Anti-Inflammatory and Antinociceptive Activity of Flavonoids Isolated from *Viscum album* ssp. *album*

Didem Deliorman Orhan, Esra Küpeli, Erdem Yesilada*, and Fatma Ergun

Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, 06330, Etiler, Ankara, Turkey. Fax: +90-312-2235018. E-mail:yesilada@gazi.edu.tr

* Author for correspondence and reprint requests

Z. Naturforsch. **61 c**, 26–30 (2006); received August 1, 2005

Viscum album L. has been used in the indigenous systems of medicine for treatment of headache and some inflammatory diseases. In order to evaluate this information, antinociceptive and anti-inflammatory activities of the five flavonoids (5,7-dimethoxy naringenin or 4′,6′-dimethoxy chalcononaringenin) derivatives, isolated from the ethyl acetate fraction of the extract from *V. album* ssp. *album*, were investigated, namely 5,7-dimethoxy-flavanone-4′-*O*-β-D-glucopyranoside (**1**), 2′-hydroxy-4′,6′-dimethoxy-chalcone-4-*O*-β-D-glucopyranoside (**2**), 5,7-dimethoxy-flavanone-4′-*O*-[2″-*O*-(5‴-*O*-*trans*-cinnamoyl)-β-D-apiofuranosyl]-β-D-glucopyranoside (**3**), 2′-hydroxy-4′,6′-dimethoxy-chalcone-4-*O*-[2″-*O*-(5‴-*O*-*trans*-cinnamoyl)-β-D-apiofuranosyl]-β-D-glucopyranoside (**4**), 5,7-dimethoxy-flavanone-4′-*O*-[β-D-apiofuranosyl-(1→2)]-β-D-glucopyranoside (**5**). For the antinociceptive activity assessment the *p*-benzoquinone-induced writhing test and for the anti-inflammatory activity the carrageenan-induced hind paw edema model in mice were used. The ethyl acetate fraction in a dose of 250 mg/kg as well as compounds **2** and **5** in a 30 mg/kg dose were shown to possess remarkable antinociceptive and anti-inflammatory activities *per os* without inducing any apparent acute toxicity as well as gastric damage.

Key words: Viscum album, Flavonoids, Anti-Inflammatory, Antinociceptive

Introduction

The genus *Viscum* L. (Loranthaceae) comprises semi-parasitic plants which grow on various host trees and shrubs (Miller, 1982). *Viscum album* L. is the most widespread species worldwide and has been reputed against cardiovascular diseases, *i.e.* hypertension and atherosclerosis; various bone and joint disorders including periarthritis, spondylitis, and arthritis; to alleviate headache; for immune system stimulation; in nervous disorders as sedative or to combat epilepsy (Bartram, 1995; Murray, 1995; Newall, *et al.*, 1996; Wichtl and Bisset, 1994).

There are three *Viscum album* subspecies in Turkey, ssp. *album*, ssp. *austriacum* (Wiesb.) Vollmann, and ssp. *abietis* (Wiesb.) Abromeit. In a previous report, the aqueous and ethanol extracts and fractions obtained from these subspecies through successive solvent extractions with petroleum ether, diethyl ether, chloroform, ethyl acetate, and *n*-butanol saturated with water were investigated for their inhibitory effects on macrophage-derived cytokines [interleukin-1α (IL-1α), interleukin-1β (IL-1ß), tumor necrosis factor-α (TNF-α)] which are considered to play a key role in inflammatory and immune responses (Yesilada *et al.*, 1998). The ethyl acetate fraction of *V. album* ssp. *album* exhibited a more potent inhibitory effect than the other fractions against IL-1α. Therefore, in the present study, we investigated the anti-inflammatory and antinociceptive activity of bioactive components from the ethyl acetate fraction of *V. album* ssp. *album.*

Materials and Methods

Plant material

V. album L. ssp. *album* was collected from *Armeniaca vulgaris* Lam. (apricot) as host plant in Ankara, Baglum in an orchard. A voucher specimen of the plant (AEF 18953) is kept in the Herbarium of Faculty of Pharmacy at Ankara University.

Chemical procedures

The dried and powdered leaves and stems of *V. album* ssp. *album* (1 kg) were extracted with 80% ethanol (25 l) several times at room temperature. The combined ethanol extract was evaporated to dryness *in vacuo*. The residue dissolved in distilled water and then extracted with ethyl acetate. The ethyl acetate-soluble portion was evaporated un-

der reduced pressure to give a residue (13.5 g), which was chromatographed over silica gel using several successive solvent systems ($CHCl_3$/CH_3OH 95:5 → $CHCl_3$/CH_3OH/H_2O 10:90:10), ($CHCl_3$/CH_3OH → $CHCl_3$/CH_3OH/H_2O → CH_3OH → CH_3OH/H_2O → H_2O), and the fractions were combined into eleven main fractions [Fr. (2–5), 0.237 g; Fr. (6), 0.35 g; Fr. (7, 8), 1.42 g; Fr. (9–12), 0.184 g; Fr. (13–15), 1 g; Fr. (16), 0.48 g; Fr. (17–19), 0.123 g; Fr. (20), 0.225 g; Fr. (21), 0.089 g; Fr. (22), 0.049 g; Fr. (26), 0.172 g] according to TLC control.

Fr. (7, 8) (1.417 g) was subjected to MPLC (medium pressure liquid chromatography) (Li-Chroprep RP-18, Merck, 18.5 × 352 mm, CH_3OH/H_2O gradient, 20–90% CH_3OH) to give Fr. (13–17) and Fr. (30–36). Fr. (13–17) (193.61 mg) and Fr. (30–36) (279.83 mg) were further applied to a series of chromatographic separations on a silica gel column with $CHCl_3$/CH_3OH 99:1 → 90:10 and $CHCl_3$/CH_3OH/H_2O 80:20:1 → 80:20:2 as mobile phase to yield 5,7-dimethoxy-flavanone-4′-*O*-*β*-D-glucopyranoside (**1**) (6.21 mg), 2′-hydroxy-4′,6′-dimethoxy-chalcone-4-*O*-*β*-D-glucopyranoside (**2**) (9.90 mg), 5,7-dimethoxy-flavanone-4′-*O*-[2″-*O*-(5‴-*O*-*trans*-cinnamoyl)-*β*-D-apiofuranosyl]-*β*-D-glucopyranoside (**3**) (11.93 mg).

Fr. (6) (0.35 g) was further chromatographed by MPLC to give 2′-hydroxy-4′,6′-dimethoxy-chalcone-4-*O*-[2″-*O*-(5‴-*O*-*trans*-cinnamoyl)-*β*-D-apiofuranosyl]-*β*-D-glucopyranoside (**4**) (10.95 mg) and **3** (52.66 mg).

Fr. (13–15) (1 g) obtained from a silica gel column was applied to MPLC (CH_3OH/H_2O gradient, 20–90% CH_3OH) to give Fr. (65–72). Then Fr. (65–72) (0.132 g) was subjected to a Sephadex LH-20 column (CH_3OH) to give 5,7-dimethoxy-flavanone-4′-*O*-[*β*-D-apiofuranosyl(1→2)]-*β*-D-glucopyranoside (**5**) (13.78 mg). The structures of these compounds were identified by spectroscopic methods (UV, IR, ^1H and ^{13}C NMR, MS) and are reported elsewhere (Deliorman, 1999). Compounds **3** and **4** are new compounds (Orhan *et al.*, 2002).

Test animals

Male Swiss albino mice (20–25 g) were purchased from the animal breeding laboratories of Refik Saydam Central Institute of Health (Ankara, Turkey). The animals left for 2 d for acclimatization to animal room conditions were maintained on standard pellet diet and water *ad libitum*. The food was withdrawn on the day before the experiment, but free access of water was allowed. A minimum of six animals was used in each group. Throughout the experiments, animals were processed according to the suggested ethical guidelines for the care of laboratory animals.

Preparation of test samples for bioassay

After suspending in a mixture of distilled H_2O and 0.5% sodium carboxymethyl cellulose (CMC) test samples were given orally to animals . The control group animals received the same experimental handling as those of the test groups except that the drug treatment was replaced by appropriate volumes of the dosing vehicle. Either indomethacin (10 mg/kg) or acetyl salicylic acid (ASA) (200 mg/kg) in 0.5% CMC was used as reference drug.

Antinociceptive activity: p-benzoquinone-induced abdominal constriction test in mice (Okun et al., 1963)

60 min after the oral administration of test samples, the mice were intraperitoneally injected with 0.1 ml/10 g body weight of 2.5% (w/v) *p*-benzoquinone (PBQ; Merck) solution in distilled H_2O. Control animals received an appropriate volume of dosing vehicle. The mice were then kept individually for observation and the total number of abdominal contractions (writhing movements) was counted for the next 15 min, starting on the 5th min after the PBQ injection. The data represent the average of the total number of writhes observed. The antinociceptive activity was expressed as percentage change from writhing controls. 100 mg/kg ASA were used as a reference drug.

Carrageenan-induced hind paw edema (Yesilada and Küpeli, 2002)

60 min after the oral administration of test sample or dosing vehicle each mouse was injected with freshly prepared (0.5 mg/25 μl) suspension of carrageenan (Sigma, St. Louis, Missouri, USA) in physiological saline (154 nM NaCl) into subplantar tissue of the right hind paw. As the control, 25 μl saline solution were injected into that of the left hind paw. Paw edema was measured every 90 min during 6 h after induction of inflammation. The difference in footpad thickness was measured by gauge calipers (Ozaki Co., Tokyo, Japan). Mean values of treated groups were compared with

mean values of a control group and analyzed using statistical methods. Indomethacin (10 mg/kg) was used as reference drug.

Acute toxicity

Animals employed in the carrageenan-induced paw edema experiment were observed during 48 h and morbidity or mortality was recorded, if happens, for each group at the end of the observation period.

Gastric-ulcerogenic effect

After the analgesic activity experiment mice were killed under deep ether anesthesia and stomachs were removed. Then the abdomen of each mouse was opened through the greater curvature and examined under a dissecting microscope for lesions or bleedings.

Statistical analysis of data

Data obtained from animal experiments were expressed as mean standard error (± SEM). Statistical differences between the treatments and the control were evaluated by ANOVA and Students-Newman-Keuls post-hoc tests. $p < 0.05$ was considered to be significant [* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$].

Results and Discussion

Inflammatory diseases are among the most common health problems treated with traditional remedies. Therefore it is crucial to evaluate the potential of herbal remedies for the discovery of novel bioactive compounds that might serve as leads for the development of potent drugs.

Carrageenan-induced inflammation is useful in determining orally active anti-inflammatory agents

Table I. Effects of flavonoids isolated from *V. album* ssp. *album* against carrageenan-induced paw edema in mice.

Material	Dose [mg/kg]	Swelling thickness ($\times 10^{-2}$ mm) ± SEM (% inhibition)			
		90 min	180 min	270 min	360 min
Control		49.8 ± 5.6	57.0 ± 5.7	63.5 ± 5.8	71.5 ± 4.9
Ethyl acetate	125	37.5 ± 3.1 (24.7)	41.8 ± 3.1 (26.7)	45.3 ± 3.3 (28.7)*	50.7 ± 3.3 (29.1)**
Ethyl acetate	250	34.5 ± 4.6 (30.7)*	37.3 ± 3.8 (34.6)*	41.0 ± 3.6 (35.4)*	44.5 ± 4.1 (37.2)**
1	30	42.3 ± 3.0 (15.1)	47.2 ± 3.4 (17.2)	51.8 ± 2.9 (18.4)	56.3 ± 2.8 (21.3)*
2	30	34.5 ± 4.1 (30.7)	38.5 ± 4.0 (32.5)*	43.0 ± 4.2 (32.3)*	47.5 ± 4.8 (33.6)**
3	30	45.8 ± 3.8 (8.0)	48.7 ± 3.8 (14.6)	51.8 ± 4.6 (18.4)	53.5 ± 4.9 (25.2)*
4	30	43.7 ± 3.9 (12.2)	48.7 ± 4.0 (14.6)	53.7 ± 4.5 (15.4)	57.7 ± 4.4 (19.3)
5	30	43.2 ± 4.67 (13.3)	46.5 ± 4.7 (18.4)	48.7 ± 3.8 (23.3)*	49.0 ± 4.3 (31.5)*
Indomethacin	10	33.2 ± 3.38 (33.3)*	36.8 ± 3.1 (35.4)*	40.0 ± 3.3 (37.0)**	41.0 ± 3.1 (42.7)***

Values are expressed as mean standard error (± SEM). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$

Material	Dose [mg/kg]	Number of writhings ± SEM	Inhibitory ratio (%)	Ratio of ulceration
Control		48.8 ± 4.2		0/6
Ethyl acetate	125	35.7 ± 3.2	26.8*	0/6
Ethyl acetate	250	32.8 ± 2.6	32.8**	1/6
1	30	39.3 ± 4.6	19.5	0/6
2	30	36.2 ± 3.0	25.8*	0/6
3	30	39.8 ± 2.6	18.4*	0/6
5	30	36.3 ± 2.4	25.6*	0/6
ASA	200	22.0 ± 2.2	54.9*	4/6

Table II. Effect of flavonoids isolated from *V. album* ssp. *album* against *p*-benzoquinone-induced writhings in mice.

Values are expressed as mean standard error (± SEM).
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(Ismail *et al.*, 1997). Edema formation due to carrageenan in the rat paw is a biphasic event. The initial phase is attributed to the release of histamine and serotonin. The edema produced at the peak (180 min) is thought to be due to the release of kinin-like substances, especially of bradykinin. The second phase of edema is due to the release of prostaglandins, protease and lysosome. The second phase is known to be sensitive to most clinically effective anti-inflammatory drugs (Olajide *et al.*, 1999).

As shown in Table I, the ethyl acetate fraction showed a remarkable and dose-dependent anti-inflammatory activity in the both phases of acute inflammation (between 24.7 to 37.2%). The same pharmacological profile was also observed for compound **2** (30.7–33.6%) from the ethyl acetate fraction. The anti-inflammatory activity of **2** was almost as potent as that of indomethacin (10 mg/kg) which exerts 33.3–42.7% anti-inflammatory activity. Other flavonoids, **1**, **3**, and **5**, isolated from the ethyl acetate fraction, also showed significant but weaker anti-inflammatory activity against the same model.

For the antinociceptive activity assessment the *p*-benzoquinone-induced writhing model was used. The ethyl acetate fraction of *V. album* ssp. *album* exhibited a significant and dose-dependent antinociceptive activity (Table II). Among the isolated compounds from the ethyl acetate fraction administered in a 30 mg/kg dose, compounds **2** and **5** were found to possess the highest and significant antinociceptive activity, but not as potent as ASA.

The ethyl acetate fraction and isolated compounds did not induce any apparent acute toxicity during the 48 h observation period. It is noteworthy that, in spite of a weak gastric lesion incidence in the higher dose of the ethyl acetate fraction (gastric lesions were observed in the stomach of 1 out of 6 rats), all the isolated flavonoids were found completely safe from the view point of gastric damage.

The chemical structures of the isolated flavonoids are given in Fig. 1. All flavonoids possess closely related chemical structures either a 5,7-dimethoxy-flavanone (**1**, **3**, **5**) or an equivalent chalcone [2′-hydroxy-4′,6′-dimethoxy chalcone] (**2**, **4**) structure with different substitutions. Among the equivalent structures **1** (flavanone) and **2** (chalcone), the anti-inflammatory activity of the latter was highest, while 2″-(5‴-*trans*-cinnamoyl-*β*-D-apiofuranosyl-) substitution, as in **3** and **4**, consid-

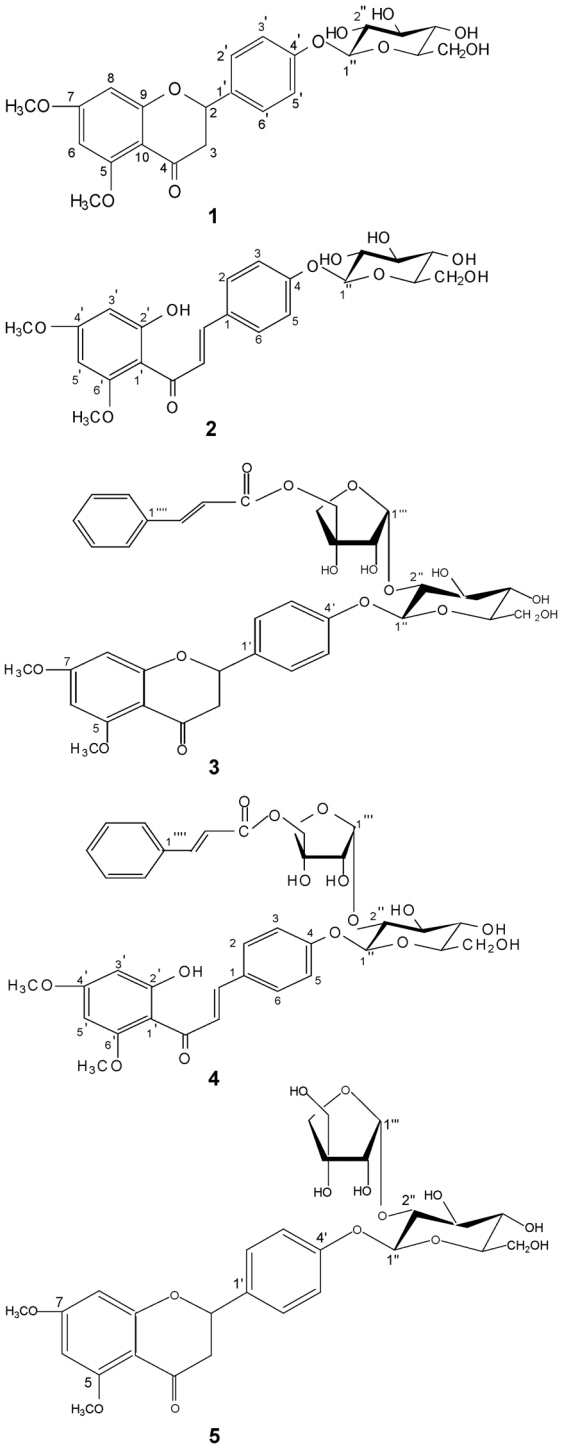

Fig. 1. Structures of investigated compounds.

erably decreased the activity. However, only 2″-β-D-apiofuranosyl substitution of **1** (as in **5**) increases both the anti-inflammatory and antinociceptive activities of the flavanone structure. (Note of the authors: Due to the low yield of compound **4** the antinociceptive activity could not be studied.)

The anti-inflammatory and antinociceptive activities of various flavonoid derivatives including flavanone and chalcone derivatives were previously reported by several authors (Pelzer *et al.*, 1998). Anti-inflammatory effects have already been demonstrated with chalcones (Hsieh *et al.*, 1998). Herencia *et al.* (1999) investigated the effects of a series of chalcone derivatives on various *in vivo* and *in vitro* inflammatory models (*i.e.* human neutrophil functions, eicosanoid release, TNF-α production, air-pouch, etc.) and reported inhibitory effects on iNOS and COX-2. Corra *et al.* (2001) reported potent antinociceptive activity for 3,4-dichlorochalcones using the writhing test in mice.

In the study of Pelzer *et al.* (1998) it was concluded that the anti-inflammatory activity of flavonoids increases depending upon the catechol or guaiacol-like substitution of ring B (3′,4′-dihydroxy or 3′-hydroxy-4′-methoxy or 3′-methoxy-4′-hydroxy). Accordingly flavanones such as eriodictyol, 7-*O*-methyleriodictyol and hesperitin showed the highest activity against carrageenan-induced edema. However, flavanone and chalcone derivatives demonstrated to possess anti-inflammatory activity in the present study were shown to possess one glucosylated hydroxy group at ring B [5,7-dimethoxy naringenin (**1**, **3**, and **5**) or 4′,6′-dimethoxy chalcononaringenin (**2**, **4**) derivatives]. Therefore, results showed herein present additional structural data for the anti-inflammatory and antinociceptive activity evaluation of chalcone and flavanone derivatives.

The results of the present study have clearly demonstrated that leaves and stems of *V. album* ssp. *album* possess significant antinociceptive and anti-inflammatory activities which support the traditional utilization of this plant and flavanone and chalcone derivatives were isolated as the active constituents of the ethyl acetate fraction. Further studies should be conducted using different test models of inflammation in order to establish its possible mechanism(s) of action.

Bartram T. (1995), Encyclopedia of Herbal Medicine. Grace, Dorsett, p. 296.

Corra R., Pereira M. A., Buffon D., Dos Santos L., Cechinel Filho V., Santos A. R., and Nunes R. J. (2001), Antinociceptive properties of chalcones. Structure-activity relationships. Arch. Pharm. **334**, 332–334.

Deliorman D.(1999), Pharmacognostical comparisons of *V. album* L. subspecies. Ph. D. Thesis, Gazi University, Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Turkey.

Herencia F., Ferrandiz L. M., Ubeda A., Guillén I., Domínguez J. N., Charris J. E., Lobo G. M., and Alcaraz M. J. (1999), Novel anti-inflammatory chalcone derivatives inhibit the induction of nitric oxide synthase and cyclooxygenase-2 in mouse peritoneal macrophages. FEBS Lett. **453**, 129–134.

Hsieh H. K., Lee T. H., Wang J. P., Wang J. J., and Lin C. N. (1998), Synthesis and anti-inflammatory effect of chalcones and related compounds. Pharm. Res. **15**, 39–46.

Ismail T. S., Gopalakrishnan S., Begum V. H., and Elango V. (1997), Anti-inflammatory activity of *Salacia oblonga* Wall. and *Azima tetracantha* Lam. J. Ethnopharmacol. **56**, 145–152.

Miller A. G. (1982), *Viscum album* L. In: Flora of Turkey and the East Aegean Islands, vol. 7 (Davis P. H., ed.). Edinburgh University Press, Edinburgh, p. 547.

Murray T. M. (1995), The Healing Power of Herbs, 2nd ed. Prima, Rocklin, CA, pp. 253–260.

Newall C. A., Anderson L. A., and Phillipson J. D. (1996), Herbal Medicines. Pharmaceutical Press, London, pp. 193–196.

Okun R., Liddon S. C., and Lasagnal L. (1963), The effect of aggregation, electric shock and adrenergic blocking drugs on inhibition of the "writhing syndrome". J. Pharmacol. Exp. Ther. **139**, 107–109.

Olajide O., Makinde J. M., and Awe S. O. (1999), Effects of the aqueous extract of *Bridelia ferruginea* stem bark on carrageenan-induced oedema and granuloma tissue formation in rats and mice. J. Ethnopharmacol. **66**, 113–117.

Orhan D. D., Çalış İ., and Ergun F. (2002), Two new flavonoid glycosides from *Viscum album* ssp. *album*. Pharm. Biol. **40**, 380–383.

Pelzer L. E., Guardia T., Juarez A. O., and Guerreiro E. (1998), Acute and chronic antiinflammatory effects of plant flavonoids. Il Fármaco **53**, 421.

Wichtl M. and Bisset N. G. (eds.) (1994), Visci herba – Mistletoe herb. In: Herbal Drugs and Phyto-pharmaceuticals. CRC Press, Stuttgart, pp. 534–536.

Yesilada E. and Küpeli E. (2002). *Berberis crataegina* DC. root exhibits potent anti-inflammatory, analgesic and febrifuge effects in mice and rats. J. Ethnopharmacol. **79**, 237–248.

Yesilada E., Deliorman D., Ergun F., Takaishi Y., and Ono Y. (1998), Effects of the Turkish subspecies of *Viscum album* on macrophage-derived cytokines. J. Ethnopharmacol. **61**, 195–200.