Antinociceptive and Anti-Inflammatory Effects of Saponin and Iridoid Glycosides from *Verbascum pterocalycinum* var. *mutense* Hub.-Mor.

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The anti-inflammatory and antinociceptive properties of four major compounds from the flowers of *Verbascum pterocalycinum* var. *mutense* were investigated. Saponin glycosides called ilwensisaponin A and C and iridoid glycosides known as ajugol and picroside IV were isolated from the methanolic extract. A dose-related anti-inflammatory and antinociceptive response were obtained in this study at doses of 100 and 200 mg/kg. The results of the evaluation of the anti-inflammatory activity induced by carrageenan and PGE_1 showed that this species possesses active constituents that could diminish the cyclooxygenase activitiy. No effects were observed in the 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ear edema model. Our results support the anti-inflammatory and analgesic effects of *Verbascum pterocalycinum* var. *mutense*. Ilwensisaponins A and C could explain in part the anti-inflammatory and analgesic activities of this species. Although antinociceptive and anti-inflammatory activities of ajugol and picroside IV were found insignificant in the statistical analysis, ilwensisaponin A and C showed notable activity without inducing any apparent acute toxicity as well as gastric damage.

Key words: *Verbascum pterocalycinum* var. *mutense*, Anti-Inflammatory Activity, Antinociceptive Activity

Introduction

The genus *Verbascum* L. (Scrophulariaceae), commonly known as "mullein", is represented by 228 species, 185 of which are endemic in the flora of Turkey (Huber-Morath, 1978). *Verbascum* extracts, decoctions and infusions have been used in traditional medicine. The leaves and flowers of *Verbascum* are reported to have expectorant, mucolytic and demulcent properties and are used to treat respiratory problems such as bronchitis, dry coughs, tuberculosis and asthma in traditional Turkish medicine (Baytop, 1999; Turker and Camper, 2002). *Verbascum* flowers are boiled in milk and are applied externally for pruritic conditions affecting the urogenital organs. In addition, as a traditional Anatolian cure for anal fistulae, the flowers are boiled in a cauldron and then the anus is exposed to the vapours. These species are reported to be midly diuretic and to have a soothing and anti-inflammatory effect on the urinary tract, as well as to act as a mild sedative. Oil made from the flowers is used to help soothing earache, and can be applied externally for eczema and other types of inflammatory skin conditions. These species are commonly used to treat hemorrhoids, rheumatic pain, superficial fungal infections, wounds and diarrhea. They are traditionally consumed as a tea to relieve abdominal pains (Tuzlaci and Erol, 1999; Sezik *et al.*, 2001; Turker and Camper, 2002).

In previous studies, the aqueous extracts from the flowers of *Verbascum thapsiforme* demonstrated a strong inhibitory effect on the elongation step of protein biosynthesis. It was suggested that the saponin fraction was responsible for the activity (Paszkiewicz-Gadek *et al.*, 1990). The protective activities of the saponins may be due to the activation of mucous membrane protective factors, inhibition of the gastric secretion volume and acid secretion. The water extract of *V. cheiranthifolium* Boiss. var. *cheiranthifolium* given orally was tested for gastric protection against an ethanol-induced

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gastric ulcer model in rats, and the extract had a promising activity (Gurbuz et *al.*, 2005).

Phytochemical studies of *Verbascum pterocalycinum* var. *mutense* have revealed the presence of oleanane-type triterpene saponins (Tatli *et al.*, 2004). The pharmacological activities of saponins in plants, such as their anti-inflammatory, antitumour, antiexudative, antiulcer, analgesic, antipyretic and immunostimulant effects, have been known for many years, while new activities are continually being discovered (Hostettmann and Marston, 1995).

In the current study, anti-inflammatory and antinociceptive activities of the isolated compounds from *Verbascum pterocalycinum* var. *mutense* were discussed using carrageenan-induced hind paw edema, PGE_1-induced hind paw edema and TPA-induced ear edema models, and *p*-benzoquinone-induced writhing in mice.

Material and Methods

Plant material

Verbascum pterocalycinum var. *mutense* Hub.-Mor. was collected from Icel, between Mut and Karaman, 930–1100 m, in July 2000. A voucher specimen has been authenticated by Prof. Dr. Hayri Duman (Gazi University, Faculty of Science, Etiler, Ankara, Turkey) and was deposited at the herbarium of the Pharmacognosy Department, Faculty of Pharmacy, Hacettepe University, Ankara, Turkey (HUEF 00184).

Chromatographic separation and isolation of the constituents

The plant material was dried under shade and powdered to a fine grade by using a laboratory scale mill. Dried flower parts of the plant material (485.6 g) were extracted with methanol by maceration at room temperature for two times (2×2 L). The combined extract was evaporated to dryness *in vacuo* to give 43.5 g methanol extract. The methanol extract was fractionated by column chromatography (CC) on silica gel (500 g) using hexane, ethylacetate, chloroform, acetone and methanol (4 L each), respectively, to yield 5 fractions (Fractions A–E). Fraction E (11.5 g), eluted with methanol, was subjected to vacuum liquid chromatography (VLC) using reversed-phase material (Sepralyte 40 μm, 750 g) and MeOH/H_2O mixtures (0–100%) to give ilwensisaponin A (mimengoside A, **1**) (283.1 mg), ilwensisaponin C (**2**) (260.1 mg), and fraction E_3 (80.9 mg). Fraction E_3 was rechromatographed on a silica gel column (65 g) and eluted with $CHCl_3$/MeOH mixtures (90:10, 85:15, 80:20) to yield ajugol (**3**) (3.4 mg). Fraction D (1.6 g), eluted with acetone, was applied to VLC using reversed-phase material (Sepralyte 40 μm, 150 g) and MeOH/H_2O mixtures (0–100%) to give 3 fractions (Fractions D_1–D_3). Fraction D_2 (945.6 mg) was subjected to a silica gel column (57 g) using $CHCl_3$ and $CHCl_3$/MeOH mixtures (95:5, 90:10, 85:15, 80:20, 70:30) to yield picroside IV (**4**) (6.3 mg).

*Structure elucidation of compounds **1**–**4***

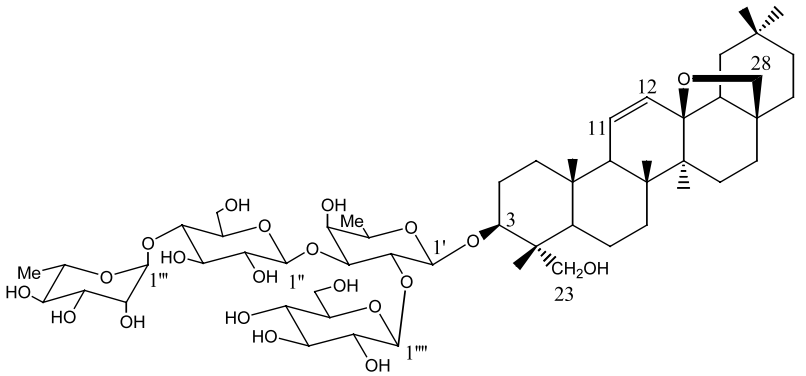
Structure elucidation of the isolated compounds **1**–**4** was carried out by spectral techniques; UV, IR, 1D- and 2D-NMR, mass spectroscopy (HR-ESIMS) and other detailed data were recently published elsewhere (Tatli *et al.*, 2004). The structures of compounds **1**–**4** were as follows (Fig. 1): ilwensisaponin A (**1**), ilwensisaponin C (**2**), ajugol (**3**) and picroside IV (**4**).

Animals

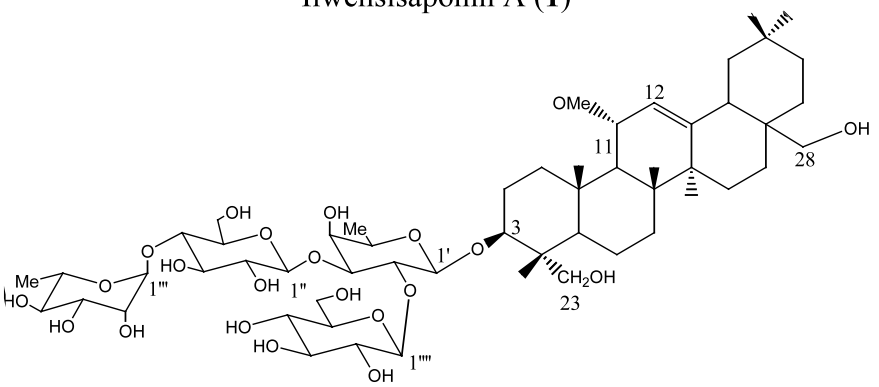
Male Swiss albino mice (20–25 g) were purchased from the animal breeding laboratories of Refik Saydam Central Institute of Health (Ankara, Turkey). The animals left for 2 d for acclimatization to animal room conditions were maintained on standard pellet diet and water *ad libitum*. The food was withdrawn 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 bioassays

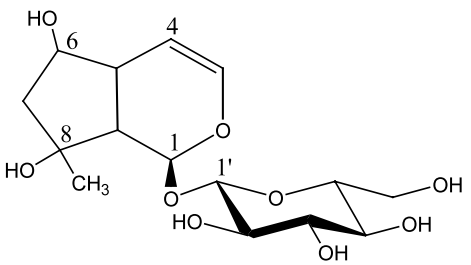
The methanolic extract and isolated compounds were administered in 100 and 200 mg/kg doses after suspending in 0.5% sodium carboxymethyl cellulose (CMC) suspension in distilled water. The control group animals received the same experimental handling as those of the test groups except that the drug treatment was replaced with appropriate volumes of the dosing vehicle. Either indomethacin (10 mg/kg and 0.5 mg/ear) or aspirin (acetyl salicylic acid; 100 and 200 mg/kg) in 0.5% CMC was used as reference drug.



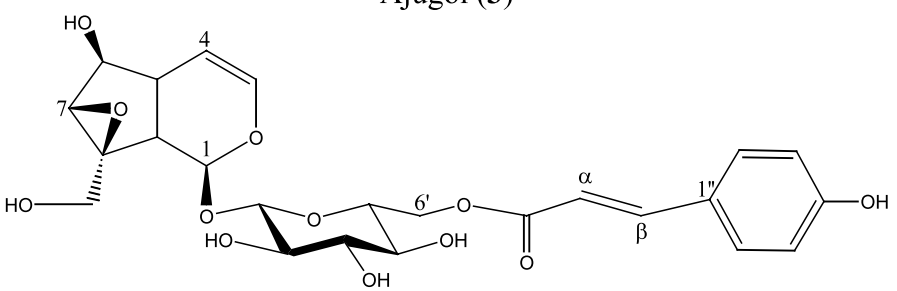
Ilwensisaponin A (**1**)



Ilwensisaponin C (**2**)



Ajugol (**3**)



Picroside IV (**4**)

Fig. 1. Chemical structures of isolated compounds **1**–**4**.

Antinociceptive activity

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 the oral administration of test samples, the mice were intraperitoneally (i.p.) 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 averages of the total number of writhes observed. The antinociceptive activity was expressed as percentage change from writhing controls. Aspirin at 100 and 200 mg/kg doses was used as the reference drug in this test.

Anti-inflammatory activity

Carrageenan-induced hind paw edema model

Carrageenan-induced hind paw edema model was used for the determination of the anti-inflammatory activity (Küpeli *et al.*, 2005). The difference in footpad thickness between the right and left foot was measured with a pair of dial thickness 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. 60 min after the oral administration of a test sample or dosing vehicle, each mouse was injected with a freshly prepared (0.5 mg/25 μL) suspension of carrageenan (Sigma, St. Louis, Missouri, USA) in physiological saline (154 nM NaCl) into the subplantar tissue of the right hind paw. As the control, 25 μL saline solution were injected into 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.). 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 the reference drug.

PGE_1-induced hind paw edema model

Prostaglandin E_1 (PGE_1)-induced hind paw edema model was used for the determination of the anti-inflammatory activity (Kasahara *et al.*, 1985). The difference in footpad thickness between the right and left foot was measured with a pair of dial thickness gauge calipers (Ozaki Co.). Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods. 60 min after the oral administration of a test sample or dosing vehicle, each mouse was injected with freshly prepared (5 μg/5 μL) suspension of PGE_1 (Fluka Chemie AG, Art. 82475) in Tyrode's solution into the subplantar tissue of the right hind paw. As the control, 5 μL Tyrode's solution were injected into the left hind paw. Paw edema was measured every 15 min during 75 min after induction of inflammation. The difference in footpad thickness was measured by a gauge calipers (Ozaki Co.). 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 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). This 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 with TPA. 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 (Ozaki Co.). The edema was expressed as the difference between the right and left ear due to TPA application and, consequently, inhibition percentage was expressed as a reduction thickness with respect to the control group.

2.) After 4 h, the animals were killed under deep ether anesthesia. Discs 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 the right and left ear 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 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 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. However, *p*-benzoquinone applied i. p. did not induce any irritation on the gastric mucosa, but anti-inflammatory agents of COX-1 inhibitors, *i.e.,* aspirin or indomethacin, orally, caused severe bleedings, without repeated administrations.

Statistical analysis

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

Verbascum species have been used since ancient times in traditional medicine for the therapy of scabies, eczema, tumours and various inflammatory affections (Turker and Camper, 2002). Therefore, the purpose of this paper was to establish the scientific basis for traditional uses of *Verbascum* species against inflammation and rheumatic pains. The results indicate that the activity of the isolated compounds from *V. pterocalycinum* var. *mutense* might contribute to the anti-inflammatory activity of *Verbascum* species.

In our previous studies, *in vivo* anti-inflammatory and antinociceptive activities of *Verbascum lasianthum* Boiss. ex Bentham flowers were investigated, which have been used in Turkish folk medicine to treat hemorrhoids. A methanolic extract of the flowers was shown to possess significant inhibitory activity in the carrageenan-induced hind paw edema model and in *p*-benzoquinone-induced writhings in mice. Through bioassay-guided fractionation and isolation procedures, a triterpenoid saponin, ilwensisaponin A, was found to be the antinociceptive and anti-inflammatory principles, without inducing any apparent acute toxicity or gastric damage, while the iridoid glucoside ajugol had no activity (Küpeli *et al.*, 2007). Ilwensisaponin A (**1**) and ajugol (**3**) were also isolated from the flowers of *V. pterocalycinum* var. *mutense* (Tatli *et al.*, 2004); therefore these compounds were not tested for their antinociceptive and anti-inflammatory activities.

As shown in Table I, although the iridoid glycoside picroside IV did not show any antinociceptive activity, the saponin glycoside ilwensisaponin C (23.2%) significantly inhibited the abdominal constriction induced by *p*-benzoquinone in mice at a 200 mg/kg dose.

In addition, we have investigated the effects of pure compounds of *V. pterocalycinum* var. *mutense* in three models of acute inflammation, the carrageenan-induced hind paw edema, PGE1-induced hind paw edema and TPA-induced ear edema.

It was reported that the anti-inflammatory effects of several agents result in the partial inhibition of inflammation-mediator release (Amadio *et al.*, 1993). Subcutaneous injection of carrageenan into the rat paw produces plasma extravazation (Szolcsanyi *et al.*, 1998) and inflammation characterized by increased tissue water and plasma protein exudation with neutrophil extravazation and metabolism of arachidonic acid by both

Table I. Effect of the materials from *Verbascum pterocalycinum* var. *mutense* against *p*-benzoquinone-induced writhings in mice.

Test sample	Dose [mg/kg]	Number of writhings ± SEM	Inhibition (%)	Ratio of ulceration
Control		40.1 ± 5.2		0/6
Ilwensisaponin C (**2**)	100	33.6 ± 2.4	16.2	0/6
	200	30.8 ± 2.1	23.2*	0/6
Picroside IV (**4**)	100	37.1 ± 3.2	7.5	1/6
	200	36.3 ± 3.5	9.5	3/6
Aspirin	100	21.5 ± 2.6	46.4***	4/6
	200	19.3 ± 2.1	51.9***	6/6

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

cyclooxygenase and lipoxygenase enzyme pathways (Gamache *et al.*, 1986). There are biphasic effects in carrageenan-induced edema. The first phase begins immediately after injection and diminishes within 1 h. The second phase begins at 1 h after injection and remains through 3 h (Garcia-Pastor *et al.*, 1999). It is suggested that the early hyperemia of carrageenan-induced edema results from the release of histamine and serotonin (Kulkarni *et al.*, 1986). On the other hand, the delayed phase of carrageenan-induced edema results mainly from the potentiating effect of prostaglandins on mediator release, especially of bradykinin. Hydrocortisone and some NSAIDs inhibit strongly the second phase of carrageenan-induced edema, but some others are effective against both phases (Kulkarni *et al.*, 1986).

In the light of these data and Table II, ilwensisaponin C (4.8–23.3%) from *V. pterocalycinum* var. *mutense* seems to be more effective in the second phase of acute inflammation than in the first phase. Therefore, *V. pterocalycinum* var. *mutense* may block the prostaglandin and/or bradykinin release better than that of the histamin and/or serotonin.

Ilwensisaponin C (4.3–21.5%) significantly inhibited hind paw edema induced by prostaglandin E_1 dose-dependent, which supports the carrageenan-induced hind paw edema hypothesis (Table III).

As shown in Table IV, the isolated compounds did not show any anti-inflammatory activity against ear edema induced by TPA. Although TPA model has limited selectivity in determining the

Table II. Effect of the materials from *Verbascum pterocalycinum* var. *mutense* against carrageenan-induced hind paw edema in mice.

Test sample	Dose [mg/kg]	Swelling thickness [$\times 10^{-2}$ mm] ± SEM (% inhibition)			
		90 min	180 min	270 min	360 min
Control		37.6 ± 4.2	41.3 ± 3.9	45.1 ± 5.2	49.7 ± 3.8
Ilwensisaponin C (**2**)	100	38.3 ± 2.9	44.1 ± 3.2	46.4 ± 2.5	51.6 ± 3.4
	200	35.8 ± 3.2 (4.8)	37.6 ± 3.6 (8.9)	35.3 ± 2.7 (21.7)*	38.1 ± 3.1 (23.3)*
Picroside IV (**4**)	100	39.2 ± 2.6	44.1 ± 3.2	47.8 ± 3.0	51.4 ± 3.5
	200	38.1 ± 4.3	42.7 ± 3.1	46.3 ± 4.3	47.4 ± 3.2 (4.6)
Indomethacin	10	29.6 ± 2.9 (21.3)	28.1 ± 2.3 (31.9)**	28.2 ± 1.7 (37.5)***	30.6 ± 2.8 (38.4)***

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

Table III. Effect of the materials from *Verbascum pterocalycinum* var. *mutense* against PGE_1-induced hind paw edema in mice.

	Dose [mg/kg]	Swelling thickness [$\times 10^{-2}$ mm] ± SEM (% inhibition)					
		0 min	15 min	30 min	45 min	60 min	75 min
Control		1.5 ± 0.7	11.6 ± 1.2	19.8 ± 1.5	25.1 ± 1.2	27.6 ± 1.4	16.1 ± 1.1
Ilwensisaponin C (**2**)	100	1.6 ± 1.1	11.8 ± 1.7	18.1 ± 1.9 (8.6)	24.7 ± 1.5 (1.6)	28.2 ± 1.7	18.3 ± 1.3
	200	1.5 ± 0.9	10.1 ± 1.3 (12.9)	16.2 ± 1.1 (18.2)	19.7 ± 1.2 (21.5)*	22.4 ± 1.3 (18.8)	15.4 ± 1.4 (4.3)
Picroside IV (**4**)	100	1.6 ± 0.3	12.1 ± 1.1	21.9 ± 1.2	26.8 ± 1.6	29.2 ± 1.2	17.4 ± 1.4
	200	1.5 ± 0.5	13.4 ± 1.4	18.5 ± 1.7 (6.6)	24.7 ± 1.4 (1.6)	25.9 ± 1.5 (6.2)	17.1 ± 1.8
Indomethacin	10	1.4 ± 0.4 (6.7)	10.1 ± 1.0 (12.9)	13.5 ± 0.9 (31.8)**	16.2 ± 1.1 (35.5)***	20.7 ± 1.3 (25.0)**	13.6 ± 1.4 (15.5)

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

Table IV. Effect of the materials from *Verbascum pterocalycinum* var. *mutense* against TPA-induced ear edema in mice.

Test sample	Dose [mg/ear]	Swelling thickness ± SEM [μm]	Inhibition (%)	Weight edema ± SEM [mg]	Inhibition (%)
Control		202.7 ± 26.8		23.8 ± 4.2	
Ilwensisaponin C (**2**)	0.5	205.4 ± 21.6	–	20.9 ± 5.2	12.2
Picroside IV (**4**)	0.5	213.9 ± 31.2	–	31.6 ± 5.7	–
Indomethacin	0.5	60.1 ± 11.9	70.3***	8.6 ± 2.6	63.9***

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

possible mechanism of action, it is appropriate for assessing the anti-inflammatory effects in a first stage trial. The phorbol ester (TPA) elicited an inflammatory response characterized by a delayed time of onset. It has been established that this agent exerts its inflammatory effect through protein kinase C activation with the subsequent cytosolic phospholipase A2 stimulation, AA mobilization, and biosynthesis of prostaglandins and leukotrienes (Nishizuka, 1988).

In contrast with the results obtained in the carrageenan-induced hind paw edema test, ilwensisaponin C failed to affect the ear edema. This could be due to the different mechanism of action of the phlogistic agents or to the fact that the systemic route of administration fails to achieve an adequate concentration of the compounds at the site of inflammation.

In a reference survey, some triterpenes have been reported to have anti-inflammatory, cytotoxic, antitumour activities and to be chemopreventive. Papillomas in mouse skin were initiated with 390 nmol of 7,12-dimethylbenz(a)anthracene and, 1 week later, were promoted 2 times a week with 4.1 nmol of 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Five saponin-related compounds used as potential anti-inflammatory agents from *Verbascum songaricum* were applied in the same position at 82 nmol. The saponin-related compounds effectively inhibited tumour formation in the sensitive mouse stock even when these compounds were given 1 h prior to TPA treatment. These results suggested that there is a general correlation between the anti-inflammatory and anti-tumour-promoting activities of saponins (Tokuda *et al*., 1991). Songarosaponins and their acylated derivatives, which had been previously isolated from *Verbascum songaricum*, have also been tested for their anti-inflammatory activity using the croton oil ear model. Acylated derivatives showed excellent anti-inflammatory activity as compared to that of saponins (Anam, 2001). Giner *et al.* (2000) also demonstrated that verbascosaponin A and verbascosaponin, isolated from *Scrophularia auriculata*, significantly inhibited the mouse paw edema induced by carrageenan and ear edema induced by single and multiple doses of TPA.

To the best of our knowledge, this is the first report on ilwensisaponin C, an oleanane-type triterpene saponin, exhibiting anti-inflammatory activity in the carrageenan-induced hind paw edema test (without apparent gastric lesion induction) and antinociceptive activity in the *p*-benzoquinone-induced abdominal constriction test. The mechanism of action, however, has not yet been established. It may be speculated that the glucose molecule in the sugar residue connected to the C-3 position of the genin is crucial for its acute anti-inflammatory effect (Hostettmann and Marston, 1995; Gepdiremen *et al*., 2005). Therefore, the anti-inflammatory and antinociceptive activity of *Verbascum pterocalycinum* var. *mutense* is contributed mainly to saponins. However, further studies are required in order to reveal the possible pharmacokinetic factors which contribute to the activity of these compounds and to evaluate the potential of the plant.

Results of the present study have clearly demonstrated that the flowers of *Verbascum pterocalycinum* var. *mutense* possess significant antinociceptive and anti-inflammatory activities which support the traditional utilization in Turkey. In connection with the role of ilwensisaponin A and C as the active principles of *V. pterocalycinum* var. *mutense*, it seems that they could be synergistic with other active substances in their fractions and extracts.

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