 (sh); NaOAc: 274, 289 (sh), 332; NaOAc–H_3BO_3: 260, 305 (sh), 358; FABMS *m/z*: 634 $[M+H\text{-}CH_2+Na]^+$, 611 $[M+H\text{-}CH_2]^+$, 575, 517, 445, 391, 329, 163, 135 [100]; ^1H and ^{13}C NMR spectra are listed in Tables 1 and 2.

Luteolin 6-C-(6″-O-trans-caffeoylglucoside) (**2**). Yellow powder, mp 224–226° (from MeOH), $[\alpha]_D$ −51.0° (MeOH; *c* 0.49), UV λ_{max}^{MeOH}nm: 245, 272 (sh), 295 (sh), 336; NaOMe: 267, 389; $AlCl_3$: 273, 302 (sh) 370 (sh), 424; $AlCl_3$–HCl: 281, 298 (sh), 339, 386 (sh); NaOAc: 274, 333; NaOAc–H_3BO_3: 261, 297 (sh), 357; FABMS *m/z*: 634 $[M+H+Na]^+$, 611 $[M+H]^+$, 531, 443, 391, 329, 299, 177, 149 [100]; ^1H and ^{13}C NMR spectra are listed in Tables 1 and 2.

Luteolin 6-C-(2″-O-trans-caffeoylglucoside) (**3**). Yellow powder, mp 213–215° (from MeOH), $[\alpha]_D$ −239.3° (MeOH; *c* 0.57), UV λ_{max}^{MeOH}nm: 246, 272 (sh), 295 (sh), 335; NaOMe: 235 (sh), 278, 337 (sh), 389; $AlCl_3$: 273, 304 (sh), 380, 420; $AlCl_3$–HCl: 261 (sh), 278, 298 (sh), 342, 386 (sh); NaOAc: 279, 330; NaOAc–H_3BO_3: 260, 305 (sh), 357; FABMS *m/z*: 633 $[M+Na]^+$, 353, 329, 193, 163 [100], 135; ^1H and ^{13}C NMR spectra are listed in Tables 1 and 2.

Luteolin 7-O-(6″-p-benzoylglucoside) (**4**). Yellow powder, mp > 300° (from MeOH), $[\alpha]_D$ −78.6° (MeOH; *c* 0.028), UV λ_{max}^{MeOH}nm: 255, 349; NaOMe: 269 (sh), 296, 402; $AlCl_3$: 268, 295 (sh), 335 (sh), 427; $AlCl_3$–HCl: 260, 294 (sh), 355 (sh), 386; NaOAc: 258, 365; NaOAc–H_3BO_3: 258, 373; FABMS *m/z*: 591 $[M+Na]^+$, 531, 429, 385, 287, 147 [100]; ^1H and ^{13}C NMR spectra are listed in Tables 1 and 2.

Bioassay of cytotoxic activity against P388 cells. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) colorimetric assay was performed in a 96-well plate [6]. The assay is based on the reduction of MTT by the mitochondrial dehydrogenase of viable cells to give a blue formazan product which can be measured spectrophotometrically. Mouse P388 leukemia cells (2×10^4 cell ml^{-1}) were inoculated in each well 100 μl ml^{-1} of RPMI-1640 medium (Nissui Pharm. Co., Ltd) supplemented with 5% fetal calf serum. (Mitsubishi Chemical Industry Co., Ltd) and kanamycin (100 μg ml^{-1}) at 37° in a humidified atmo-

sphere of 5% CO_2. Various drug concs (10 μl) were added to the cultures at day 1 after transplantation. At day 3, 20 μl of the MTT soln (5 mg ml^{-1}) per well was added to each cultured medium. After a further 4 hr of incubation 100 μl of 10% sodium dodecyl sulphate-0.01 N HCl soln was added to each well and the formazan crystals in each well were dissolved by stirring with a pipette. The optical density measurements were made using a microplate reader (Tohso MPR-A4i) at two wavelengths (550 and 700 nm). In all these experiments 3 replicate wells were used to determine each data point.

Acknowledgements—The authors are grateful to Professor Dan Palevitch (Action Director, The Northern Research Center, Agricultural Research Organization, Israel) for supplying authentic *Vitex agnus-castus*, and also to Israel Nature Reserve Authority, Wildlife and Nature Protection Wing for the collection permit.

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