Anti-Inflammatory and Antinociceptive Activity of Coumarins from *Seseli gummiferum* subsp. *corymbosum* (Apiaceae)

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n-Hexane and ethyl acetate extracts as well as coumarin derivatives obtained from the *n*-hexane extract of the aerial parts of *Seseli gummiferum* Pall. ex Sm. subsp. *corymbosum* (Boiss. & Heldr.) P.H. Davis (Apiaceae) were evaluated *in vivo* for their anti-inflammatory and antinociceptive activities. The *n*-hexane and ethyl acetate extracts of the species were shown to possess significant inhibitory activity against the carrageenan-induced hind paw edema and *p*-benzoquinone-induced writhing models in mice. Among the isolated coumarin derivatives; (−)-(3'S,4'S)-3'-acetoxy-4'-isovaleryloxy-3',4'-dihydroseselin (**1**), (−)-(3'S,4'S)-3'-acetoxy-4'-angeloyloxy-3',4'-dihydroseselin (**2**), (+)-(3'S,4'S)-3'-hydroxy-4'-angeloyloxy-3',4'-dihydroseselin (D-laserpitin) (**3**), (−)-(3'S,4'S)-3'-angeloyloxy-4'-hydroxy-3',4'-dihydroseselin (**4**), and osthole (**5**), only the 3'-acetoxy derivatives **1** and **2** were found to possess potent antinociceptive and anti-inflammatory activities, *per os*, without inducing any apparent acute toxicity as well as gastric damage, while all other compounds and extracts were found to be ineffective in the TPA-induced mouse ear edema model assay.

Key words: Apiaceae, Anti-Inflammatory Activity, Antinociceptive Activity

Introduction

The Apiaceae family is one of the well-known aromatic herbs families with economically important species. Some of the members are used as foods, spices, condiments and ornamentals (Lawrence, 1969; Pimenov and Leonov, 1993).

Several biological activity tests have been carried out so far, in particular focused on the essential oil contents of *Seseli* species (Tosun *et al.*, 2003), *e.g.* on the fungitoxic and antimicrobial activity (Chaturvedi and Tripathi, 1989). Tandan *et al.* (1990) reported a significant and dose-dependent anti-inflammatory and analgesic activity for *S. indicum* seeds. Recently, the antimicrobial, anti-inflammatory and antinociceptive activities of several Turkish *Seseli* species have been reported (Küpeli *et al.*, 2006). In that study, the ethyl acetate extracts of some *Seseli* species showed potential activity against the carrageenan-induced hind paw edema and *p*-benzoquinone-induced writhing models. The results prompted us to investigate the compounds isolated from the active *Seseli* species. Among these species *Seseli gummiferum* subsp.

corymbosum is a perennial and monocarpic plant growing in southern Anatolia, Turkey (Hedge and Lamond, 1972). In our previous studies, five angular-type dihydropyranocoumarins and a simple coumarin, osthole (Fig. 1), were isolated from the *n*-hexane extract of aerial parts of the plant (Tosun *et al.*, 2003, 2005a). In the current study, anti-inflammatory and antinociceptive activities of some of these coumarins were investigated together with the crude *n*-hexane and ethyl acetate extracts by using carrageenan-induced hind paw edema, TPA-induced ear edema and *p*-benzoquinone-induced writhing models in experimental animals.

Experimental

Plant material

The plant material was collected at Antalya-Akseki, Pinarbasi Village, Turkey. A voucher specimen identified by Prof. H. Duman, Department of Biology, Faculty of Science and Art, Gazi University, Ankara, Turkey, is deposited at the

Herbarium of the Faculty of Pharmacy of Ankara University (AEF), Turkey (Herbarium Number AEF 21701).

Preparation of plant extracts

Aerial parts of the plants were dried in shade and powdered by using a laboratory scale mill. Powdered aerial parts (10 g) were extracted with *n*-hexane and ethyl acetate (200 ml), respectively, at room temperature and continuous stirring for 8 h. The solvents were removed under reduced pressure to dryness to give *n*-hexane and ethyl acetate extracts [5.7% and 4.7% (w/w), respectively].

Chromatographic separation and isolation of the constituents

In a previous study (Tosun *et al.*, 2003, 2005a), aerial parts of *S. gummiferum* subsp. *corymbosum* were extracted with *n*-hexane, Et$_2$O, EtOAc and MeOH, respectively, in a Soxhlet apparatus for 8 h; then the solvents were removed under vacuum to obtain the respective crude extracts. The *n*-hexane extract of the plant was submitted to silica gel column chromatography eluted with *n*-hexane/EtOAc mixtures of increasing polarity to yield angular-type pyranocoumarins together with a simple coumarin (Fig. 1). Further purification of the compounds was carried out by preparative HPLC under the following conditions: TOSOH-prep-HPLC instrument equipped with a HPLC packed column of Senshu-Pak silica 4251-N (250 mm × 10 mm) and detection with a TOSOH UV-8010 instrument at 320 nm.

Structure elucidation of the compounds

The chemical structures of the compounds were determined as (−)-(3'*S*,4'*S*)-3'-acetoxy-4'-iso-valeryloxy-3',4'-dihydroseselin (**1**), (−)-(3'*S*,4'*S*)-3'-acetoxy-4'-angeloyloxy-3',4'-dihydroseselin (**2**), (+)-(3'*S*,4'*S*)-3'-hydroxy-4'-angeloyloxy-3',4'-di-hydroseselin (**3**), (−)-(3'*S*,4'*S*)-3'-angeloyloxy-4'-hydroxy-3',4'-dihydroseselin (**4**), and osthole (**5**) (Tosun *et al.*, 2003, 2005a) by spectroscopic and physical analyses, and comparison of the data with those of known compounds previously reported (Gonzales *et al.*, 1979; Murray *et al.*, 1982; Swager and Cardellina, 1985; Ikeshiro *et al.*, 1993; Fan *et al.*, 2000). Compounds **1**−**5** are shown in Fig. 1.

HPLC analysis of the extracts

HPLC analysis was carried out with a Hitachi Model chromatograph equipped with a Hamilton Model Rheodyn 7125 injector (20 μl loop) and a photodiode array ultraviolet detector L-7450H operated at 320 nm. The temperature was maintained constant at 40 °C. The injector volume was 10 μl and the pressure was 34 kg/cm^2. A prepacked analytical column [Senshu-Pak Silica (1251-N), 4.6 × 250 mm I.D.] was used as chromatographic column.

Elution of the *n*-hexane extract was carried out with a gradient of *n*-hexane and ethyl acetate. Solvent A consisted of *n*-hexane/ethyl acetate (9:1) and solvent B of ethyl acetate. The gradient was applied as follows: 0−20 min, 80% A/20% B (isocratic); 10 min, increasing amount of solvent B; 10 min 100% B. At the end, the column was washed with ethyl acetate for 10 min. The flow rate was kept at 0.8 ml/min.

For the elution of the ethyl acetate extract, *n*-hexane/ethyl acetate (2:1) was used for 0−20 min and the ethyl acetate ratio was increased up to 50 min and held for 10 min in the same ratio; the analysis was completed after 75 min. Before the application, the ethyl acetate extract was filtered through Sep-Pak® Cartridges (Waters) (Tosun *et al.*, 2007). Solvents were obtained from Fisher Chemical and were of HPLC grade. All solvents were filtered through Shibata 1311−25100 (25G/P100) filters and all samples were filtered through membrane filters (0.45 μ) prior to analysis.

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 to animal room conditions for acclimatization were maintained on standard pellet diet and water *ad libitum*. The food was withdrawn on the day before the experiment, but free access to 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

All plant materials were administered in 100 mg/kg body wt. doses suspended in 0.5%

sodium carboxymethyl cellulose (CMC) in distilled water. 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 body wt. for carrageenan-induced hind paw edema; 0.5 mg/ear for TPA-induced ear edema) or acetyl salicylic acid (Aspirin, ASA) (100 mg/kg body wt.) in 0.5% CMC were used as reference drugs.

Antinociceptive activity

The *p*-benzoquinone-induced abdominal constriction test (Okun *et al.*, 1963) was performed on mice for the determination of the antinociceptive activity. According to the method, 60 min after oral administration of the test samples, the mice were intraperitoneally injected with 0.1 ml/10 g body wt. 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 PBQ injection. The data represent the average of the total number of writhings observed. The antinociceptive activity was expressed as percentage change from writhing controls. ASA at an 100 mg/kg body wt. dose was used as the reference drug in this test.

Anti-inflammatory activity

Carrageenan-induced hind paw edema model

The carrageenan-induced hind paw edema model was used for the determination of the anti-inflammatory activity (Yesilada and Küpeli, 2002). 60 min after oral administration of the test sample or dosing vehicle, into the subplantar tissue of the right hind paw of each mouse a freshly prepared (0.5 mg/25 μl) suspension of carrageenan (Sigma, St. Louis, Missouri, USA) in physiological saline (154 nM NaCl) was injected. As the control, 25 μl saline solution were injected into the subplantar tissue 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 a 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 body wt.) was used as the reference drug.

TPA-induced mouse ear edema

Each mouse received 2.5 μg of TPA (12-*O*-tetradecanoylphorbol-13-acetate*)* dissolved in 20 μl of 70% EtOH (De Young *et al.*, 1989), which was applied by an automatic pipette in 20 μl volumes to both anterior and posterior surfaces of the right ear. The left ear (control) received the same volume of solvent (70% EtOH) simultaneously. Indomethacin (0.5 mg/ear) was used as a standard drug. For the evaluation of the activity, two different ways were followed up.

1. The thickness of each ear was measured 4 h after induction of inflammation using a gauge calipers. The edema was expressed as the difference between right and left ears due to TPA application and, consequently, inhibition percentage was expressed as the reduction thickness with respect to the control group.

2. After 4 h, the animals were killed under deep diethyl ether anesthesia. Disks of 6 mm diameter were removed from each ear and weighed in balance. The swelling was estimated as the difference in weight between the punches from right and left ears, and expressed as an increase in ear thickness.

Acute toxicity

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

Gastric-ulcerogenic effect

After the antinociceptive activity experiment, mice were killed under deep diethyl 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 ($\pm$ SEM). Statistical differences between the treatments and the control were evaluated by ANOVA and Stu-

dents-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

The *n*-hexane and ethyl acetate extracts, and five coumarins from *S. gummiferum* subsp. *corymbosum* growing in Turkey were evaluated for their *in vivo* anti-inflammatory activity, using carrageenan-induced hind paw edema and TPA-induced mouse ear edema models, and for their antinociceptive activity, using the *p*-benzoquinone-induced abdominal constriction test in mice at a dose of 100 mg/kg body weight. The experimental results are shown in Tables I, II and III.

As shown in Table I, **1** and **2** obtained from the *n*-hexane extract showed significant antinociceptive activity; 38.2% and 28.1% inhibition were achieved, respectively, in *p*-benzoquinone-induced abdominal contractions without inducing any ap-

parent gastric damage. While ASA, the reference compound, showed 53.6% inhibition at the same dose, 4 out of 6 mice suffered from severe gastric damage.

The *n*-hexane and ethyl acetate extracts, and **2** also exhibited notable inhibition, ranging between 20.1–31.6%, 24.6–29.9%, and 22.5–29.9%, respectively, in the carrageenan-induced hind paw edema model, and the activity was quite comparable to that of reference compound indomethacin (31.5–36.4% inhibition) (Table II). However, all extracts and compounds were found to be completely ineffective against the TPA-induced ear edema model (Table III). On the other hand, neither antinociceptive nor anti-inflammatory activity was observed with the rest of the compounds and the methanol extract of this plant (Table I and II). The acute toxicity assessment has also revealed that all the extracts and compounds were safe in the administered dose.

Table I. Effect of compounds **1–5** against *p*-benzoquinone-induced writhings in mice.

Compound	Dose [mg/kg]	Number of writhings ± SEM	Inhibitory ratio (%)	Ratio of ulceration
Control		57.6 ± 5.1		0/6
1	100	35.6 ± 3.7	38.2**	0/6
2	100	41.4 ± 3.4	28.1*	0/6
3	100	47.6 ± 2.9	17.4	0/6
4	100	50.2 ± 2.7	12.8	0/6
5	100	44.3 ± 4.1	23.1	0/6
ASA	100	26.7 ± 2.5	53.6***	4/6

*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
ASA, acetyl salicylic acid; SEM, standard error mean.

Table II. Effect of compounds **1–5** against carrageenan-induced paw edema in mice.

Compound	Dose [mg/kg]	Swelling thickness ($\cdot 10^{-2}$ mm) ± SEM (% inhibition)			
		90 min	180 min	270 min	360 min
Control		51.2 ± 4.6	59.6 ± 4.3	63.4 ± 3.8	71.2 ± 3.9
1	100	39.7 ± 2.6 (22.4)	44.9 ± 2.9 (24.7)*	47.8 ± 3.9 (24.6)	55.9 ± 4.1 (21.5)
2	100	37.8 ± 3.0 (26.2)*	41.8 ± 3.3 (29.9)**	47.4 ± 3.7 (25.2)	55.2 ± 4.0 (22.5)
3	100	53.4 ± 3.3	61.1 ± 3.7	67.9 ± 3.1	73.3 ± 3.6
4	100	55.7 ± 5.2	65.8 ± 4.9	64.8 ± 4.1	77.9 ± 3.4
5	100	47.4 ± 3.1 (7.4)	55.4 ± 3.7 (7.0)	59.8 ± 3.2 (6.0)	65.8 ± 3.9 (7.6)
Indomethacin	10	34.5 ± 2.7 (32.6)**	40.4 ± 3.2 (32.2)**	43.4 ± 2.6 (31.5)**	45.3 ± 2.4 (36.4)***

*$p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.
SEM, standard error mean.

Table III. Effects of compounds **1–5** against TPA-induced ear edema in mice measured as swelling thickness and weight of edema.
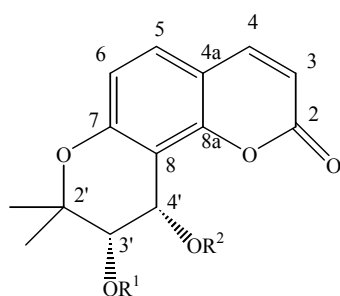
Compound	Dose [mg/ear]	Swelling thickness [μm] $\pm$ SEM	Inhibition (%)	Weight of edema [mg] $\pm$ SEM	Inhibition (%)
Control		234.7 $\pm$ 34.8		31.7 $\pm$ 4.7	
1	0.5	215.9 $\pm$ 20.8	8.0	28.4 $\pm$ 3.7	10.4
2	0.5	201.6 $\pm$ 23.9	14.1	25.7 $\pm$ 3.1	18.9
3	0.5	242.9 $\pm$ 27.8	–	29.8 $\pm$ 3.4	5.9
4	0.5	235.4 $\pm$ 30.2	–	37.1 $\pm$ 2.9	–
5	0.5	254.3 $\pm$ 31.2	–	35.6 $\pm$ 4.8	–
Indomethacin	0.5	69.7 $\pm$ 18.9	70.3***	14.2 $\pm$ 1.7	55.2***

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ significant from control.
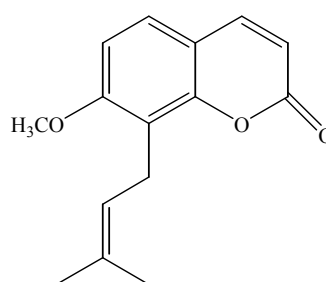SEM, standard error mean.

Various phytochemical studies have been carried out on the various components of several *Seseli* species, *i.e.*, coumarins (Gonzales *et al.*, 1979; Banerjee *et al.*, 1987; Lemmich and Shabana, 1984; Barrero *et al.*, 1992; Ceccherelli *et al.*, 1990; Glowniak *et al.*, 1991; Tosun, 2006; Tosun *et al.*, 2003, 2005a, 2006a), cinnamic acid derivatives (Banerjee *et al.*, 1987), sesquiterpene lactones, phenylpropanoids (Barrero *et al.*, 1992, 1994), and essential oils (Baser *et al.*, 2000; Bader *et al.*, 2003; Kaya *et al.*, 2003; Tosun *et al.*, 2005b, 2006b; Dogan *et al.*, 2006; Chaturvedi and Tripathi, 1989). Previously, Tandan *et al.* (1990) reported that a coumarin, seselin, from the seeds of *S. indicum* was shown to possess significant and dose-dependent anti-inflammatory and analgesic activities. Egan *et al.* (1990) reported that coumarin inhibited the release of histamine from mast cells and also possesses mild adrenergic activity. Higher inhibitory rates were also observed for the active coumarin derivatives **1** and **2** in the early stages of the carrageenan-induced edema model where histamine and related metabolites were released (Olajide *et al.*, 1999) (Table II).

Garcia-Argaez *et al.* (2000) have also proven the anti-inflammatory effect of several coumarins, including seselin, using a TPA-induced ear edema model in mice and concluded that the anti-inflammatory activity of coumarins depend on the individual substitutions on the aromatic ring rather than the coumarin skeleton itself. They have further suggested that the activity might possibly be due to their ability to inhibit lipid peroxidation and to act as a radical scavenger.

	R^1	**R**^{2}
1	COCH$_3$	COCH$_2$CH(CH$_3$)$_2$
2	COCH$_3$	COC(CH$_3$)=CHCH$_3$
3	H	COC(CH$_3$)=CHCH$_3$
4	COC(CH$_3$)=CHCH$_3$	H

5 Osthole

Fig. 1. Chemical structures of the investigated coumarins.

Although, Garcia-Argaez *et al.* (2000) reported a poor dose-activity relationship for seselin, this compound showed 53.8% inhibition against TPA-induced ear edema at a dose of 0.5 mg/ear, the similar dose level for the coumarins administered in the present study. Since we did not observe any remarkable activity for the seselin derivatives **1** and **2** in the present study against TPA-induced ear edema, it may be proposed that hydroxylation and/or substitution of 3'- and 4'-positions in the seselin-type coumarins may have a negative effect on the inhibitory potential in the TPA-induced ear edema model. Since the mechanism of TPA-induced edema is at least partially involved in the lipoxygenase pathway of the inflammatory processes, this substitution may have a reducing effect on the action of this type of coumarins (Lloret and Moreno, 1995).

As a result, less polar extracts such as *n*-hexane and ethyl acetate extracts, and coumarins **1** and **2**, among the isolated components from the *n*-hexane extract, showed remarkable anti-inflammatory and antinociceptive activities. The chemical structures of the isolated coumarins are shown in Fig. 1. The main distinguishing chemical characteristic of the active coumarins **1** and **2** is the acetoxy group at the 3'-position of the seselin ring. This substitution seems crucial for the anti-inflammatory and antinociceptive activity of the seselin derivatives of coumarins; however, this suggestion needs further scientific evidence. Since some *Seseli* species are used as vegetable in Turkey as well as in folk medicine (Baytop, 1999), investigation of the active components in the *Seseli* species may motivate future studies to isolate other therapeutic components with reasonable safety profiles.

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