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Anti-inflammatory activity of extracts and a saponin isolated from *Melilotus elegans*

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Received May 17, 2004, accepted June 20, 2004

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Pharmazie 60: 310–312 (2005)

The crude methanol extract of *Melilotus elegans* Ser. (Fabaceae), a plant widely used in Ethiopian traditional medicine for the treatment of asthma, haemorrhoid and lacerated wounds showed a significant anti-inflammatory activity against carrageenin-induced rat paw oedema. At a dose corresponding to 333.3 mg per kg body weight of dry plant material, the methanol extract displayed a strong inhibitory effect that was comparable to the inhibitory effect of 1 mg/kg of indomethacin in the same test system. Bioassay guided fractionation of the alcoholic extract led to the isolation of an oleanene-type triterpene saponin identified as azukisaponin V (**1**) ((3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl(1→2)-β-D-glucuronopyranosyl]-soyasapogenol B). The structure of the compound was identified by using MS and extensive one- and two-dimensional NMR experiments (^{1}H, ^{13}C, COSY, HMQC, HMBC and NOESY). One hour after injection of carrageenin, inhibition of oedema exerted by **1** was approximately ten times higher than that of indomethacin on a molar basis.

1. Introduction

Melilotus elegans Ser. (syn. *M. abyssinica* Bak., *M. lippoldiana* Lowe) is one of the five species of the genus *Melilotus* (Fabaceae) that are known to occur in Ethiopia (Thulin 1983). The mature plant of this erect annual herb is about 1 m in height and has a wide distribution, particularly in highlands where the altitude ranges between 1700–2800 m above sea level. The plant is known by several vernacular names one of which indicates that the twigs are used to perfume clothes and ointments due to their pleasant scent (Thulin 1989). In folk medicine the powdered leaves of *M. elegans* are mixed with butter and applied topically against haemorrhoids, mouth inflammation and lacerated wounds. The drug can also be taken orally for the above diseases as well as for bronchial asthma in which case it is covered with fresh butter to mask the unpleasant taste.

Prior to our studies, there appears to have been only one study published on *M. elegans*, in which screening for the presence of tertiary and quaternary alkaloids is described (Viladomat et al. 1986). Previously we reported the isolation and anti-inflammatory activity of two flavonol glycosides, kaempferol-3-*O*-(6″-α-L-rhamnosyl)-β-D-galactoside-7-*O*-α-L-rhamnoside (robinin) and kaempferol-3-*O*-β-D-galactoside-7-*O*-α-L-rhamnoside from the aqueous extracts of the leaves of *M. elegans* (Asres et al. 2000).

In the present study we report the isolation of a saponin, which possessed a powerful anti-inflammatory activity when tested on carrageenin-induced rat paw oedema.

2. Investigations, results and discussion

In Ethiopian traditional medical practices *M. elegans* is used for the treatment of diseases including asthma, lacerated wounds and haemorrhoids. In view of the fact that these diseases are known to contain an inflammation component, both polar and non-polar solvent fractions prepared from the leaves of the plant were examined for their anti-inflammatory activity against carrageenin-induced rat paw oedema. Results of the activity tests indicated that the hexane, chloroform and acetone extracts did not show a significant anti-inflammatory activity whilst the methanol extract displayed a strong inhibition of oedema (Fig. 1). At a dose corresponding to 333.3 mg/kg body weight of dried plant material, 1 h after carrageenin injection, the MeOH extract exerted 42.0% inhibition, compared with the control group (Fig. 1). These results were better than the inhibitory effect of 1 mg/kg of indomethacin, which possessed 31.6% inhibition.

Thus, it was concluded that the anti-inflammatory principles of *M. elegans* were hydrophilic substances. For further investigations, the bioactive methanol extract was subjected to solid phase extraction on Isolute C-18 columns using hydroalcoholic solutions containing different proportions of MeOH and H_2O as eluant. Eluates obtained from water, 50% and 75% MeOH in water were shown to be active while the 25% MeOH in H_2O fraction was devoid of any significant action (Fig. 2).

The isolation of two flavonoids from the 50% MeOH/H_2O fraction of extracts of *M. elegans*, one of them exhibiting a significant anti-inflammatory activity, was reported else-

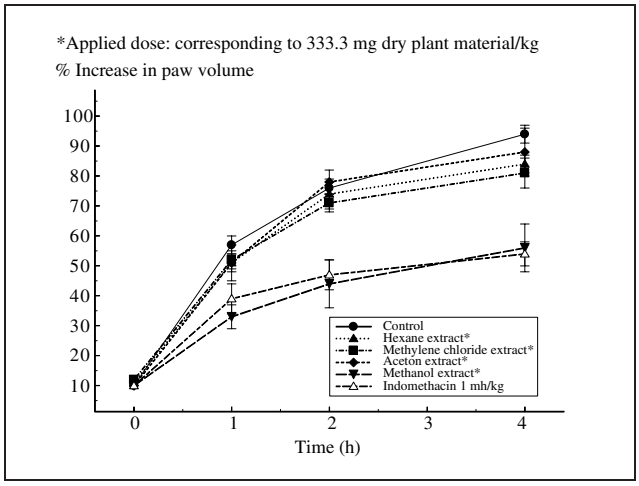


Fig. 1: Inhibition of carrageenin-induced rat paw oedema by different extracts of *Melilotus elegans* [values represent mean ± SEM (n = 5)]

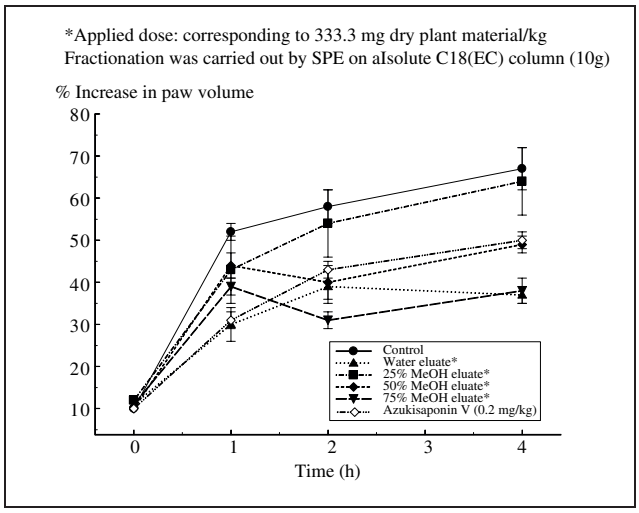
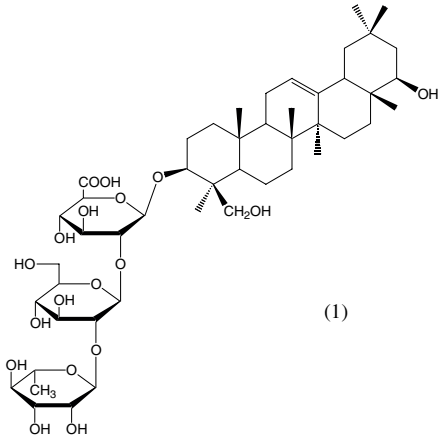


Fig. 2: Inhibition of carrageenin-induced rat paw oedema by azukisaponin V and different fractions obtained by SPE of the MeOH extract of *Melilotus elegans* [values represent mean ± SEM (n = 5)]

where (Asres et al. 2000). However, the compound isolated from the 75% MeOH in water fraction displayed a much higher activity than the flavonoids previously reported. The identity of the compound was confirmed as azukisaponin V (**1**) (3-*O*-[α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl-(1→2)-β-D-glucuronopyranosyl]-soya-sapogenol B) by means of MS and extensive 1- and 2-dimensional NMR experiments. Thus a combination analysis of ^{1}H and ^{13}C NMR, ^{1}H-^{1}H shift correlation spectroscopy (COSY), heteronuclear multiple bond correlation (HMBC), heteronuclear multiple coherence (HMQC) and nuclear overhauser enhancement correlation spectroscopy (NOESY) were carried out. The ^{13}C-NMR chemical shift values were found to be consistent with those reported in the literature (Kang et al. 1988). The monosaccharide mixture obtained by acid hydrolysis revealed the presence of glucuronic acid, glucose and rhamnose by TLC and by GC-MS analysis of the trimethylsilyl (TMS) derivatives of the respective sugars. The sugar sequence was established by tandem ESI-MS analysis of the glycoside. Direct infusion of the methanolic solution of (**1**) in a concentration of 0.02 mg/ml into the ESI source coupled to an ion trap mass analyser gave a quasi-molecular ion at m/z 941 $[M - H]^-$ (ESI neg.) and m/z 965 $[M + Na]^+$ (ESI pos.). Stepwise fragmentation showed



(1)

splitting off of M-146, M-146-162, and M-146-162-176 fragments, respectively, indicating that the compound was a triglycoside with the aglycone directly attached to glucuronic acid-glucose-rhamnose. Exact linkage of the sugar units was established through HMBC correlations, which showed H-1‴ (rha) at 5.22 ppm correlated with C-2″ (glu) at 78.7 ppm and H-1″ (gluc) at 4.92 ppm correlated with C-2′ (glcA) at 78.4 ppm.

When tested on carrageenin-induced rat paw oedema, Compound **1** displayed a marked activity (Fig. 2) inhibiting oedema (41.1%) at a concentration of 0.2 μmol/kg (0.2 mg/kg) of body weight, 1 h after injection of carrageenin. Under similar conditions the sodium salt of indomethacin exerted an inhibitory effect of only 31.6% at a concentration of 2.6 micromole/kg. Thus, the anti-inflammatory action of **1** was faster and also stronger than that of indomethacin. However, 4 h after injection of carrageenin, the inhibitory effect of indomethacin (42.6%) was higher than that of **1** (25.5%) although the concentration of indomethacin used was more than 10 times than that of **1**.

Previously, azukisaponin V (**1**) was isolated from yellow sweet clover, *Melilotus officinalis* (Kang et al. 1988) and the Japanese azuki bean, *Vigna angularis* (Kitagawa et al. 1983), both plants belonging to the family Fabaceae. Kang et al. have indicated that the compound possesses potent inhibitory action on leukocyte migration (Kang et al. 1988). The leaves of *M. elegans* were also shown to contain a high concentration of robinin, a flavonoid glycoside with a pronounced anti-inflammatory activity (Asres et al. 2000). Furthermore, robinin has been reported to possess a diuretic action in both intact and water-loaded animals (Vasil'chenko and Sokolova 1973). In addition to flavonoids and saponins, *M. elegans* leaves produce a series of coumarins (Kraxner 2001). In other studies, extracts of *M. officinalis* containing 0.25% of coumarins (Plesca-Manea et al. 2002), and the hydroalcoholic extracts of *Justicia pectoralis* whose main constituents are coumarin and umbelliferone were proved to display a strong inhibitory effect on acute inflammation (Lino et al. 1997). It therefore appears that the anti-inflammatory action of *M. elegans* leaves is due to the presence of mixtures of natural products.

In conclusion, the reputed applications of *M. elegans* leaves for the treatment of wounds and haemorrhoids in traditional medical practice seem to be well founded. Like most plants used in traditional medicine, the activity of the plant is attributed not to a single compound but to a combination of secondary metabolites which in this case belong to at least three classes of plant metabolites namely, coumarins, flavonoids and saponins.

3. Experimental

3.1. General techniques

Preparative HPLC was carried out on a Varian Rainin HPLC system. Column Dynamax 100 Å, C-18, 8 µm, 41.4 × 250 mm, mobile phase AcCN – water 38 : 62, TFA 0.01%, flow rate 80.0 ml/min, detection UV 210 nm. NMR spectra were recorded at 500 MHz (^{1}H) and 125 MHz (^{13}C) on a Bruker AVANCE 500 spectrometer in pyridine-d_5. GC-MS analyses were carried out on a HP 5890 Series II Plus gas chromatograph interfaced to a HP 5989B mass spectrometer on an Ultra 2 fused silica capillary (ID: 0.20 mm, film thickness: 0.10 µm, length: 46 m). ESI-MS^n analysis was carried out on a LCQ Deca XP (Thermo-Finnigan) ion trap mass spectrometer in negative and positive ESI mode. Sugars were identified after acid hydrolysis (2 N HCl) by GC-MS analysis of the TMS derivatives, sugar sequence was established by ESI-MS^n analysis of the glycoside. Sugar linkage was established through HMBC correlations.
TMS derivatives were prepared by adding Sigma-Sil A reagent (Sigma) to the dried residue of the acidic aqueous fraction after hydrolysis of the saponin.

3.2. Plant material

The plant material was collected in October 1996 in and around the city of Addis Ababa, Ethiopia. Its identity was confirmed by Dr. Insermu Kelbesa, The National Herbarium, Department of Biology, Addis Ababa University, Addis Ababa, Ethiopia. A voucher specimen is deposited at the Department of Pharmacognosy, Addis Ababa University.

3.3 Preparation of extracts for pharmacological testing

Powdered leaves (150 g) were exhaustively extracted successively in Soxhlet apparatus using hexane, methylene chloride, acetone and methanol. Solvents were removed under reduced pressure to yield the following amounts of residues: hexane 7.4 g; methylene chloride 2.8 g; acetone 5.1 g and methanol 24.4 g.

3.4. Fractionation of the methanolic extract and isolation of saponin

The lyophilized methanolic extract (10 g) was dissolved in 20 ml of MeOH–H_2O 1 : 4 (v/v) and fractionated by column chromatography on a Sephadex G-25 column (560 × 45 mm) using 20% MeOH in H_2O as eluant. Flavonoids and saponins were eluted between 1140–1820 ml and this fraction was designated Fraction A. Fraction A was further separated by solid phase extraction on Isolute C-18 (EC) columns (10 g, IST, Hengoed, UK). Elution was carried out by increasing the amount of MeOH in H_2O. Analytical TLC indicated that flavonoids were present in 25% and 50% MeOH in H_2O fraction whilst the 75% MeOH in H_2O eluate contained the saponin.
Purification of the saponin was done by preparative HPLC (R_t value of **1** = 20.1 min).

3.5. Hydrolysis of saponin

Azukisaponin V (**1**) (2 mg) was dissolved in 5 ml of 2 M HCl in a 25-ml round bottom flask and heated (under condenser) on a steam bath for 60 min. After complete hydrolysis as shown by TLC, the hydrolysate was extracted several times with EtOAc (by shaking vigorously in a test-tube). The sugars in the remaining acidic solution were identified as glucuronic acid, glucose and rhamnose by GC-MS analyses of their TMS derivatives and by comparison with authentic samples (Roth, Germany).

3.6. Anti-inflammatory testing using carrageenin-induced rat paw oedema

The testing was carried out as described previously (Bucar et al. 1998), where female Sprague-Dawley rats (180–200 g) were used after being deprived of food for 20 h prior to experiments. Before the experiments the rats were also anaesthetized with pentobarbitone (50 mg/kg, i.p.). Two hours before injection of carrageenin, different groups of rats were given extracts, fractions or pure compounds in aqueous solutions, which were made blood isotonic by adding appropriate amounts of sodium chloride. One control group of rats (negative control) received the vehicle and another group (positive control) received 1 mg/kg indomethacin (as the sodium salt, in 0.9% saline). Extracts, fractions, vehicle, pure compounds and indomethacin were administered by an oral tube. Thirty minutes prior to injection of carrageenin (0.1 ml, 2%) into the plantar region of the right hind paw, the volumes of the paws were measured in a Perspex cell conductometrically with a plethysmometer (Ugo Basile, Italy). The left paw (control) was injected with 0.1 ml of 0.9% saline immediately after injection of carrageenin. Oedema was determined by subtracting the volume of the control paw from that of the treated paw. The significance of differences between means (against controls) was assessed by two-sample t-test, with a significance level of $p < 0.05$.

Acknowledgements: W. Sametz (†) and H. Juan, Institute of Biomedical Research, Medical University of Graz, are thanked for carrying out animal experiments. Financial support by Dr.-Heinrich-Jörg-Stiftung, University of Graz, is gratefully acknowledged.

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