FABAD J. Pharm. Sci., 33, 11–16, 2008

RESEARCH ARTICLE

Antioxidant Potency of Flavonoids from *Vitex agnus-castus* L. growing in Turkey

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Summary

*Our aim was to investigate the isolation, structure elucidation and determination of radical scavenging properties of flavonoids from Vitex agnus-castus L. growing in Turkey. Four flavone glycosides, namely isoorientin (**1**), 2″-O-trans-caffeoylisoorientin (**2**), 6″-O-trans-caffeoylisoorientin (**3**) and luteolin 7-O-glucoside (**4**) were isolated from the methanol extract of the flowering stems of V. agnus-castus. The structure elucidation of the isolated compounds were made by spectroscopic methods (1D and 2D NMR and MS (ESI and DCI)) The radical scavenging activities of compounds **1-4** on DPPH were found to be very high.*

Key Words: *Vitex agnus-castus; Verbenaceae; Flavone C and O-glycosides, isoorientin, 2″-O-trans-caffeoylisoorientin, 6″-O-trans-caffeoylisoorientin, Luteolin 7-O-glucoside, Radical Scavenging Activity.*

Received: 21.08.2009
Revised: 12.01.2010
Accepted: 15.01.2010

Türkiye'de Yetişen Vitex agnus-castus L.'nin Antioksidan Etkili Flavonoitleri

Özet

*Bu çalışmada Türkiye'de yetişen Vitex agnus-castus L.'nin içerdiği flavonoitlerin izolasyonlarının, yapı tayinlerinin yapılması ve serbest radikal süpürücü özelliklerinin saptanması amaçlanmıştır. Vitex agnus-castus L.'nin çiçekli dal uçlarının metanol ekstresinden dört flavon glukoziti, izoorientin (**1**), 2″-O-trans-kafeoilizoorientin (**2**), 6″-O-trans-kafeoilizoorientin (**3**) ve luteolin-7-O-glukozit (**4**) izole edilmiştir. Bileşiklerin yapıları spektroskopik yöntemlerle (1D ve 2D NMR ve MS (ESI ve DCI)) saptanmıştır. **1-4** nolu bileşiklerin DPPH radikal süpürücü aktiviteleri çok yüksek bulunmuştur.*

Anahtar Kelimeler: *Vitex agnus-castus; Verbenaceae; Flavon C ve O-glukozitleri, izoorientin, 2″-O-trans-kafeoilizoorientin, 6″-O-trans-kafeoilizoorientin, luteolin 7-O-glukozit, Serbest Radikal Süpürücü Aktivite.*

INTRODUCTION

The genus *Vitex* (Verbenaceae) is represented by two species in the flora of Turkey (1). *Vitex agnus-castus* L. is used not only as a diuretic, digestive, antifungal agent but against anxiety, early birth and stomachache in Anatolia (2,3). *V. agnus-castus* is reported to have medicinal importance in the world. It also has a hormone-like effect. Therefore, it is used for the treatment of premenstrual problems and hyperprolactinemia (4,5). Investigations on *Vitex agnus-castus* have led to the isolation of iridoids (6-9), diterpenoids (10-12), essential oils (13), ketosteroids (14) and flavonoids (7,15-21).

Previous studies have shown that C-glycosyl flavones are common in genus *Vitex*. Hirobe et al. (15) have isolated four new flavonoids from the root bark of *Vitex*

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agnus-castus for the first time in nature, namely 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 compounds, namely 4′,5-dihydroxy-3,3′,6,7-tetramethoxyflavone, luteolin, artemetin and isorhamnetin. In addition, some flavonol and flavone derivatives (apigenin 3, 8-di-C-glycosides, eupatorin, casticin, penduletin, vitexin, isovitexin, isovitexin xyloside, orientin, isoorientin, luteolin 7-*O*-glucoside) were also isolated from *V. agnus castus* (7,15-17, 20-21).

Previously, we have also reported the isolation and structure elucidation of three new iridoids, namely 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-*epi*-loganic acid (agnucastoside C) in addition to four known iridoids (aucubin, agnuside, mussaenosidic acid and 6′-*O*-*p*-hydroxybenzoylmussaenosidic acid) and one known phenylbutanone glycoside (myzodendrone) from *Vitex agnus-castus* (9). In this study, isolation, structure elucidation and radical scavenging activity of flavonoids isolated from methanol extract of the flowering stems of *V. agnus-castus* were given.

MATERIALS AND METHODS

General Experimental Procedures

The UV (MeOH) spectra were recorded on Varian Cary 3E. IR spectra were recorded on a Perkin-Elmer 1600 spectrophotometer. ^{1}H NMR, ^{13}C NMR, APT, ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY spectra were determined on Varian Inova 500, Varian U 300, Bruker AMX 300. Chemical shifts were expressed in δ values (ppm), relative to TMS. Solvent resonances were used as internal references. ESI/DCI-MS were obtained using a Finnigan LC-Q instrument. Silica gel 60 (0.063-0.200 mm, Merck) and Sephadex LH-20 (Fluka) were used for open column chromatographic separations. TLC analyses were carried out on pre-coated Kieselgel 60 F_{254} aluminium sheets (Merck) and compounds were detected by UV fluorescence and spraying 1% vanillin-H_2SO_4 reagent, followed by heating at 105°C for 1-2 min. DPPH (2,2-diphenyl-1-picrylhydrazyl) was used for free radical scavenging activity test.

Plant Material

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

Extraction and isolation

Dried and powdered flowering stems of *V. agnus-castus* (700 g) were extracted three times with MeOH (3.5 L) at 45 °C and the combined extracts were evaporated under reduced pressure 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 phase (25 g) was fractionated on a silica gel column eluting with a gradient solvent system ($CHCl_3$-MeOH) to give nine main fractions (Frs. A-I). Fraction G (3.3 g) was further chromatographed over silica gel column eluting with EtOAc-MeOH-H_2O (100:5:2 to 100:17:13) to yield eight main fractions (Frs. G_{1-8}). Fr G_6 was applied to repeated column chromatographies (CC) over Sephadex LH-20 eluted with MeOH to afford compound **1** (isoorientin (=Luteolin 6-*C*-glucoside), 67 mg) and compound **4** (luteolin 7-*O*-glucoside**,** 5 mg). Fr. G_2 was subjected to column chromatography on Sephadex LH-20 using MeOH to give compound **2** (2′′-*O*-*trans*-caffeoylisoorientin**,** 17 mg). Fractionation of Fr. F (10.2 g) by open CC on silica gel using EtOAc-MeOH-H_2O (100:17:13) yielded subfractions F_{1-6} . Fr. F_2 was submitted to Sephadex LH-20 CC (MeOH) to afford pure compound **3** (6′′-*O*-*trans*-caffeoylisoorientin**,** 7 mg).

DPPH Radical Scavenging Activity Assay

The DPPH assay was carried out using the method described by Cavin et al. (22). The free radical scavenging effect of the isolated compounds **1-4** was assessed by the decoloration of MeOH solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH)

spectroscopically and ascorbic acid were used as standard. Each MeOH solution (230 μl) of the tested compounds at various concentrations (100, 50, 25, 10, 5, 1 μg/ml) was added to DPPH solution (50 μl, 0,022% in MeOH). The mixture was allowed to react for 30 min at room temperature. The absorbance of the solution was read at 517 nm with a spectrophotometer. Radical scavenging activity was determined by comparing the absorbance with that of a blank (100%) containing only DPPH and solvent. The percentage of radical scavenging activity (RSA%) was calculated as follows: RSA% = $((A_c - A_t)/ A_c) \times 100\%$, where A_c is the average absorbance of the control and A_t is the absorbance of the test compounds. All the analyses were made in dublicates.

RESULTS AND DISCUSSION

In this study, compounds **1-4** were isolated from the methanol extract of the flowering stems of *Vitex agnus-castus* L. by open column chromatographic methods on silica gel and Sephadex LH-20. The structure of the compounds were elucidated as isoorientin (**1**), 2′′-*O*-trans-caffeoylisoorientin (**2**), 6′′-*O*-trans-caffeoylisoorientin (**3**) and luteolin 7-*O*-glucoside (**4**) by spectroscopic methods (1D and 2D NMR and MS (ESI and DCI)) and by comparison of their physical and spectroscopical data with literature values (18, 23-26).

Isoorientin (=Luteolin 6-*C*-glucoside) **(1)**: UV (MeOH) λ_{max} 255, 270, 348. IR (KBr) ν_{max} 3390 (OH), 1650 (C=C), 1450 (aromatic ring), 1265, 1083 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ_H = 7.35 (1H, *dd*, *J* = 9.0 / 1.5 Hz, H-6′), 7.32 (1H, *d*, *J* = 1.5 Hz, H-2′), 6.92 (1H, *d*, *J* = 9.0 Hz, H-5′), 6.50 (1H, *s*, H-3), 6.45 (1H, *s*, H-8), 4.88 (1H, *d*, *J* = 9.0 Hz, H-1′′), 4.17 (1H, *t*, *J* = 9.0 Hz, H-2′′), 3.88 (1H, *dd*, *J* = 12.0 / 8.0 Hz, H_B-6′′), 3.74 (1H, *dd*, *J* = 12.0 / 5.0 Hz, H_A-6′′), 3.46 (3H, *m*, H-3′′, H-4′′, H-5′′). ^{13}C NMR (300 MHz, CD_3OD): δ_C = 184.0 (C-4), 166.2 (C-2), 164.9 (C-7), 162.0 (C-5), 158.7 (C-9), 151.0 (C-4′), 147.0 (C-3′), 123.5 (C-1′), 120.3 (C-6′), 116.8 (C-5′), 114.1 (C-2′), 109.1 (C-6), 105.1 (C-10), 103.9 (C-3), 95.2 (C-8), 82.6 (C-5′′), 80.1 (C-3′′), 75.3 (C-1′′), 72.6 (C-2′′), 71.8 (C-4′′), 62.9 (C-6′′). ESI-MS: *m/z* = 471 $[M+Na]^+$, $C_{21}H_{20}O_{11}$.

2′′-O-trans-caffeoylisoorientin **(2)**: UV (MeOH) λ_{max} 245, 336. IR (KBr) ν_{max} 3390(OH), 1650(C=C), 1578, 1450 (aromatic ring), 1267, 1079 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ_H = 7.36 (1H, *d*, *J* = 16.0, H-7′′′), 7.29 (1H, *d*, *J* = 1.5 Hz, H-2′), 7.28 (1H, *dd*, *J* = 9.0 / 1.5 Hz, H-6′), 6.90 (1H, *d*, *J* = 2.0 Hz, H-2′′′), 6.84 (1H, *d*, *J* = 9.0 Hz, H-5′), 6.80 (1H, *dd*, *J* =8.0 \ 2.0 Hz, H-6′′′), 6.69 (1H, *d*, *J* = 8.0 Hz, H-5′′′), 6.44 (1H, *s*, H-3), 6.39 (1H, br. *s*, H-8), 6.05 (1H, *d*, *J* = 16.0 Hz, H-8′′′), 5.66 (1H, br. *s*, H-2′′), 5.10 (1H, *d*, *J* = 10.0 Hz, H-1′′), 3.92 (1H, *dd*, *J* = 12.5 / 2.0 Hz, H_B-6′′), 3.79 (1H, *dd*, *J* = 12.5 / 5.5 Hz, H_A-6′′), 3.72 (1H, *t*, *J* = 9.0 Hz, H-3′′), 3.60 (1H, *t*, *J* = 9.0 Hz, H-4′′), 3.50 (1H, *m*, H-5′′). ^{13}C NMR (300 MHz, CD_3OD): δ_C = 183.8 (C-4), 168.3 (C-9′′′), 166.1 (C-2), 164.8 (C-7), 161.7 (C-5), 158.7 (C-9), 150.9 (C-4′), 149.4 (C-4′′′), 146.9 (C-7′′′,C-3′*), 146.6 (C-3′′′*), 127.6 (C-1′′′),123.4 (C-1′), 122.9 (C-6′′′), 120.3 (C-6′), 116.7 (C-5′), 116.4 (C-5′′′), 115.0 (C-2′′′), 114.8 (C-8′′′), 114.1 (C-2′), 107.9 (C-6), 104.9 (C-10), 103.8 (C-3), 95.2 (C-8), 82.8 (C-5′′), 77.9 (C-3′′), 73.8 (C-2′′), 73.2 (C-1′′), 71.7 (C-4′′), 62.8 (C-6′′); * signals are interchangeable. ESI-MS: *m/z* = 633 $[M+Na]^+$, $C_{30}H_{26}O_{14}$.

6′′-O-trans-caffeoylisoorientin **(3)**: UV (MeOH) λ_{max} 245, 336, IR (KBr) ν_{max} 3390(OH), 1650(C=C), 1578, 1450 (aromatic ring), 1267, 1079 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ_H = 7.55 (1H, *d*, *J* = 16.0, H-7′′′), 7.36 (1H, *dd*, *J* = 8.5 / 1.5 Hz, H-6′), 7.34 (1H, *d*, *J* = 1.5 Hz, H-2′), 7.02 (1H, *d*, *J* = 2.0 Hz, H-2′′′), 6.92 (1H, *dd*, *J* =8.0 \ 2.0 Hz, H-6′′′), 6.88 (1H, *d*, *J* = 8.5 Hz, H-5′), 6.75 (1H, *d*, *J* = 8.0 Hz, H-5′′′), 6.53 (1H, *s*, H-3), 6.48 (1H, *s*, H-8), 6.28 (1H, *d*, *J* = 16.0 Hz, H-8′′′), 4.90 (1H, *d*, H-1′′), 4.52 (1H, *dd*, *J* = 12.0 / 2.0 Hz, H_B-6′′), 4.34 (1H, *dd*, *J* = 12.0 / 5.5 Hz, H_A-6′′), 4.23 (1H, *t*, *J* = 9.0 Hz, H-2′′), 3.65 (1H, *m*, H-5′′), 3.51 (2H, *m*, H-3′′, H-4′′). ^{13}C NMR (300 MHz, CD_3OD): δ_C = 183.9 (C-4), 169.2 (C-9′′′), 166.1 (C-2), 165.2 (C-7), 162.1 (C-5), 158.7 (C-9), 151.1 (C-4′), 149.6 (C-4′′′), 147.1 (C-7′′′), 147.0 (C-3′), 146.7 (C-3′′′), 127.7 (C-1′′′),123.4 (C-1′), 123.1 (C-6′′′), 120.3 (C-6′), 116.8 (C-5′), 116.4 (C-5′′′), 115.1 (C-2′′′), 114.9 (C-8′′′), 114.1 (C-2′), 108.8 (C-6), 105.0 (C-10), 103.8 (C-3), 95.2 (C-8), 79.9 (C-3′′, C-5′′), 75.4 (C-1′′), 72.4 (C-2′′), 71.8 (C-4′′), 65.0 (C-6′′). ESI-MS: *m/z* = 633 $[M+Na]^+$, $C_{30}H_{26}O_{14}$.

Luteolin 7-*O*-glucoside **(4)**: UV (MeOH) λ_{max} 255, 336, IR (KBr) ν_{max} 3389 (OH), 1654 (C=C), 1445 (aromatic ring), 1070 cm^{-1}. ^1H NMR (300 MHz, CD_3OD): δ_H = 7.43

(1H, dd, J = 8.0 / 2.0 Hz, H-6′), 7.40 (1H, br. s, H-2′), 6.88 (1H, d, J = 8.0 Hz, H-5′), 6.77 (1H, d, *J* = 2.0 Hz, H-8), 6.71 (1H, s, H-3), 6.43 (1H, d, J = 2.0 Hz, H-6), 5.06 (1H, d, *J* = 7.0 Hz, H-1′′), 3.71 (1H, br. d, *J* = 10 Hz, H_B-6′′), 3.48 (1H, br. dd, *J* = 14.5 / 5.5 Hz, H_A-6′′), 3.46 (1H, m, H-5′′), 3.30 (1H, m, H-3′′), 3.28 (1H, t, J = 7.5 Hz, H-2′′). ^{13}C NMR (300 MHz, CD_3OD): δ_C = 181.8 (C-4), 164.5 (C-2), 162.9 (C-7), 161.1 (C-5), 156.9 (C-9), 150.2 (C-4′), 145.8 (C-3′),121.1 (C-1′), 119.1 (C-6′), 115.9 (C-5′), 113.4 (C-2′), 105.3 (C-10), 103.0 (C-3), 99.9 (C-1′′), 99.5 (C-6), 94.7 (C-8), 77.1 (C-5′′), 76.4 (C-3′′), 73.1 (C-2′′), 69.6 (C-4′′), 60.6 (C-6′′). DCI-MS: m/z = 449 $[M+H]^+$, $C_{21}H_{20}O_{11}$.

Compounds **1** - **4** were isolated as yellow amorphous powders. Their UV and IR spectra were characteristic of a flavone system.

Molecular formula of compound **1** was determined as $C_{21}H_{20}O_{11}$ by the NMR spectra and ESI-MS. In the ESI mass spectrum of the compound **1**, peak $[M+Na]^+$ was observed at m/z 471 . In the ^{1}H NMR spectrum of the compound **1,** H-6′and H-2′ gave dublet (*J*=9 Hz) and singlet at δ 7.35 and 7.32, resp. H-5′ proton signal was observed at 6.92 ppm as a dublet (*J*=9 Hz). H-3 and H-8 protons were at δ 6.50 (1H, s) and δ 6.45 (1H, s) respectively. Anomeric proton (H-1″) appeared at δ 4.88 as dublet with *J*=7 Hz to indicate β-glucosyl unit. Comparing with the reported data, the ^{1}H NMR and ^{13}C NMR data are in agreement with those of isoorientin in articles (23-26). Therefore, the structure of the compound **1** was determined as isoorientin.

The ESI-MS of compound **2** and **3** which exhibited $[M+Na]^+$ at *m/z* 633 showed that they had the same molecular formula ($C_{30}H_{26}O_{14}$). The NMR spectra of **2** and **3** showed the presence of a flavone skeleton with one caffeoyl and one C-glucosyl group. The C-glucose was assigned to C-6 from the cross peak between C6/H-1″, C7-H-1″ and C5-H-1″ in the HMBC spectrum. The caffeoyl groups were assigned to C-2″ for compound **2** and C-6″ for compound **3** from the cross peak between C-9′″- H-2″ and H-6″, resp. in the HMBC spectrum and the *trans-geometry* was elucidated from the coupling constants between H-7′″ and H-8′″ (*J* = 16.0 Hz). The NMR spectra of **2** were similar to those of **3**, but the chemical shifts of C-2″ and C-5″ were shifted downfield in comparison with those of **3**, 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 **2** were shifted downfield in comparison with those of **3**, but those of H-5″ and H-6″ were shifted upfield. These results demostrated that there were caffeoyl groups at C-2″ in **2** and C-6″ in **3**. Consequently, the structures of compounds **2** and **3** were determined as 2′′-*O*-trans-caffeoylisoorientin and 6′′-*O*-trans-caffeoylisoorientin, respectively (15, 23-26).

The molecular formula of compound **4** was determined as $C_{21}H_{20}O_{11}$ by DCI-MS, which exhibited a molecular ion peak at *m/z* 449 $[M+H]^+$. The ^{1}H- and ^{13}C-NMR spectral data of compound **4** indicated a luteolin bearing one glucose moiety. The site of glycosylation in **4** was determined to be the C-7 position based on the chemical shifts of H-6 and H-8 in comparison to that reported for luteolin (26) and from the cross peak between C-7 and H-1″ (δ_C 162.9 and δ_H 5.06) in the HMBC spectrum for compound **4**. The chemical shift value of the anomeric proton and carbon atom for compounds **4** as well as the C resonances of the hydroxyl group indicated that the β-glucose unit were connected at C-7. In comparison with the literature, data of luteolin were found to be in good agreement with those recorded for luteolin-7-*O*-glucoside in our study. Therefore, we conclude that compound **4** is luteolin 7-*O*-glucoside (23-26).

In addition, we tested the isolated flavonoids for their free radical scavenging activities by comparison with ascorbic acid as reference. DPPH radical scavenging activities of the compounds **1**-**4**, isolated from methanol extract are shown in *Table 1*. Compounds **1**-**4** were found to have significant free radical scavenging activity.

CONCLUSION

V. agnus-castus is reported to have medicinal importance. Previous studies of the secondary metabolites from *V. agnus-castus* have shown that C-glycosyl flavones are common in genus *Vitex* (27).

Table 1. *Scavenging activities of the compounds* **1-4** *on DPPH radical.*

Compounds	IC_{50}*
1	16.9
2	23.6
3	23.4
4	21.8
Ascorbic acid[a]	12.0

*IC_{50} values were calculated from regression lines using six different concentrations in dublicate.

[a] Positive control

We now report the results of chemical and biological examination of methanol extract of the flowering stems of *V. agnus-castus*. And this is the first report of the isolation of flavonoids from the flowering stems of *V. agnus-castus* growing in Turkey.

Oxidative stress has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases (Parkinson's and Alzheimer's). Flavonoids may provide a protection against these diseases. *V. agnus-castus* is rich in flavonoid derivatives, which have high antioxidant properties.

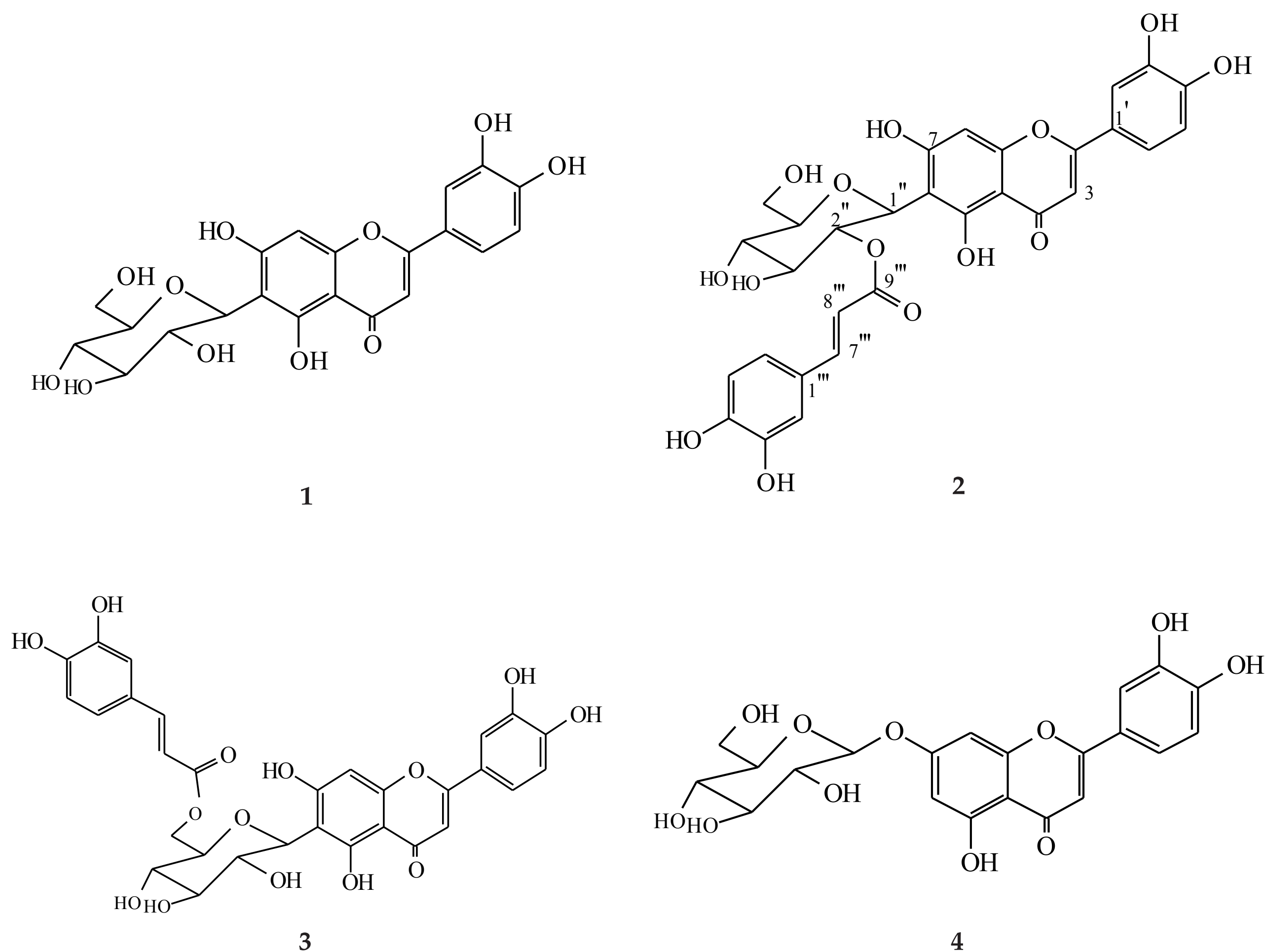

Figure 1. Flavonoid glycosides of *Vitex agnus-castus.*

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