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Bioassay-guided isolation of the antinociceptive compounds motiol and β-sitosterol from *Scorzonera latifolia r*oot extract

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Scorzonera latifolia (Asteraceae) is a plant widely distributed in Central and East Anatolia. A mastic, named *yakı sakızı*, is prepared from the latex and roots of *S. latifolia* and similar species. This latex is used in Turkish folk medicine for its analgesic activity, as anthelmintic and against infertility. The aim of this study was to isolate the compounds responsible for the antinociceptive activity of *S. latifolia* using bioassay-guided fractionation. The methanolic extract of the *S. latifolia* roots was prepared and subjected to chromatographic purification. Isolated active compounds were identified by means of MS and NMR techniques. Writhing and tail-flick tests were used to determine antinociceptive activity. Motiol and β-sitosterol were isolated as compounds with promising antinociceptive activity. It is suggested that antinociceptive activity of the plant extract is probably caused by the synergic interaction of the isolated compounds.

1. Introduction

Scorzonera L. genus, a member of Asteraceae family primarily originates from the Mediterranean Region and widely expanded to the arid regions of Eurasia and northern Africa with more than 175 species (Bohm and Stuessy 2007; Hamzaoğlu et al. 2010). In Europe, 28 species of *Scorzonera* have been found distributed from Northern Russia to Spain and Crete (Paraschos et al. 2001). In Turkey, *Scorzonera* genus is represented by 49 species including new species described (Hamzaoğlu et al. 2010; Davis 1975; Ünal and Göktürk 2003; Duran and Hamzaoğlu 2004; Özhatay and Kültür 2006). Some *Scorzonera* species have been widely used as food in Turkey and in some European countries. Especially *Scorzonera* roots and green buds are eaten freshly or after cooking (Tsevegsuren et al. 2007; Wang et al. 2009).

Scorzonera species are also used as medicinal plants in European, Chinese, Mongolian and Turkish folk medicine for different purposes. *S. hispanica* L. (black salsify, vipers grass), is used as stomachic, diuretic, antipyretic and as a herbal medicine against pulmonary diseases as well as colds (Tsevegsuren et al. 2007; Zidorn et al. 2000a) in Europe. *S. humilis* L., known as humble vipers grass, has been utilized to treat wounds and gastro-intestinal disorders (Zidorn et al. 2000b, 2003, 2002). *S. cretica* Willd. is known as an appetitive agent in Greek cuisine (Paraschos et al. 2001; Davis 1975). In Mongolian traditional medicine, *S. divaricata* Turcz. roots and herb are known as antipyretic, antidote and an herbal remedy for treatment of poisonous ulcers as well as malignant stomach neoplasia. *S. pseudodivaricata* Lipsch is widely used as diuretic and against diarrhea, parasitic diseases, lung oedema and fever (Tsevegsuren et al. 2007). *S. radiata* Fisch. is also reported to have medicinal usage in Mongolian medicine for the treatment of poisonous ulcer and fever accompanying bacterial and viral infections as well for its diuretic and galactagogic properties (Wang et al. 2009). *S. mongolica* Maxim. and *S. austriaca* Willd. are utilized as medicinal herbs in Chinese and Tibetian folk medicine due to their antipyretic and anti-inflammatory activities against the breast inflammation and the abscesses (Jiang et al. 2007; Zhu et al. 2009). Members of *Scorzonera* genus are also used to treat a variety of illnesses in Turkish folk medicine: arteriosclerosis, kidney diseases, hypertension, diabetes mellitus and rheumatism, as well for pain relief and healing of different injuries (Sezik et al. 1997; Baytop 1999).

S. latifolia (Fisch. and Mey.) DC. is one of the best described *Scorzonera* species. It is widely distributed in Central and East Anatolia. A mastic, named *yakı sakızı*, is prepared from the latex and roots of *S. latifolia* and similar species. This latex is used in Turkish folk medicine as analgesic, as well for treatment of infertility and as anthelmintic (Baytop 1999; Turan et al. 2003). *S. latifolia, S. tomentosa, S. suberosa* ssp. *suberosa*, *S. mollis* ssp. *szowitsii*, and *yakı sakızı* have been investigated for their antinociceptive activities in order to verify this claimed traditional usage in our previous study. All of the extracts were found to possess significant antinociceptive activity; *S. latifolia* extract was established as the most active species and further purification procedures were carried out on *S. latifolia* roots methanolic extract (Bahadir et al. 2010). Bioassay-guided fractionation approach was used to obtain the main compounds responsible

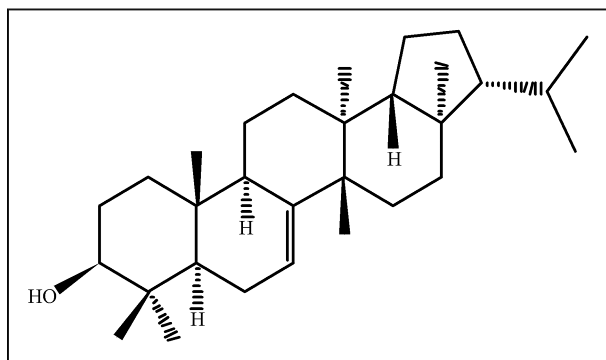

Fig. 1: Fern-7-en-3-β-ol (motiol) (**1**).

for the antinociceptive activity. Three compounds were isolated from the *n*-hexane portion of extract and identified as taraxasteryl myristate, taraxasteryl acetate and fern-7-en-3-*β*-one. Both isolated taraxasterol derivatives showed promising antinociceptive activities when compared to reference compounds but there was found only a slight activity of fern-7-en-3-*β*-one (Bahaddir et al. 2012). However, our results revealed that significant antinociceptive activities were also observed in other subfractions of *n*-hexane fraction. Therefore, as a continuation of our study, we report the bioassay guided isolation of motiol and *β*-sitosterol as further antinociceptive compounds present in *S. latifolia* *n*-hexane fraction in the current study.

2. Investigation, results and discussion

We report two analgesic constituents **1** and **2** from the *n*-hexane fraction of *S. latifolia* root extract. Subfractions 34–44 and 54–67 were selected for isolation procedures depending on their TLC profile and antinociceptive activity potentials. Subfractions 34–44 and 54–67 exerted notable activity in acetic acid induced writhing test model (92.1 and 95.5 % respectively) as well as statistically significant effect in tail flick test at each time point (Bahadir et al. 2010). Two pure compounds **1** and **2** were isolated from these subfractions and their structures were elucidated as motiol (fern-7-en-3-β-ol, **1**) (Fig. 1) and β-sitosterol (**2**) (Fig. 2) by comparison of their ^{1}H, ^{13}C 2D NMR (COSY, TOCSY, NOESY, HMBC, HSQC) and MS data with those reported in literature (Kariyone et al. 1957; Seca et al. 2000; Villasenor et al. 2002).

Compounds **1** and **2** were subsequently tested in acetic acid induced writhing and tail-flick test to evaluate their antinociceptive effects according to previously published methods (Bahadir et al. 2010, 2012). As given in Tables 1 and 2, compounds **1** and **2** displayed statistically significant antinociceptive activities in acetic acid induced abdominal stretching at 10 mg/kg dose with values 92.1 % and 94.3 %, respectively, compared to control, while acetylsalicylic acid

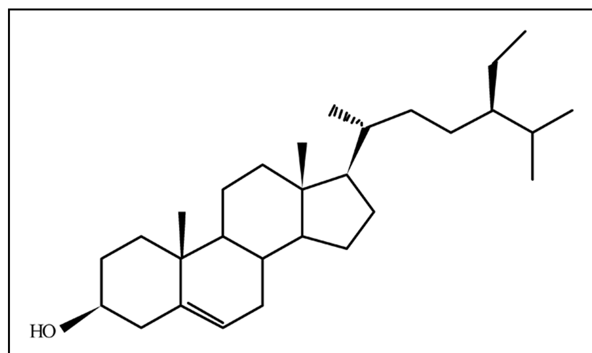

Fig. 2: β-Sitosterol (**2**).

(300 mg/kg) showed abdominal stretching inhibition with a value of 44.5 % only. Significant analgesia was also observed after treatment of animals with **1** and **2** in the tail flick test (both in doses of 10 mg/kg). The analgesic effect of **1** reached its peak 30 min after injection and decreased slowly. Similarly to **1**, the *i.p.* application of **2** induced its maximal analgesic effect 30 min and its effect was later reduced slowly. However, at 10 mg/kg dose, **2** exhibited higher activity than **1**. In tail flick test, only week antinociceptive activity of acetylsalicylic acid was observable in the 30th min of the assay, the most potent activity was observed later, in 150th minute, whereas morphine showed remarkable antinociceptive activity in 30 th and 90th min and only a slight activity remained in 150th min.

Treatment with **1** and **2** was found to be more effective than treatment with acetylsalicylic acid (300 mg/kg) in both tail flick and acetic acid induced writhing tests. Moreover, at the dose applied (10 mg/kg), antinociceptive activities of **1** and **2** were found to be close to morphine in the tail-flick test. Taraxasteryl acetate and taraxasteryl myristate, isolated in our previous study as analgesic compounds from the most active subfraction of the *n*-hexane extract, showed significant inhibition on writhing responses (61 % and 77 %, respectively) but these effects were not found to be higher than those observed for fern-7-en-3-*β*-ol (**1**) and *β*-sitosterol (**2**). Both isolated compounds **1** and **2** decreased abdominal constriction numbers more effectively compared to taraxasteryl acetate and taraxasteryl myristate, until present, the most active compound among those isolated from *S. latifolia* has been established to be *β*-sitosterol (**2**) with 94.3 % inhibition in the acetic acid-induced writhing test. Moreover, in the tail-flick test, administration of taraxasteryl acetate and *β*-sitosterol (**2**) resulted in a significant increase in the tail-flick response latency at each time point when compared to the control animals, but taraxasteryl myristate (Bahadir et al. 2010) and fern-7-en-3-β-ol (**1**) produced relatively significant alteration in the reaction time at 30th and 90th min.

β-Sitosterol is the principal phytosterol in many higher plants. It is used to decrease the pathologically raised level of cholesterol. In addition, it has anti-inflammatory, antipyretic, antineoplastic, immunomodulating and blood sugar-controlling effects (Ju et al. 2004). Analgesic activity of *β*-sitosterol was also reported previously – *β*-sitosterol and its glucoside were isolated as analgesic constituents of *Mentha cordifolia* leaves (Villasenor et al. 2002). Results of our study support these findings; *β*-sitosterol was revealed as promising antinociceptive agent. However, no reports about the analgesic activity of fern-7-en-3-*β*-ol (**1**) have been found to date. Compound **1** exhibited significant antinociceptive activities, whereas another fernane derivative isolated from *S. latifolia*, fern-7-en-3-*β*-on, showed low activity only in acetic acid-induced writhing test and no activity in tail-flick test (Bahadir et al. 2010). The only difference between these two fernane derivatives is the functional group at C-3, therefore the analgesic activity could be in part related to the presence of hydroxyl group at position 3 of **1**. The observation of the different activity observed between the two fernane derivatives may underline the need for the exploration of further related compounds for a systematic evaluation of the structure activity relationships for the antinociceptive fernane compounds, which may lead to achieve more effective compounds by synthesis or semi-synthesis. Further studies are needed to establish a possible mechanism of action.

This study confirmed the ethnomedicinal usage of *S. latifolia* for its analgesic effect. It is suggested that antinociceptive activity of *S. latifolia* and especially of the *n*-hexane fraction of extract can be attributed to synergic interaction of the content compounds. The highest activity exhibiting *β*-sitosterol (**2**) is considered to possess promising antinociceptive properties.

3. Experimental

3.1. Plant material

S. latifolia (Fisch. & Mey.) DC was collected in Kars, Arpaçay in August 2005. Roots were separated from aerial parts and dried at room temperature. The taxonomic identification of the plant was confirmed by H. Duman, a plant taxonomist of the Department of Biological Sciences, Faculty of Art and Sciences, Gazi University. Voucher specimen is kept in the herbarium of Ankara University, Faculty of Pharmacy (AEF 23830).

3.2. Extraction and isolation

The extraction and solvent-solvent fractionation was performed that we described in our previous study (Bahadir et al. 2010). *n*-Hexane fraction which was selected for further separation subjected to column chromatography to obtain subfractions as reported previously (Bahadir et al. 2010).
Precoated silica gel 60 TLC sheets (Merck 5724, 0.25 mm, 5 cm × 20 cm and Merck 1.05744, 0.5 mm, 20 cm × 20 cm) were used for the separation of SFr. 34–44 and n-hexan: ethylacetate (8:2, v/v) solvent system was used. Further purification was performed using a column chromatography on silica gel 40 (Fluka 60734, 0.015–0.035 mm) under the nitrogen overpressure. Isocratic benzene: acetone (20:1 v/v) solvent system was used to obtain 20 subfractions. Compound **1** (4.7 mg) was obtained from SSFr. 11–15 after crystallization with addition of MeOH.
Compound **2** (53 mg) was isolated from SFr. 54–67 using *n*-hexane: ethylacetate (7.5:2.5, v/v) solvent system. Precoated silica gel 60 TLC sheets (Merck 5724, 0.25 mm, 5 cm × 20 cm and Merck 1.05744, 0.5 mm, 20 cm × 20 cm) were used for preparative TLC separation.

3.3. Structural analysis of isolated active compounds

^{1}H NMR, ^{13}C NMR and 2D NMR techniques (HMBC, HMQC, COSY, TOCSY, NOESY, DEPT) were used for the structure elucidation of the isolated active compounds using Varian Mercury 400, 400 MHz High Performance Digital FT-NMR Spectrometer (in $CDCl_3$). Structures were determined as fern-7-en-3-β-ol (motiol) (**1**) (Fig. 1) and β-sitosterol (**2**) (Fig. 2). The purity of both compounds was established to be higher than 95 %.

3.4. Bio-activity assays

3.4.1. Test animals

Male Balb/C mice (20–24 g) were maintained in the Animal House of Yuzuncu Yil University, Faculty of Medicine. The animals were housed in standard cages (48 cm × 35 cm × 22 cm) at room temperature (22 ± 2 °C) with artificial light from 7:00 AM to 7:00 PM, and provided with pelleted food (Van Animal Feed Factory, Van, Turkey) and water *ad libitum*. The protocol for the study was approved by the Ethical Committe of Yuzuncu Yil University Faculty of Medicine Animal Breeding and Research.

3.4.2. Preparation of test samples

Compounds **1** and **2** were dissolved in DMSO and were administered by *i.p.* injection.

3.4.3. Acetic acid-induced writhing test

The slightly modified method of Koster et al. (1959) was used. The animals were kept in a temperature-controlled environment (22 ± 2 °C) with a 12 h light-dark cycle. Food and water were freely available. Abdominal writhing was induced by *i.p.* injection of acetic acid (6%, 60 mg/kg). Animals were pre-treated with the extract by intraperitoneal administration 5 min before the acetic acid injection and 5 min after the acetic acid administration the test had been started. Control animals received the same volume of isotonic saline solution (ISS)/ 15 % Tween 80/DMSO. Acetylsalicylic acid at a dose of 300 mg/kg, which is the preferential dose in such as studies was administered *p.o.* by gastric probe and used as a standard for comparison (Koster et al. 1959). After drug application, pairs of mice were placed into a standard glass cages. The number of stretching occurring for 15 min immediately after the acetic acid injection was recorded. Six mice were used per group. Animals were sacrificed immediately after experiment. The results were evaluated by calculating the mean number of stretching per group, and were represented as the percentage of stretching movements inhibition compared to the control group:

$$\%\ \text{antinociceptive activity}:\ (n - n')n \times 100$$

where n is average number of stretching of control group, and n' is average number of stretching of test group.

3.4.4. Tail-flick Test

Analgesic response was assessed with a tail-flick apparatus (LSI Letica LE 7106, Spain) using a method previously described in the literature (Hunskaar et al. 1985; D'Amour and Smith 1941). The animals were gently immobilized using a glove, and the radiant heat was focused on a blackened spot 1–2 cm from the tip of the tail. Beam intensity was adjusted to give a tail flick latency of 5–8 s in control animals. Measurement was terminated if the latency exceeded the end of time (15 s) to avoid tissue damage. In all the experiments, each mouse was tested twice. The measurement started 30 min before drug administration to determine the baseline latency and then continued in interval of 30, 90 and 150 min after drug administration. Acetylsalicylic acid (300 mg/kg, p. o.) and morphine hydrochloride (10 mg/kg, s. c.) were used as reference standards (Parimala et al. 2003; Matsumoto et al. 2004). Isotonic saline solution (ISS)/ 15 % Tween 80/DMSO was given to the control group. Test samples were given to the animals by *i. p.* injection.

3.4.5. Statistical analysis

Results are expressed as mean ± SEM (standard error of the mean). SPSS Statistics 17.0 Base Win computer program was used for statistical analysis. The total variation was analysed by performing one-way analysis of variance (ANOVA). Tukey's HSD (honestly significant difference) test was used for determining significance. $p < 0.05$ was considered to be significant.

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