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# The evaluation of sterile solutions of Ilwensisaponin A and C from *Verbascum pterocalycinum* var. *mutense* Hub.-Mor. on antiviral, antinociceptive and anti-inflammatory activities

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## ABSTRACT

Antiviral, antinociceptive and anti-inflammatory activities of the 1% sterile solutions of Ilwensisaponin A and C isolated from the methanolic extract of the flowers of *Verbascum pterocalycinum* var. *mutense* Hub.-Mor. were investigated. Antiviral activities of sterile solutions were evaluated against *Bovine Herpes Virus Type-1* (BHV-1) (Cooper strain). Both solutions showed cytotoxic effects. However, none of the sterile solutions showed antiviral activity with CPE values. Anti-inflammatory and antinociceptive response were obtained at doses of 100 mg/kg. The results of the evaluation of the anti-inflammatory activities induced by carrageenan showed that these sterile solutions possess active constituents and diminish cyclooxygenase activity. In addition, antinociceptive activities using *p*-benzoquinone-induced writhing model in mice of tested solutions were found to show notable activity in statistical analysis without inducing any apparent acute toxicity as well as gastric damage. To the best of our knowledge, this study is the first investigation of antiviral, anti-inflammatory and antinociceptive activities of sterile solutions of Ilwensisaponin A and C isolated from *Verbascum pterocalycinum* var. *mutense* Hub.-Mor. growing in Turkey.

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## 1. Introduction

The genus *Verbascum*, commonly known as “mullein”, is a widespread genus of the family Scrophulariaceae, which comprises more than 2500 species worldwide. The genus is represented by 233 species, 196 of which are endemic in Turkish Flora (Huber-Morath, 1978; Davis et al., 1988; Ekim, 2000). Various preparations of some species of this genus have been used as expectorant, mucolytic, sedative, diuretic and constipate; as well as they have been used for the respiratory disorders such as bronchitis, dry

coughs, tuberculosis and asthma in traditional Turkish medicine. *Verbascum* species are used to treat hemorrhoids, rheumatic pain, superficial fungal infections, wounds and diarrhea. In addition, they have inhibitory activities against the murine lymphocytic leukemia and influenza viruses A2 and B. The oil made from the flowers is used to help soothe earache and can be applied externally for eczema and other types of inflammatory skin conditions. They are traditionally consumed as a tea to relieve abdominal pains. A decoction of roots febrifuge is used to alleviate toothache and to relieve cramps, convulsions and migraines. The leaves, roots and the flowers are also anodyne, antiseptic, antispasmodic, astringent, emollient, nervine, vulnerary, analgesic, antihistaminic, anticancer, antioxidant, antiviral, bactericide, cardiodepressant, oestrogenic, fungicide, hypnotic and sedative. In addition to the above-mentioned common uses, these species have been used for pruritic conditions in urogenital organs. Moreover, the commercial popularity of *Verbascum* species has been increasing for the past few years with the growing interest in herbs and preference for the

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'greener' lifestyle. Today in health food stores in the United States, one can easily find dried leaves and flowers, swallow capsules, alcohol extracts and flower oil of mullein (Baytop, 1999; Turker and Camper, 2002; Turker and Gurel, 2005).

*Verbascum* species contain biologically active compounds, such as flavonoids, phenylethanoid and neolignan glycosides, iridoid glycosides and specially oleanane type triterpene saponins (Tatli and Akdemir, 2004). The pharmacological activity of saponins in plants, such as their anti-inflammatory, antitumor, antiexudative, antiulcer, analgesic, antipyretic and immunostimulant effects, has been known for many years, while new activities are continually being discovered (Hostettmann and Marston, 1995). In our previous studies, Ilwensisaponin A and C, oleanane type triterpene saponins, have shown anti-inflammatory activity using the carrageenan-induced hind paw edema test (without apparent gastric lesion induction) and antinociceptive activity using the *p*-benzoquinone-induced abdominal constriction test (Kupeli et al., 2007).

Inflammatory diseases are treated currently with steroidal and nonsteroidal anti-inflammatory drugs (NSAIDs). NSAIDs exert their effects by inhibiting the metabolism of arachidonic acid by both cyclooxygenase and lipoxygenase enzyme pathways (Insel, 1996). Despite their widespread use, NSAIDs are often associated with severe adverse effects, the most common being gastrointestinal bleeding (Fung and Kirschenbaum, 1999). Although antiviral drugs are available for most viral disease, there are very few active substances that have specific effects, and drug resistance is an emerging threat to the clinical utility of antiviral drugs. Because of these effects, safer compounds and their pharmaceutical forms are needed (Razonable, 2011).

In the current study, antiviral, anti-inflammatory and antinociceptive activities of the 1% sterile solutions of Ilwensisaponin A and C were discussed for their anti-inflammatory activities using carrageenan-induced hind paw edema model, antinociceptive activities using *p*-benzoquinone-induced writhing model in mice, and for antiviral activities against BHV-1.

## 2. Material and methods

### 2.1. 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).

### 2.2. Isolation and structure elucidation of the constituents

Chromatographic separation, isolation and structure elucidation of Ilwensisaponin A and C were previously submitted elsewhere (see Fig. 1) (Tatli et al., 2004).

### 2.3. Preparation of sterile solutions

The formulation was prepared in aseptic conditions. For the preparation of 1% sterile solution, compounds were dissolved in distilled water and then the solution filtered through a 0.22  $\mu$ m membrane filter, and the sterile filtrate collected. Final product was autoclaved for 20 min at 121  $^{\circ}$ C, 1 atm pressure (British Pharmacopoeia, 2002).

### 2.4. Biological activity tests

#### 2.4.1. Animals

Male, Sprague-Dawley rats (160–180 g) and Swiss albino mice (20–25 g) were purchased from the animal breeding laboratories of Refik Saydam Central Institute of Health (Ankara, Turkey).

The animals were left for 3 days at room conditions for acclimatization. They were maintained on standard pellet diet and water *ad libitum* throughout the experiment. A minimum of six animals were used in each group. Throughout the experiments, animals were processed according to the suggested international ethical guidelines for the care of laboratory animals under the audit of Gazi University Commission of Animal Ethics.

#### 2.4.2. Preparation of test samples for bioassay

Test samples were given orally to test animals after suspending in a mixture of distilled H<sub>2</sub>O and 0.5% sodium carboxymethyl cellulose (CMC) for anti-inflammatory and antinociceptive activities. The control group animals received the same experimental handling as those of the test groups except the drug treatment was replaced with appropriate volumes of dosing vehicle. Indomethacin (10 mg/kg) in 0.5% CMC was used as a reference drug.

#### 2.4.3. Anti-inflammatory activity

**2.4.3.1. Carrageenan-induced hind paw edema model.** Carrageenan-induced hind paw edema model was used for determination of anti-inflammatory activity (Yesilada and Kupeli, 2007). 60 min after the parenteral administration of a test sample or dosing vehicle, each mouse was injected with freshly prepared suspension of carrageenan (0.5 mg/25  $\mu$ L) in physiological saline (154 mM NaCl) into subplantar tissue of the right hind paw. As the control, 25  $\mu$ L saline solutions were injected into that of the left hind paw. Paw edema was then measured in every 90 min during 6 h after induction of inflammation. The difference in footpad thickness was mea-

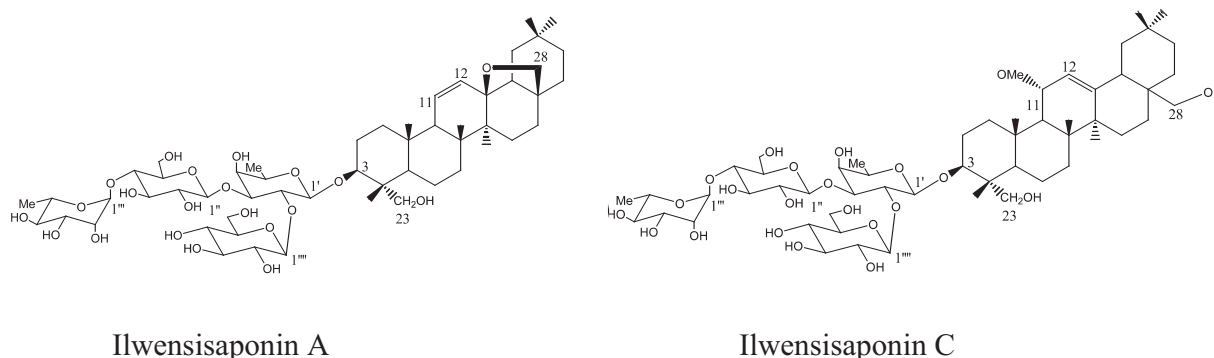


Fig. 1. Chemical structures of Ilwensisaponin A and C isolated from *Verbascum pterocalycinum* var. *mutense*.

sured by a gauge calipers (Ozaki Co., Tokyo, Japan). Mean values of treated groups were compared with those of a control group and analyzed by using statistical methods. Indomethacin (10 mg/kg) was used as the reference drug.

#### 2.4.4. Antinociceptive activity

2.4.4.1. *p*-Benzoquinone-induced abdominal constriction test. *p*-Benzoquinone-induced abdominal constriction test (Okun et al., 1963) was performed on mice for the determination of antinociceptive activity. According to the method evaluated, 60 min after the parenteral administration of a test sample, the mice were intraperitoneally injected with 0.1 ml/10 g body weight of 2.5% (w/v) *p*-benzoquinone (PBQ) solution in distilled water. Control animals received an appropriate volume of dosing vehicle. The mice were then kept individually for the observation and the total number of the abdominal contractions (writhing movements) was counted for the following 15 min, starting 5 min after the PBQ injection. The data represent the average of the total number of writhes observed. Antinociceptive activity was then expressed as the percentage change from writhing controls. Acetylsalicylic acid (ASA) was used at 100 and 200 mg/kg doses as the reference drug (Kupeli and Yesilada, 2007).

#### 2.4.5. 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 observation period.

#### 2.4.6. Gastric-ulcerogenic effect

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

#### 2.4.7. Statistical analysis of the data

The data on percentage wound healing was statistically analyzed using one-way analysis of variance (ANOVA). The values of  $p \leq 0.05$  were considered statistically significant. Histopathologic data were considered to be nonparametric; therefore, no statistical tests were performed.

#### 2.4.8. Antiviral effect

BHV-1 (Cooper strain) was used for determination of antiviral activity. Test virus and Madin-Darby Bovine Kidney (MDBK) cells were obtained from the Department of Virology, Faculty of Veterinary, Ankara University (Turkey). The cells were grown Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 5% fetal calf serum (Bio Whittaker Europe, Germany), 100 IU/mL penicillin and 100 mg/mL streptomycin. The cells were incubated in 5% CO<sub>2</sub> at 37 °C until the monolayer formations occurs and harvested using dose (TCID<sub>50</sub>). 50 µL test virus was added into each well of the microplates and incubated in 5% CO<sub>2</sub> at 37 °C for 2 h. After that 50 µL cell suspension of 300.000 cells/mL were added and incubated in 5% CO<sub>2</sub> at 37 °C for 48 h. At the end of this duration, cytopathogenic effect (CPE) of test virus on the cells was evaluated using cell culture microscope (Olympus CKX41 × 400) by comparison with treated and untreated control cultures and with acyclovir. Antiviral activities of sterile solutions expressed as maximum CPE concentrations. In addition that maximum non-toxic concentrations (MNTCs) of each sample were determined by comparing treated and untreated cultures based on cellular morphologic alteration. Acyclovir (Nobel Co.) was used as standard drug (Ozcelik et al., 2005).

### 3. Results

Although there are some studies representing the biological activities of Ilwensisaponin A and C, there is no study about antiviral, anti-inflammatory and antinociceptive activities of sterile solutions of these compounds. We, therefore, studied these activities for both solutions.

Antiviral effect of the sterile solutions was determined against BHV-1. In antiviral activity test, cytotoxic effects of the sterile solutions were observed, and the results are expressed as MNTC values in Table 1. The sterile solutions had lower cytotoxicity than acyclovir as expressed by their MNTC values. The most potent cytotoxic effect was observed in 1% sterile solution of Ilwensisaponin A (MNTC: 125 µg/mL). None of the solutions displayed antiviral activity in certain range of CPE values. At the same time, selection of solvent (distilled water) was not considered to have an effect on the toxicity of the solutions.

As shown in Table 2, the sterile solutions of Ilwensisaponin A and C were found to show significant inhibitory effect on carrageenan-induced hind paw edema in mice. These compounds were administered at doses of 100 mg/kg according to their molecular weight. Furthermore, the potency of the solution of ilwensisaponin A was similar to that of indomethacin in acute anti-inflammatory experimental models and consistent, i.e. the effect was not reduced with time. 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). There are biphasic effects in carrageenan-induced edema. The first phase begins immediately after injection and diminishes in 1 h. The second phase begins at 1 h 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. 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 in Table 2, the sterile solution of Ilwensisaponin A (9.6–36.6%) seem more effective in the second phase of acute inflammation than in the first phase.

To determine antinociceptive activity, the sterile solutions of the saponins were studied using the *p*-benzoquinone-induced writhing method in mice. As shown in Table 3, 1% sterile solutions of Ilwensisaponin A and C were found to possess significant antinociceptive activity without inducing any apparent acute toxicity or gastric damage.

### 4. Discussion

Along with the increasing population in the world, natural resources are used in the protection of human and animal health.

**Table 1**

Cytotoxicity and antiviral activity results of 1% sterile solutions of Ilwensisaponin A and C (µg/mL).

Compounds	MNTC	CPE Inhibitory Concentration
Ilwensisaponin A (1%)	125	–
Ilwensisaponin C (1%)	62.5	–
Acyclovir	500	3.90–0.48

MNTC: Maximum non-toxic concentration.

CPE: Cytopathogenic effect.

–: No activity observed.

**Table 2**

Anti-inflammatory effects of 1% sterile solutions of Ilwensisaponin A and C on carrageenan-induced paw edema in mice.

Test compounds	Dose (mg/kg)	Swelling thickness $\pm$ S.E.M. ( $\times 10^{-2}$ mm) (inhibitory %)			
		90 min	180 min	270 min	360 min
Control		30.1 $\pm$ 3.7	34.9 $\pm$ 3.2	41.0 $\pm$ 3.8	45.9 $\pm$ 4.4
1% sterile solution of Ilwensisaponin A	100	27.2 $\pm$ 3.0 (9.6)	27.9 $\pm$ 3.2 (20.1)	31.0 $\pm$ 2.1 <b>(24.4)<sup>†</sup></b>	29.1 $\pm$ 3.2 <b>(36.6)<sup>***</sup></b>
1% sterile solution of Ilwensisaponin C	100	25.8 $\pm$ 2.9 (14.3)	30.2 $\pm$ 3.4 (13.5)	32.7 $\pm$ 3.0 (20.2)	34.2 $\pm$ 3.1 <b>(25.5)<sup>†</sup></b>
Indomethacin	10	21.3 $\pm$ 2.4 <b>(29.2)<sup>**</sup></b>	24.5 $\pm$ 2.1 <b>(29.8)<sup>**</sup></b>	29.7 $\pm$ 2.3 <b>(27.6)<sup>**</sup></b>	28.8 $\pm$ 2.5 <b>(37.3)<sup>***</sup></b>

†p &lt; 0.05, \*\*p &lt; 0.01, \*\*\*p &lt; 0.001 significant from the control values; S.E.M.: standard error mean.

**Table 3**

Antinociceptive effects of 1% sterile solutions of Ilwensisaponin A and C on p-benzoquinone-induced nociception in mice.

Test compounds	Dose (mg/kg)	Number of writhes $\pm$ S.E.M.	Inhibitory %	Gastric lesion <sup>a</sup>
Control		47.6 $\pm$ 4.2		0/6
1% sterile solution of Ilwensisaponin A	100	30.1 $\pm$ 2.9	<b>36.8<sup>**</sup></b>	0/6
1% sterile solution of Ilwensisaponin C	100	34.0 $\pm$ 3.3	<b>28.6<sup>†</sup></b>	0/6
Acetyl salicylic acid	100	25.8 $\pm$ 3.0	<b>45.7<sup>***</sup></b>	5/6

†p &lt; 0.01, \*\*\*p &lt; 0.001 significant from the control values; S.E.M.: standard error mean.

<sup>a</sup> Number of mice with gastric lesions or bleeding.

Saponins, have many biological activities in many plants and are generally amorphous and colorless, sometimes crystalline and white colors, and are soluble in polar solvents such as water, ethyl alcohol etc (Hostettmann and Marston, 1995). Therefore, in our study, we aimed to determine whether saponin type compounds would be effective as a pharmaceutical form by preparing sterile solutions. The results presented that both compounds (Ilwensisaponin A and C) showed meaningful anti-inflammatory and antinociceptive activities in the form of sterile solution, when compared our previous data mentioned moderate activity without any formulation (Kupeli et al., 2007). Eventually, in the present study, sterile solutions of two compounds (especially 1% sterile solution of Ilwensisaponin A) administrated as parenteral, showed significant anti-inflammatory and antinociceptive activities without any acute toxicity or gastric damage.

According to survey of literatures, *in vitro* antiviral activity studies on sterile solutions of Ilwensisaponin A and C have not been reported before. Although saponins have antiviral activity (Simoes et al., 1999), the expected results could not be obtained and none of the solutions exhibited any antiviral activity against BHV-1 (Cooper strain) in this study, while both solutions showed cytotoxic effects. In this case, it would be critical and important that isolated saponins have been evaluated for their correlation with cytotoxicity. The assessment of the cytotoxicity of an antiviral compound is also clearly an important part of the evaluation of a potential chemotherapeutic agent, since it should show neither acute nor long-term toxicity against the host (Simoes et al., 1999). When also evaluated their structure-activity relationship, it could be said that cytotoxic effect of these two different oleanane type triterpenoid saponins is affected by increasing sugar moieties, hydroxyl substitution at C-28, methoxyl group at C-11 and the double bond between C-12 and C-13 positions (Simoes et al., 1999).

In conclusion, our results support the use of isolated saponins from *Verbascum* species as potential drugs, especially in treatment of infected diseases by the preparation of pharmaceutical forms. These results will assist in the search new drugs, used for prevention and treatments of infectious diseases. Further biological activities and/or other pharmaceutical forms can be evaluated as a potential agent.

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