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Short Communication

Flavonoids from the Aerial Parts of *Artemisia biennis* Willd

Kazhal Rostami Dehmoradkhani[1], Azizollah Jafari[2], Yalda Shokoohinia[3], Seyed Ahmad Emami[4], Mahdi Mojarrab[3*]

[1]*Department of Chemistry, Faculty of Sciences, University of Yasouj, Yasouj, Iran.*
[2]*Department of Biology, Faculty of Sciences, University of Yasouj, Yasouj, Iran.*
[3]*Pharrmaceutical Sciences Research Center, Health Institute, Kermanshah University of Medical Sciences, Kermanshah, Iran.*
[4]*Department of Pharmacognosy, School of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.*

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ABSTRACT

Background: The current phytochemical study was carried out on a fraction of dried polar extract of aerial parts of *Artemisia biennis* Willd. which was previously reported to decrease the viability of the rat pheochromocytoma cell line PC12 in cell-based antioxidant assays.
Methods: A combination of solid phase extraction (SPE) and high pressure liquid chromatography (HPLC) of the hydroethanolic extract was used to purify the compounds. Structures of the isolated compounds were elucidated by spectroscopic means, including ^{1}H-NMR and MS analyses.
Results: Three isolated and identified flavonoids in this study were luteolin, kaempferol and apigenin.
Conclusion: The cytotoxic potential of flavone aglycones, as the major components of selected fraction in the hydroethanolic extract of *A. biennis* might partly be related to the high death rate of PC12 cells.

Introduction

Artemisia biennis Willd. (Compositae) is one of 34 *Artemisia* species growing wild in Iran.[1] *Artemisia* spp are used in Iranian folk medicine as eupeptic, emmenagogue, antispasmodic, haemostatic and laxative.[2,3] Previous phytochemical investigations on *A. biennis* focused on volatile constituents which led to identification of camphor and (E)-beta-farnesene as the major components of essential oils obtained from Iranian and Canadian habitats, respectively.[4,5] Dichloromethane and ethanolic fractions of *A.biennis* were reported to have cytotoxicity and leishmanicidal activities, respectively [6-8] while no sesquiterpene lactone has been detected in the terpenoid extract of the species.[9] Another studies on different extracts of *A. biennis* showed the superiority of hydroethanolic and ethyl acetate extracts in antioxidant and antimalarial cell free assays, respectively.[10,11] In another research, antioxidant capacity was evaluated in seven fractions (A-G) of defatted hydroethanolic extract of *A. biennis* aerial parts via cell-based methods (in the rat pheochromocytoma cell line PC12). The results indicated that particularly a fraction named F (which was eluted by 80% methanol in water in a reversed phase vacuum liquid chromatography (VLC) system,), possessed notable inhibitory effect on viability of PC12 cell line.[12]

Here, we report the isolation and identification of three flavonoids (1 – 3) from this fraction of the defatted hydroethanolic extract of *A. biennis* aerial parts.

Materials and Methods

Chemicals

LiChroprep® RP-18 (15-25 µm) was purchased from Merck and all the solvents used for extraction, fractionation and purification from Scharlau. Deuterated methanol was obtained from Armar AG.

General experimental procedures

HPLC was carried out on a Young Lin apparatus equipped with a binary pump (YL 911S) and a PDA detector (YL 9160) using Vertica (RP_{18}, 250×30 mm i.d., 10 µm) column. A Supelco (RP_{18}, 250×10 mm i.d., 5 µm) column was replaced for final purification. Electron Impact Mass Spectrometry (EIMS) were performed on a 5973 Network Mass Selective Detector (Agilent technology). The NMR spectra were recorded on a Bruker Avance AV 400, using CD_3OD as the solvent and residual solvent signal used as internal standard.

Plant material

Aerial parts of *Artemisia biennis* Willd. were collected from Zoshk (Razavi Khorasan, Iran) in September 2010. Sample was identified by Dr V. Mozaffarian (Research Institute of Forest and Rangelands, Tehran, Iran). The voucher specimen (No. 12570) has been deposited in the herbarium, Department of Pharmacognosy, Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran.

***Corresponding Author:** Mahdi Mojarrab, E-mail: mmojarrab@kums.ac.ir

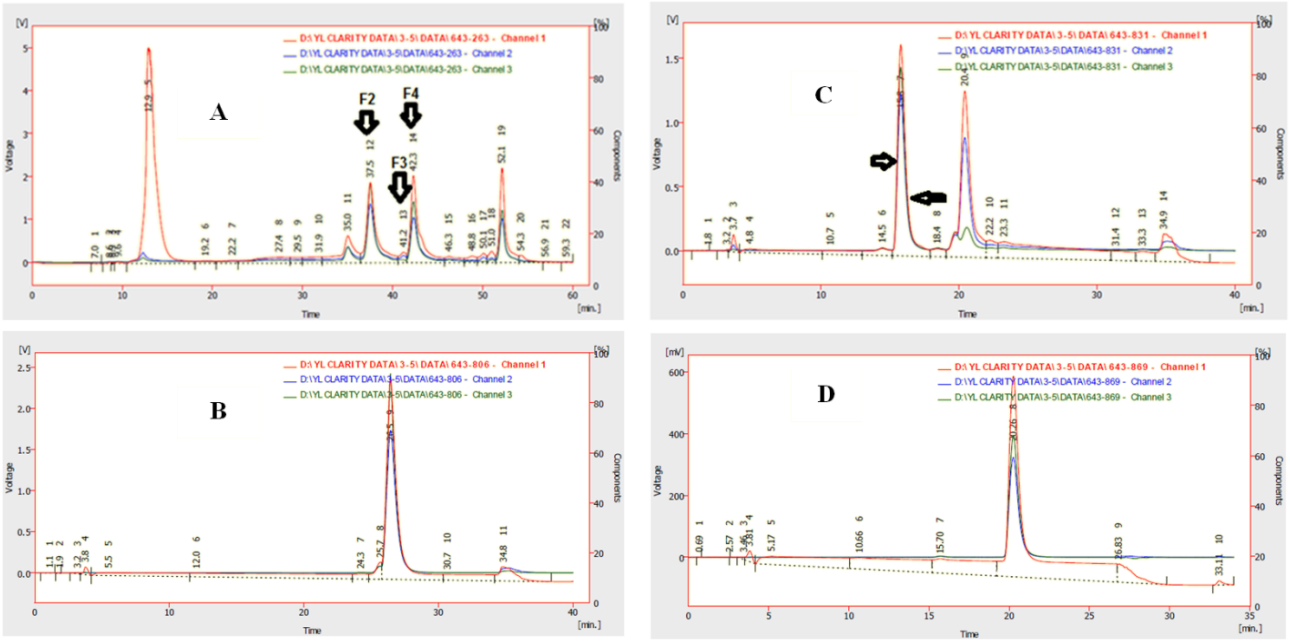


Figure 1. Chromatograms of (A) primary purification of the fraction F, (B) purification of the compound 1, (C) purification of the compound 2 and an additional amount of the compound 1,and (D) purification of the compound 3.

Extraction and fractionation

In this study, the fraction residues obtained in the previous studies[10,12] were used. Briefly, hydroethanolic extract was obtained by maceration after successive extraction of dried and grounded aerial parts with petroleum ether (40-60), dichloromethane, ethyl acetate, ethanol, and equal mixture of water-ethanol. The fractionation of hydroethanolic extract was carried out by reversed phase VLC method with rising ratios of methanol in water as a mobile phase. A fraction (F) which was eluted by 80% methanol in water was used for further phytochemical study.

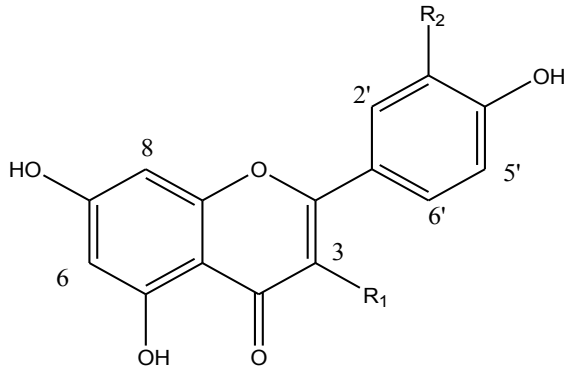
Isolation procedure

Primary purification of the fraction F (660 mg) by semi-preparative HPLC (mobile phase: 0-5 min, 50 % MeOH in H_2O, 0.1 % formic acid; 5–45 min, MeOH from 50 to 90% in H_2O, 0.1 % formic acid; 45–48 min, 90% MeOH in H_2O, 0.1 % formic acid; 48–49 min, MeOH from 90 to 100% in H_2O, 0.1 % formic acid; 49–55 min, 100% MeOH, flow rate 8 ml/min, detection at 220, 254 and 360 nm) yielded eight subfractions F1-F8 (retention times= 35.0, 37.5, 41.2, 42.3, 43.5, 50.1, 51.0 and 52.1 min, respectively). Further purification of F2 (13.3 mg) by semi-preparative HPLC (mobile phase: 0–30 min, MeOH from 40 to 60 % in H_2O, 0.1 % formic acid; 30–31 min MeOH from 60 to 100% in H_2O, 0.1 % formic acid; 31–37 min 100% MeOH, flow rate 3 ml/min, detection at 220, 254 and 360 nm), yielded compound 1 (0.9 mg; t_R= 26.6 min). Final purification of the F3 (41.0 mg) by semi-preparative HPLC (mobile phase: 0–22.5 min, MeOH from 50 to 65 % in H_2O, 0.1 % formic acid; 22.5–23 min MeOH from 65 to 100% in H_2O, 0.1 % formic acid; 23–30 min 100% MeOH, flow rate 3 ml/min, detection at 220, 254 and 360 nm) afforded an additional amount of compound 1 (8.0 mg; t_R= 15.6 min) and compound 2 (1.2 mg; t_R= 15.8 min). The same procedure was followed to isolate and purify compound 3 (23.5 mg; t_R= 20.3 min) from F4 (140.2 mg) (Figure 1). Structure elucidation of the isolated compounds was achieved using spectroscopic techniques including EIMS and ^{1}H-NMR experiments.

Results and Discussion

Using combination of solid phase extraction (SPE) and HPLC methods, three flavonoids were isolated from the defatted hydroethanolic extract of *A. biennis* and their structures were elucidated by means of spectroscopic analysis including EIMS and ^{1}H-NMR. All spectroscopic data were in agreement with those given in the literature.[13-15]

Compounds 1 to 3 were defined to be luteolin, kaempferol and apigenin, respectively (Figure 2).



R_1 = H, R_2= OH — Luteolin
R_1 = OH, R_2= H — Kaempferol
R_1 = R_2 = H — Apigenin

Figure 2. Chemical structures of isolated flavonoids from the aerial parts of *A. biennis*.

Compound 1

Luteolin [=2-(3,4-dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone); yellow crystalline solid,^{1}H-NMR (CD_3OD, 400 MHz): 6.22 (1H, d, J = 2.0 Hz, H-6), 6.45 (1H, d, J = 2.0 Hz, H-8), 6.55 (1H, s, H-3), 6.92 (1H, d, J = 8.8 Hz, H-5′), 7.39 (1H, d, J = 2.0 Hz, H-2′), 7.40 (1H, dd, J = 2.0, 8.8 Hz H-6′].
EIMS: *m/z* 286 $[M]^+$.

Compound 2

Kaempferol [=3,5,7-trihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one); yellow crystalline solid, ^{1}H-NMR (CD_3OD, 400 MHz): 6.20 (1H, d, J = 2.0 Hz, H-6), 6.41 (1H, d, J = 2.0 Hz, H-8), 6.93 (2H, d, J = 8.4 Hz, H-3′,5′), 8.11 (2H, d, J = 8.4 Hz, H-2′, 6′].
EIMS: *m/z* 286 $[M]^+$.

Compound 3

Apigenin [=5,7-Dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one); yellow crystalline solid, ^{1}H-NMR (CD_3OD, 400 MHz): 6.22 (1H, d, J = 2.0 Hz, H-6), 6.47 (1H, d, J = 2.0 Hz, H-8), 6.61 (1H, s, H-3), 6.95 (2H, d, J = 8.8 Hz, H-3′, 5′), 7.87 (2H, d, J = 8.8 Hz, H-2′, 6′].
EIMS: *m/z* 270 $[M]^+$.

The EIMS molecular ion peak of compounds 1 and 2 observed at *m/z* 286 a.m.u, consistent with the molecular formula of $C_{15}H_{10}O_6$. In the ^{1}H-NMR spectrum of compound 1, the presence of a singlet with the chemical shift of 6.55 ppm was indicative of a flavone structure. Substitution patterns of hydroxyl groups in ring A were similar for both compounds while they differed in number of hydroxyl substitution groups on ring B. The EIMS molecular ion peak of compound 3 observed at *m/z* 270 a.m.u, suggesting the molecular formula of $C_{15}H_{10}O_5$. In the ^{1}H-NMR spectrum of compound 3, the presence of a singlet with the chemical shift of 6.61 ppm was indicative of a flavone structure. Substitution patterns of hydroxyl groups in rings A and B were similar to those of compound 2.

These facts, along with the comparison of the rest of spectroscopic data with those reported in literature,[13-15] allowed identification of 1, 2 and 3 as luteolin, kaempferol and apigenin, respectively. There is no previous report on the presence of three flavonoid aglycones in *A. biennis*, but these phytochemicals have been proven to be present in some other species of the genus like *A. sacrorum, A. anomala, A. vulgaris* and *A. ludoviciana.*[16-19] Luteolin, apigenin and quercetin are regarded as the most common flavonoids in the tribe Anthemideae. Interestingly, among more than 160 isolated individual flavonoid components in the genus *Artemisia*, one third of them are derivatives of the flavones luteolin and apigenin.[20] In the current study, they have been isolated from a fraction of hydroethanolic extract which had the most powerful inhibitory effect on viability of PC12 cell line, based on a previous report.[12] In a study on neuroprotective effects of six phenolic compounds on H_2O_2-induced oxidative damage in PC12 cells, the cell viabilities of all experimental groups increased except the apigenin treated group. Apigenin nearly exhibited no effect on H_2O_2-induced PC12 cells damage.[21] Survival (%) of undifferentiated PC12 cells preincubated with apigenin prior to exposure to linoleic acid hydroperoxide in MTT assay was reported to be lower than that of negative control. Mean survival percentages of differentiated PC12 cells preincubated with the same condition and determined by the trypan blue exclusion test were the same for apigenin and negative control.[22]

The viability of the rat cardiac myoblast cell line H9C2 treated with 13.5 µg/ml of apigenin was significantly lower compared with the control.[23] Apigenin induced micronuclei in a dose dependent manner in peripheral human lymphocytes and it may have potential clastogenic activity on human genetic material *in vitro*.[24] Clastogenicity of luteolin and apigenin in Chinese hamster lung cell line V79 has been demonstrated as well.[25] Time - dependent viability of human peripheral blood lymphocytes treated *in vitro* with the flavonoids apigenin , kaempferol and luteolin administered at different concentrations was studied and it was indicated that 2.86 µg /ml of kaempferol had the highest cytotoxic effect (after 18 h of incubation), followed by luteolin and apigenin. The ability of the flavonoids to cause oxidative damage in lymphocyte DNA was assumed as a reason for these observations.[26] Cytotoxicity of isolated flavonoids in the current study has been previously evaluated against normal cell lines including human lung embryonic fibroblast cell line TIG-1 and human umbilical vein endothelial cell line HUVEC. In comparison with kaempferol, apigenin (similar IC_{50} values of 29.7 µg/ml in both of the assays) and luteolin (IC_{50} values of 30.6 and 16.3 µg/ml, respectively) have demonstrated stronger cytotoxicity toward the cell lines.[27]

Defatted hydroethanolic extract of *A. biennis* which showed potent free radical scavenging effect and metal chelating activity [10] was fractionated and all the fractions were subjected to cell–free and cell-based antioxidant assays.[10,12] Fraction F demonstrated disappointing results in metal chelating activity and viability of PC12 cell line.[10,12] In the present study, three flavonoids were isolated and identified from this fraction which corroborated previous reports on the biological effects of the these compounds and their distribution in the genus *Artemisia*.

Conclusion

The presence of apigenin, luteolin and kaempferol might partly be related to the reported decrease in viability of PC12 cell line after exposure to a fraction of hydroethanolic extract of *A. biennis.* Flavone aglycones seem to be dominant in this fraction.

Acknowledgments

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Conflict of interests

The authors claim that there is no conflict of interest.

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