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Phytochemistry 63 (2003) 959–964
PHYTOCHEMISTRY
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Glucosides from *Vitex agnus-castus*

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Received 1 February 2003; received in revised form 13 April 2003

Abstract

The methanolic extract of the flowering stems of *Vitex agnus-castus* yielded three new iridoids: 6′-*O*-foliamenthoylmussaenosidic acid (agnucastoside A), 6′-*O*-(6,7-dihydrofoliamenthoyl)mussaenosidic acid (agnucastoside B) and 7-*O*-*trans*-*p*-coumaroyl-6′-*O*-*trans*-caffeoyl-8-epiloganic acid (agnucastoside C) in addition to four known iridoids (aucubin, agnuside, mussaenosidic acid and 6′-*O*-*p*-hydroxybenzoylmussaenosidic acid) and one known phenylbutanone glucoside (myzodendrone). The structure elucidations were mainly done by spectroscopic methods (1D and 2D NMR spectra) and MS data interpretation. The purified compounds were tested for biological activities against various microorganisms and cancer cell lines.

Keywords: *Vitex agnus-castus*; Verbenaceae; Iridoid glucosides; Aucubin; Mussaenosidic acid; Agnucastosides A–C; Myzodendrone

1. Introduction

Vitex agnus-castus L. (Verbenaceae) is a small tree or shrub, which is widely distributed along the Anatolian coastal lane (Davis, 1982). This plant has important medicinal properties and is especially used for treatment of premenstrual problems and hyperprolactinemia because of it is hormone-like effect (Milewicz et al., 1993; Odenthal, 1998; Lucks et al., 2002). In Anatolian folk medicine, *V. agnus-castus* is used as diuretic, digestive, antifungal and also against anxiety, early birth and stomachache (Baytop, 1984; Honda et al., 1996).

V. agnus-castus contains iridoids (Hänsel and Winde, 1959; Gomaa et al., 1978; Görler et al., 1985), flavonoids (Sirait et al., 1962; Hänsel and Rimpler, 1963; Gomaa et al., 1978; Wollenweber and Mann, 1983; Hirobe et al., 1997; Hoberg et al., 2001), diterpenoids (Hoberg et al., 1999; Li et al., 2002), essential oils (Sorensen and Katsiotis, 2000) and ketosteroids (Saden-Krehula et al., 1990). In the previous studies on the leaves and fruits of *V. agnus-castus,* agnuside, aucubin and 10-*p*-coumaroylaucubin (eurostoside) have been isolated (Hänsel and Winde, 1959; Gomaa et al., 1978; Görler et al., 1985). However, the investigations of some other *Vitex* species have resulted in the isolation of iridoid glycosides named agnuside, eurostoside, negundoside (2′-*p*-hydroxybenzoylmussaenosidic acid), 6′-*p*-hydroxybenzoylmussaenosidic acid, nishindaside and isonishindaside from leaves; agnuside and 10-*O*-vanilloylaucubin from fruits; agnuside, limoniside and pedunculariside from stem bark (Rimpler, 1972; Sehgal et al., 1982, 1983; Dutta et al., 1983; Kouno et al., 1988; Iwagawa et al., 1993; Ono et al., 1997; Okuyama et al., 1998; Suksamrarn et al., 1999, 2002; Santos et al., 2001).

The iridoids of *V. agnus-castus* have not been studied sufficiently. Our investigation deals with the isolation, characterization and biological evaluation of glucosidic components in flowering stems (flowers, leaves and twigs) of *V. agnus-castus*.

2. Results and discussion

The methanolic extract of the air dried and powdered flowering stems (flowers, leaves and twigs) of *V. agnus-castus* L. was divided into $CHCl_3$ and *n*-BuOH soluble fractions. The *n*-BuOH fraction was subjected to a combination of silica gel, Sephadex LH-20 and RP-18 column chromatographies (CC) to afford seven iridoid

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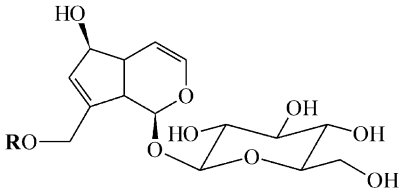
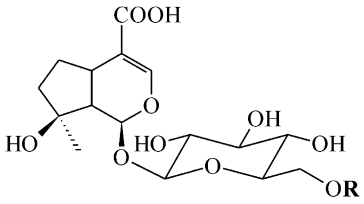
0031-9422/03/$ - see front matter © 2003 Elsevier Ltd. All rights reserved.
doi:10.1016/S0031-9422(03)00285-1

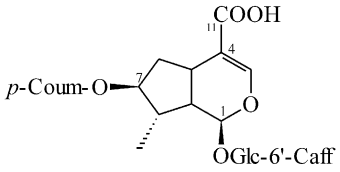
glycosides (**1**–**7**), among three new ester iridoids (**5**–**7**), and a known phenolic glucoside (**8**). Glucosides **1**–**4** and **8** were identified as aucubin (**1**) (Görler et al., 1985; Nicoletti, 1983), agnuside (**2**) (Dutta et al., 1983; Görler et al., 1985; Okuyama et al., 1998), mussaenosidic acid (**3**) (Damtoft et al., 1984), 6′-*O*-*p*-hydroxybenzoylmussaenosidic acid (**4**) (Sehgal et al., 1983), and myzodendrone (**8**) (Kouno et al., 1988; Pabst et al., 1990) by comparison with spectroscopical data (^{1}H, ^{13}C, COSY, HMQC and HMBC NMR experiments) and MS values in literature.



1: R=H
2: R= *p*-OH-Ph-CO-



3: R=H
4: R= *p*-OH-Ph-CO-
5: R= foliamenthoyl
6: R= 6,7-dihydrofoliamenthoyl



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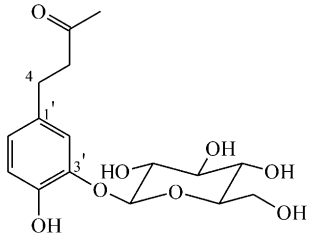

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Compounds **5** and **6** were obtained as amorphous powders. Their ESI-MS exhibited pseudomolecular ions $[M+Na]^+$ at *m*/*z* 565 and 567, respectively. The molecular formulae of **5** and **6** were determined as $C_{26}H_{38}O_{12}$ for **5** and $C_{26}H_{40}O_{12}$ for **6** in conjunction with the NMR spectra (^{1}H and ^{13}C). The spectroscopical data of compounds **5** and **6** were quite similar. Their UV (220 nm) and IR spectra (1650 cm^{-1}, 1703 cm^{-1}) revealed the presence of typical conjugated carboxylic enol-ether systems of iridoids. The ^{1}H NMR spectra (Table 1) [δ_H 7.30 (*s*) and 7.38 (*s*) for 3-H of compounds **5** and **6**, respectively] indicated the presence of a 4-substituted enol-ether system, and comparison with published data (Damtoft et al., 1984) showed that both compounds were derivatives of **3**. Furthermore, in both cases a C_{10} acid were attached at C-6′ of the mussaenosidic acid moiety as seen by the expected NMR shifts for this part of the molecule [e.g. for **5**: downfield shifts for C-6′ (δ_C 64.3, $\Delta\delta_C = +1.4$ ppm) and 6′-H_2 (δ_H 4.27, $\Delta\delta_H = +0.64$ and δ_H 4.49, $\Delta\delta_H = +0.60$ ppm), upfield shifts for C-5′ (δ_C 75.7, $\Delta\delta_C = -2.4$ ppm) and 5′-H (δ_H 3.50, $\Delta\delta_H = +0.22$ ppm), caused by esterification of the 6′-hydroxy group (Dutta et al., 1983)]. The additional proton signals together with the ^{13}C NMR values (Table 1) could clearly be attributed to a foliamenthoyl (8-hydroxy-2,6-dimethyl-2,6-octadienoic acid) moiety (Sehgal et al., 1983). Therefore, compound **5** was identified as 6′-*O*-foliamenthoylmussaenosidic acid (agnucastoside A).

The 1D NMR and 2D NMR spectral data of **6** were in good agreement with those of **5**. When comparing their NMR data (Table 1), the only difference in the ^{13}C NMR spectrum of **5** was the presence of two olefinic carbons [δ_C 125.8 (C-7″) and δ_C 138.3 (C-6″)] instead of two alkyl carbons at δ_C 30.6 (C-6″) and 40.6 (C-7″) in **6**. Namely, compound **6** is a mussaenosidic acid derivative with a saturated ($\Delta^{6,7}$) foliamenthoyl unit [6′-*O*-(6,7-dihydrofoliamenthoyl)mussaenosidic acid], named agnucastoside B. Due to the small amounts of compounds **5** and **6,** it was not possible to determine the configuration of the 6″,7″-double bond of **5** and the stereochemistry at C-6″ of **6**, respectively. However, as previously noted as a general rule for the 6,7-double bond in foliamenthic acids (chemical shift difference between C-5 and C-10: $\Delta_{C\text{-}5,C\text{-}10} > 20$ ppm for an (*E*)-configuration, $\Delta_{C\text{-}5,C\text{-}10} < 10$ ppm for a (*Z*)-configuration), the shift difference in the ^{13}C NMR spectrum of **5** ($\Delta_{C\text{-}5'',C\text{-}10''} = 23$ ppm) indicates an (*E*)-configuration of the 6″,7″-double bond. So far, most compounds with a 6,7-dihydrofoliamenthic acid moiety like in **6** have been reported with a 6(*S*)-configuration (Arslanian et al., 1990; Damtoft et al., 1997).

Although some iridoids esterified with a foliamenthic acid have been found in nature previously (Junior, 1983; Stenzel et al., 1986; Damtoft et al., 1997), it is the first report of iridoid glucosides substituted with a foliamenthic acid isolated from Verbenaceae.

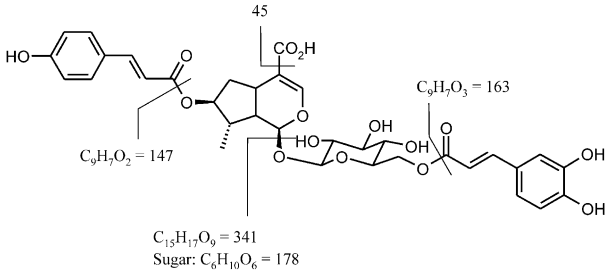
Compound **7,** which was obtained as an amorphous powder has a molecular formula of $C_{34}H_{36}O_{15}$ as determined by analysis of ESI-MS (pseudomolecular ion $[M+Na]^+$ at *m*/*z* 707) and 1D NMR spectroscopic data. Its UV and IR spectra again suggested the presence of conjugated carboxylic enol–ether system (219 nm and 1694 cm^{-1}, 1634 cm^{-1}) that is typical for an

Table 1
^{1}H and ^{13}C NMR spectral data for compounds **5** and **6** (CD_3OD, 300 MHz)

Atom numbers	5		6	
1	5.19 (1H, *d*, *J* = 6.0 Hz)	95.4	5.18 (1H, *d*, *J* = 6.0 Hz)	95.4
3	7.30 (1H, *s*)	150	7.38 (1H, *s*)	151.1
4	–	116.4	–	114.5
5	3.20 (1H, *m*)	33.7	3.20 (1H, *m*)	33.6
6	1.40 (1H, *m*)	31.1	1.38 (1H, *m*)	31.1
	2.30 (1H, *m*)		2.30 (1H, *m*)	
7	1.67 (2H, *m*)	40.1	1.67 (2H, br. *t*, *J* = 7.5 Hz)	40
8	–	81.1	–	81.1
9	2.10 (1H, *m*)	52.3	2.10 (1H, *dd*, *J* = 8.5, 6.0 Hz)	52.2
10	1.30 (3H, *s*)	25.2	1.31 (3H, *s*)	25.2
11	–	171.3	–	171.3
1′	4.69 (1H, *d*, *J* = 8.0 Hz)	99.7	4.70 (1H, *d*, *J* = 8.0 Hz)	99.7
2′	3.20 (1H, *t*, *J* = 8.0 Hz)	74.8	3.21 (1H, *m*)	74.8
3′	3.39 (1H, *m*)	77.8	3.39 (1H, *t*, *J* = 9.0 Hz)	77.9
4′	3.35 (1H, *m*)	71.7	3.34 (1H, *dd*, *J* = 10.0, 8.5 Hz)	71.7
5′	3.50 (1H, *m*)	75.7	3.55 (1H, *m*)	75.7
6′	4.27 (1H, *dd*, *J* = 12.0, 5.5 Hz)	64.3	4.28 (1H, *dd*, *J* = 12.0, 6.0 Hz)	64.4
	4.49 (1H, *dd*, *J* = 12.0, 2.0 Hz)		4.49 (1H, *dd*, *J* = 12.0, 2.0 Hz)	
1″	–	169.3	–	169.4
2″	–	128.8	–	128.5
3″	6.78 (1H, *dt*, *J* = 8.5, 1.5 Hz)	143.6	6.78 (1H, *dt*, *J* = 7.5, 1.5 Hz)	144.4
4″	2.34 (2H, *m*)	28	2.22 (2H, *m*)	27.2
5″	2.15 (2H, *m*)	39.2	1.28 (1H, *m*)	37
			1.46 (1H, *m*)	
6″	–	138.3	1.62 (1H, *m*)	30.6
7″	5.38 (1H, br. *t*, *J* = 6.5 Hz)	125.8	1.36 (1H, *m*)	40.6
			1.60 (1H, *m*)	
8″	4.09 (2H, *d*, *J* = 6.5 Hz)	59.4	3.60 (2H, *m*)	61
9″	1.83 (3H, *s*)	12.6	1.83 (3H, *d*, *J* = 1.0 Hz)	12.5
10″	1.68 (3H, *s*)	16.2	0.93 (3H, *d*, *J* = 6.5 Hz)	19.8

iridoid skeleton. Additionally, absorptions due to aromatic rings (313 nm and 1443 cm^{-1}) were observed. The ^{1}H and ^{13}C NMR data of **7** (Table 2) were similar to those reported for 8-epiloganic acid (Nakamoto et al., 1988; Nishimura et al., 1989). The ^{13}C NMR spectrum of **7** (Table 2) showed 34 signals, sixteen of them could be attributed to 8-epiloganic acid, while the remaining 18 of them were identical with those reported for coumaroyl (Otsuka et al., 1989) and caffeoyl units (Garcia and Chulia, 1986 and 1987). The C-6′ signal was seen to be shifted significantly downfield by 1.4 ppm as well as 6′-H_2 by approximately 0.70 when compared with the data of the glucose moiety and with those of an epiloganic acid derivative (Nakamoto et al., 1988). A similar situation can be seen in the case of C-7 (δ_C shifted downfield by 4.2 ppm) and 7-H (δ_H shifted downfield by 0.80 ppm) when compared with the data of 8-epiloganic acid and related compounds described in literature, confirming a second esterification point at C-7 (Damtoft et al., 1984; König and Rimpler, 1985; Nakamoto et al., 1988; Nishimura et al., 1989).

The HMBC experiment with **7** revealed a significant interaction between 3-H and a carbon atom at δ_C 170.6, giving evidence of C-11 even though it was not observable in the ^{13}C NMR experiment. It was not possible to establish the linkage from the coumaroyl and caffeoyl units either to the oxygen atom at C-7 or to the oxygen atom at C-6′ due to the same chemical shift of the ester carbonyl groups at δ_C 169.0. We decided to solve this problem with ESI-MS/MS experiments (Scheme 1). The fragmentation of the pseudomolecular ion $[M+Na]^+$ at *m*/*z* 707 resulted in four main fragments at *m*/*z* 689 $[707-H_2O]^+$, 663 $[707-CO_2]^+$, 543 $[707-164\ (C_9H_8O_3)]^+$ and 365 $[707-342\ (C_{15}H_{18}O_9)]^+$. A second fragmentation of the ion at *m*/*z* 543 let to three fragments at *m*/*z* 525 $[543-H_2O]^+$, 499 $[543-CO_2]^+$ and



Scheme 1. ESI-MS/MS fragmentation pattern of compound **7**.

Table 2
^1H and ^{13}C NMR spectral data for compound **7** (CD_3OD, 300 MHz)

Atom numbers	7	
1	5.33 (1H, *d*, *J* = 5.5 Hz)	96
3	7.41 (1H, *s*)	152.4
4	–	115.9
5	3.06 (1H, *q*, *J* = 8.0 Hz)	32.6
6	1.92 (1H, *m*)	39.5
	2.17 (1H, *m*)	
7	4.90 (1H, *m*)	82.3
8	2.42 (1H, *m*)	43.1*,a
9	2.48 (1H, *m*)	43.4*,a
10	1.08 (3H, *d*, *J* = 7.0 Hz)	14.4
11	–	170.6
1′	4.73 (1H, *d*, *J* = 8.0 Hz)	99.9
2′	3.30 (1H, *m*)	74.8
3′	3.40 (1H, *t*, *J* = 6.5 Hz)	77.8
4′	3.39 (1H, *t*, *J* = 6.5 Hz)	71.7
5′	3.55 (1H, *m*)	75.7
6′	4.40 (1H, *dd*, *J* = 12.0, 6.0 Hz)	64
	4.53 (1H, *dd*, *J* = 12.0, 2.0 Hz)	
1″	–	127.7*,b
2″	7.04 (1H, *d*, *J* = 2.0 Hz)	114.8
3″	–	149.6
4″	–	147.3
5″	6.72 (1H, *d*, *J* = 8.0 Hz)	116.4
6″	6.91 (1H, *dd*, *J* = 8.0, 2.0 Hz)	123.2
7″	7.57 (1H, *d*, *J* = 16.0 Hz)	146.4*,c
8″	6.29 (1H, *d*, *J* = 16.0 Hz)	115.4*,d
9″	–	169
1‴	–	127.2*,b
2‴	7.43 (1H, *d*, *J* = 8.5 Hz)	131.2
3‴	6.78 (1H, *d*, *J* = 8.5 Hz)	116.8
4‴	–	161.2
5‴	6.78 (1H, *d*, *J* = 8.5 Hz)	116.8
6‴	7.43 (1H, *d*, *J* = 8.5 Hz)	131.2
7‴	7.56 (1H, *d*, *J* = 16.0 Hz)	146.4*,c
8‴	6.29 (1H, *d*, *J* = 16.0 Hz)	115.1*,d
9‴	–	169

*a,b,c,dAssigments bearing the same superscript may be exchanged.

365 [543−178 ($C_6H_{10}O_6$)]$^+$. This fragmentation pattern was only possible for a linkage of the caffeoyl unit to the hydroxy group at C-6′ and of the coumaroyl unit to the hydroxy group of C-7. Therefore the structure of the compound **7** was elucidated to be 7-*O*-*trans*-*p*-coumaroyl-6′-*O*-*trans*-caffeoyl-8-epiloganic acid (agnucastoside C). This is the first occurrence of an epiloganic acid derivative being reported for Verbenaceae.

Previously, *Vitex trifolia*, which contains flavonoids, sterols and terpenoids, has exhibited moderate inhibiting activity against most of tested Gram positive and Gram negative bacteria (Hossain et al., 2001). In this study, we have found that the above described compounds of *V. agnus-castus* showed no important antibacterial activity against the tested microorganisms and no cytotoxic activity against the tested cell lines.

3. Experimental

3.1. General

UV: Varian Cary 3E. IR: FT-IR Perkin-Elmer 1600. Optical rotation: Perkin-Elmer 343. ESI-MS: Finnigan LC-Q. ^1H NMR, ^{13}C NMR, APT, ^1H–^1H COSY, HSQC, HMBC and NOESY spectra: Varian Inova 500, Varian U 300, Bruker AMX 300. Chemical shifts are expressed in δ values (ppm), relative to TMS. Solvent resonances were used as internal references.

3.2. Plant material

The flowering stems (flowers, leaves and twigs) of *Vitex agnus-castus* L. were collected from Fethiye, Muğla, Turkey in June 2001. It was identified by one of the authors, Dr. Ayşe Kuruüzüm-Uz. A voucher specimen (no 01031) has been deposited in Faculty of Pharmacy, Hacettepe University, Ankara, Turkey.

3.3. Extraction and isolation

Dried and powdered flowering stems (700 g) of *V. agnus-castus* were extracted with MeOH (3×3.5 l, 45 °C) and the MeOH extract was evaporated to yield 300 g syrupy residue. The MeOH extract was dissolved in H_2O and partitioned with $CHCl_3$ (36 g) followed by *n*-BuOH (74 g). A part of the *n*-BuOH soluble fraction (25 g) was applied to a column of Si gel and eluted with $CHCl_3$ containing increasing amounts of MeOH to give nine fractions. The fractions 4–7 were further chromatographed to give **1** (14 mg), **2** (130 mg), **3** (52 mg), **4** (78 mg), **5** (5 mg), **6** (6 mg), **7** (26 mg) and **8** (6 mg).

Separations and purifications of compounds were carried out repeatedly on Si gel CC eluting with EtOAc:MeOH:H_2O (100:5:2 to 100:17:13) and $CHCl_3$:MeOH:H_2O (90:10:1 to 60:40:4), Sephadex LH-20 CC using MeOH and RP-18 CC with stepwise gradients of MeOH in H_2O.

3.3.1. Agnucastoside A (5)

Amorphous powder; $[\alpha]_D^{20} = -52°$ (MeOH, $c = 0.1$); IR (KBr) $\nu_{max} = 3425$, 2927, 1703, 1650, 1561, 1543, 1524, 1457, 1440 (sh), 1419 (sh), 1404, 1276, 1072, 1017, 841, 913, 854 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 220 (4.25) nm; for ^1H NMR and ^{13}C NMR spectral data see Table 1. ESI–MS: *m*/*z* 565 [M + Na]$^+$, $C_{26}H_{38}O_{12}$.

3.3.2. Agnucastoside B (6)

Amorphous powder; $[\alpha]_D^{20} = -64°$ (MeOH, $c = 0.1$); IR (KBr) $\nu_{max} = 3425$, 2929, 1700, 1649, 1559, 1541, 1527, 1459, 1442 (sh), 1424 (sh), 1404, 1385 (sh), 1277, 1207, 1159, 1154, 1070, 1022, 941, 912, 855, 745 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 220 (4.27) nm; for ^1H NMR and

^{13}C NMR spectral data see Table 1. ESI-MS: *m/z* 567 $[M+Na]^+$, $C_{26}H_{40}O_{12}$.

3.3.3. Agnucastoside C (7)

Amorphous powder; $[\alpha]_D^{20} = -28^\circ$ (MeOH, $c = 0.2$); IR (KBr) $\nu_{max} = 3423$, 2927, 1694, 1634, 1605, 1515, 1443, 1382, 1273, 1170, 1115, 1075, 1019, 981, 915, 857, 832 cm^{-1}; UV (MeOH) λ_{max} (log ε) = 219 (4.51), 313 (4.54) nm; for ^{1}H NMR and ^{13}C NMR spectral data see Table 2. ESI-MS: *m/z* 707 $[M+Na]^+$, $C_{34}H_{36}O_{15}$.

3.3.4. Assay for antimicrobial activity

The pure compounds were tested against four different strains: *Escherichia coli*, *Bacillus subtilis*, *Staphylococcus aureus* and *Candida albicans*. Penicillin G (*E. coli*, *B. subtilis* and *S. aureus*) and nystatin (*C. albicans*) were used as references. Paper discs (diameter: 6 mm) were soaked with the solutions of the pure compounds (solved in MeOH, $c = 1$ mg/ml), dried under sterile conditions and then placed on agar plates, which were incubated with the different strains. The agar plates were incubated 24 h at 25 °C (*C. albicans*) and 37 °C (*E. coli*, *B. subtilis* and *S. aureus*), respectively.

3.3.5. Assay for cytotoxic activity

Cytotoxic effect of the pure compounds (up to 10 μg/ml) were tested against three cancer cell lines [HM02 (stomach carcinoma), HepG2 (liver carcinoma) and MCF7 (mamma carcinoma)] according to NCI-directives (Grever et al., 1992).

Acknowledgements

The authors are thankful to Prof. Dr. W. Beil (Medizinische Hochschule Hannover) for cytotoxicity tests.

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