Different approaches to study the traditional remedy of ‘hierba del canto’, *Sisymbrium officinale* (L.) Scop.

[Diferente enfoques en el estudio del remedio tradicional ‘hierba del canto’ -*Sisymbrium officinale* (L.) Scop.]

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**Abstract**

The infusion of the semi-fresh flowering aerial parts of *Sisymbrium officinale* (L.) Scop. (Brassicaceae), commonly known as hierba del canto in Spanish, is a popular remedy for the treatment of sore throats, coughs, and hoarseness, but little is known about its chemistry and pharmacology. HPLC-UV/PDA analyses of the dichloromethane and methanol extracts of both the fresh and the dried plant material showed the occurrence of some biochemical reactions during the drying process. The freeze-dried residue of the infusion (SOw) prepared according to the traditional recipe, was re-dissolved in methanol obtaining a soluble fraction (SOw_s) and a precipitate (SOw_p). Further fractionation of SOw_s allowed the identification of adenine, adenosine, and guanosine that were present in significant quantities only in the traditionally prepared aqueous extract. Polysaccharides were detected in the SOw_p fraction and their molecular weight evaluated by DOSY experiments. When they all were tested for the topical anti-inflammatory activity in the murine Croton oil-induced ear oedema model, only a modest effect was observed at high concentrations. Saturation Transfer Difference-NMR experiments for the study of the binding activity between the plant metabolites and the *Lens culinaris* lectin gave partial data on the active principles. This work affords new data on the phytochemistry of the plant but the question about the identity of the active principles responsible for the traditional use of *S. officinale* remains open.

**Key words:** Diffusion-edited experiments, anti-inflammatory activity, *Sisymbrium officinale*, traditional recipe.

**Resumen**

La infusión de partes aéreas floridas semi-frescas de *Sisymbrium officinale* (L.) Scop. (Brassicaceae), conocida como ‘hierba del canto’, es un remedio popular para el tratamiento de dolores de garganta, tos y ronquera, pero poco o nada se conoce de su química y farmacología. El confronto analítico de los extractos crudo de dichlorometano y metanol de material vegetal fresco y seco por HPLC-UV/PDA mostraron la presencia de algunas reacciones bioquímicas que ocurren durante el proceso de secado. El residuo seco de la infusión (SOw) preparada acorde la receta tradicional, se redissolvió en metanol obteniendo una fracción soluble (SOw_s) y un precipitado (SOw_p). Tras fraccionar SOw_s se identificaron adenina, adenosina y guanosina, las cuales se encuentran solo presentes en cantidades significativas en el extracto acuoso preparado a la manera tradicional. Se detectó la presencia de polisacáridos en la fracción insoluble (SOw_p) y su peso molecular fue evaluado mediante experimentos DOSY. Tras ensayar todas estas fracciones en busca de actividad antiinflamatoria tópica, (edema en oreja de ratón inducido por aceite de Croton) solo se observaron efectos muy modestos a altas concentraciones. Con experimentos de RMN con transferencia diferencial de saturación para detectar la unión entre metabolitos de la planta y la lectina de *Lens culinaris* sólo se pudo obtener información parcial de los posibles ligandos. Si bien este trabajo aporta nuevos datos sobre la química de la planta, la cuestión acerca de la identidad de los principios antiinflamatorios que justifica el uso popular de la planta aun sigue sin esclarecerse.

**Palabras clave:** actividad antiinflamatoria, experimentos de difusión editada, receta tradicional, *Sisymbrium officinale*.
INTRODUCTION

Sisymbrium officinale (L.) Scop. (Brassicaceae), commonly known as hedge mustard (English), herbe aux chantres (French), hierba del canto (Spanish) or erisimo (Italian), is an annual plant very common in bare ground, on roadsides, dumps and edges of fields in Europe, Western Asia, and Northern Africa. There are limited and certainly not up-to-date phytochemical and pharmacological investigations assessing the claimed therapeutic effect of this medicinal plant. With respect to the dried aerial part of the plant, standardization of S. officinale showed the presence of 10.9% mucilages, 8.9% itols, 0.5% flavonoids, 9.2% ash, and 0.63% glucosinolates (Carnat et al., 1998) of which up to a 65% is isopropyl-glucosinolate (Griffiths, et al. 2001). Traditionally, it is recommended to use the semi-fresh flowered aerial parts of the plant in order to prepare an infusion having the therapeutic effects above mentioned (Benigni, et al. 1964; Font-Quer, 1995). Singers also use it in case of loss of voice and its aerial parts and different extracts are currently sold in the market to treat sore throats, coughs, and hoarseness (Carnat, et al. 1998). From the main text of Font-Quer (1995) it is possible to read about S. officinale: “Se emplea principalmente contra las inflamaciones y catarros de la laringe, sobre todo para combatir la ronquera, así como contra la tos, los catarros pulmonares, etc., amen del escorbuto. Se usa de preferencia la planta fresca. Con la hierba recién colectada se prepara una tisana, como si se tratara de té, es decir, poniendo a hervir un cacito de agua y echando en ella cosa de 1 onza de la planta, tallos y hojas, por cada cuatro tazas; se toma cuando queda templada, y después de echar el azúcar que se desee.”

In the present work we intend to study its anti-inflammatory activity, the identity of its active principles and the differences between the dried and fresh material to gain inside the popular indications and methods to prepare this remedy.

MATERIALS AND METHODS

Plant material

The flowering aerial parts of Sisymbrium officinale (L.) Scop. (Brassicaceae) were collected in the edges of Lake Lehman (Lausanne, Switzerland) on May 2002, and identified following standard procedures (Pignatti, 1982). A voucher was deposited in the personal herbarium of the main author (MP).

Chemicals.

High purified, salt free, lyophilized Lens culinaris lectin (MW ca 25 kD), adenine, adenosine, guanosine, Croton oil, indomethacin, usrosic acid and oleanolic acid were obtained from Sigma (Milano, Italy; St. Louis, Missouri, USA). Ketamine hydrochloride was purchased from Virbac S.r.l. (Milano, Italy). The other reagents and solvents, of analytical grade, were purchased from Carlo Erba (Milano, Italy).

Extraction and fractionation

A sample of S. officinale was collected and frozen in situ by immersion into liquid nitrogen and subsequently lyophilised (‘fresh plant’). Another sample of this species was collected and dried at room temperature in the dark for two weeks (‘dried plant’). 3 grams of each, fresh and dried plant material, were consecutively extracted with dichloromethane (DCM) and methanol (yield around 10% in all cases) and both types of organic extracts were directly analysed by LC-UV/PDA (the analysis of the DCM extracts is not shown).

A third sample of S. officinale (150 g) was used to prepare the traditional remedy. This sample was extracted with hot water 2 hours after its collection (“semi-fresh plant”). Few millilitres of this infusion were used for the HPLC-UV/PDA analysis using the same condition adopted for the methanol extracts (see below). The remained infusion was freeze dried obtaining 9 g of solid residue. This solid residue (labelled SOW) was extracted with methanol obtaining around 4 g of the soluble part (SOW_s) and 4 g of precipitate (SOW_p). 3.5 g of the soluble fraction SOW_s were further fractionated using size exclusion chromatography on Sephadex LH-20 using MeOH as eluent, flow 0.8 ml/min; 9 fractions were obtained with the following yields expressed in mg: I) 132.6; II) 72.8; III) 207.8; IV) 1334.0; V) 913.0; VI) 222.5; VII) 40.0; VIII) 129.1; IX) 86.1. Semi-preparative HPLC separations were conducted on a Shimadzu LC-8A series pumping system equipped with a Waters R401 refractive index detector and with a Waters μ-Bondapak C18 300 x 7.8 mm column and Shimadzu injector. Adenine and guanosine were isolated from fraction VII using MeOH/H2O 12% as eluent while adenosine was purified from fraction VIII using MeOH/H2O 30% as eluent (flow rate of 2 ml/min in both cases).
LC-UV/PDA analysis

Analytical HPLC-UV/PDA experiments were performed with a Symmetry C18 column (250 x 4.6 mm i.d., 5 µm, Waters) on a HP-1100 Series LC chromatograph (Agilent ChemStation software), equipped with an auto-sampler and a photodiode array detector. 20 mg/ml solutions of the crude extracts dissolved in acetonitrile (MeCN) were injected. The flow rate was 1 ml/min with the following MeCN/H2O gradient: 0-30 min from 5% to 50% acetonitrile; 30-40 min from 50% to 100% acetonitrile; 40-50 min 100% acetonitrile. UV/PDA spectra were recorded between 210-500 nm.

NMR analysis of the extracts

A Bruker DRX-600 NMR spectrometer, operating at 599.19 MHz for 1H and 150.86 MHz for 13C, using the UXNMR software package, and a Varian VXR-300 NMR spectrometer operating at 300 MHz for 1H, were used for 1H and 13C NMR experiments; chemical shifts are expressed in δ (ppm) referring to the solvent peaks δH 3.34 and δC 49.0 for CD3OD. STD, DOSY, and TOCSY NMR experiments were performed with a Bruker Avance 500-MHz spectrometer at 298 K, equipped with a triple resonance 1H, 13C, 15N probe. Chemical shift are in ppm with respect to the 0 ppm point of the manufacturer indirect referencing method. For the Diffusion Ordered Spectroscopy (DOSY) experiments, the standard Bruker protocol was used for processing; with the ledpg2s pulse sequence, and a linear gradient of 32 steps between 2 % to 95 %, and a diffusion time (big delta) of 0.2 s. The sum of the projections in the diffusion dimension was displayed by using the standard f1sum command over the whole 2D-DOSY spectra following a previously proposed protocol (Politi, et al., 2006). 2D-TOCSY (mixing time, 70 ms) experiments were performed using standard sequences, with 256 increments of 16 scans each, separated by a 1.5 s relaxation delay, and spectral widths of 12 ppm, with 2K real data points in f2.

Protein binding studies by NMR spectroscopy

These experiments were conducted as previously described by Politi et al (2005). Briefly, high purified, salt free, lyophilized Lens culinaris lectin (MW ca 25 kD) was used without any other purification. Before performing of the NMR experiments, the affinity of this lectin towards a variety of known ligands was tested to ensure that the activity of the protein was intact (Data not shown). For the preparation of the samples, each NMR tube contained the fraction or extract at a concentration of 4 mg/ml in 0.5 mL of phosphate buffer D2O solution (75 mM, pH = 6.9) and NaCl (75 mM). The saturation transfer difference (STD) experiments were recorded (number of scans 4096) by use of essentially the sequence proposed by Mayer and Meyer (1999). A cascade of soft Gaussian shaped pulses of 50 ms (with a power level of 50 Hz) was used for the 2.5 s saturation time. On-resonance irradiation was carried out at 0 ppm, while the off resonance was set at δ = +50 ppm. A short spin-lock period (15 ms) was used prior to the acquisition in order to eliminate the background protein signals.

Topical anti-inflammatory activity

The topical anti-inflammatory activity was evaluated as inhibition of the Croton oil-induced ear oedema in mice (Tubaro et al., 1985). All animal experiments complied with the Italian D.L. n. 116 of 27 January 1992 and associated guidelines in the European Communities Council Directive of 24 November 1986 (86/609 ECC). Animals were kept for one week before the experiment, at constant conditions of temperature (21 ± 1°C) and humidity (60-70 %), with a fixed artificial light cycle (7:00-19:00 h). Inflammation was induced always in the late morning (10.00 - 12.00 h), in order to avoid variations in the inflammatory response due to circadian fluctuations in endogenous corticosteroid levels as recorded by Soliman et al (1983). Male CD-1 mice (28-32 g; Harlan-Italy, Udine, Italy) were anaesthetised with ketamine hydrochloride (145 mg kg⁻¹, intraperitoneally). Inflammation was induced on the inner surface of the right ear (surface: about 1 cm²) of anaesthetised mice by application of 80 µg of Croton oil dissolved in an appropriate vehicle (42 % aqueous ethanol for the extracts and the relevant controls; acetone for the fractions and the compounds and their controls). Control animals received only the irritant solution, whereas the other animals received both the irritant and the substances under testing. At the maximum of the oedematous response, six hours later, mice were sacrificed and a plug (6 mm ∅) was removed from both the treated (right) and the untreated (left) ears to determine the oedematous response. Oedema was quantified as the weight difference between the two plugs. The anti-inflammatory activity was expressed as percentage of the oedema reduction in treated mice compared to the control mice. As a
Figure 1. Analytical HPLC-UV/PDA comparison between the crude MeOH extracts of the fresh and the dried plant, and the crude extract SOw. Some biochemical reactions occurs during the drying process as indicated with black spots (major not shared peaks). SOw extract showed a similar profile concerning the flavonoidic derivatives but also new peaks in the chromatogram (compounds 1-3).

Figure 2. Complete fractionation scheme of the crude extract SOw.
Figure 3. 2D-DOSY experiments of fraction SOw_s and SOw_p; molecular weigh evaluation and chemical characteristics are indicated.

Figure 4. STD NMR experiments for the screening by NMR of fractions SOw_s and SOw_p to detect the binding activity with the lectin from *Lens culinaris*; interacting sugars are observed in the STD spectra of fraction SOw_s.
Studies on *Sisymbrium officinalis* Politi et al.

**RESULTS AND DISCUSSION**

The analytical HPLC-UV/PDA comparison between the methanol extracts of the fresh and the dried plant are shown in Figure 1. This comparison indicates the occurrence of changes in the chemistry of the plant metabolites during the drying process. Major changes are the presence of a peak at 7.8 min in the methanol extract of the fresh plant and the appearance of peaks at 10.5 and 14.7 min in the flavonoidic region of the methanol extract of dried material. The relative abundance of many other metabolites is changed in the later.

Comparison between the methanol extracts with the chromatogram of the infusion prepared with semi-fresh *S. officinale* allowed the detection of three new peaks between 4 and 8 min (compounds 1, 2, and 3 in Figure 1). These results underpin the importance of following the traditional recipes, in this particular case the infusion of semi-fresh material, in order to validate the ethnopharmacological uses of medicinal plants. Many authors work with organic extracts that chemically speaking have nothing to do with the real popular remedies inspiring their work.

Fractionation of the crude water extract SOw into two major fractions, soluble (SOw_s) and insoluble (SOw_p) in MeOH was performed (Figure 2 shows the complete fractionation scheme of the SOw extract). By measuring the values of the diffusion dimension in the 2D-DOSY experiments (Politi, et al. 2006) on fractions SOw_s and SOw_p, it was possible to evaluate the molecular weight of the metabolites contained in both fractions (Figure 3). From the corresponding proton NMR spectra, partial chemical data were acquired. Fraction SOw_p
contains oligosaccharides of around 1 KDa; proton signals belonging to lower molecular weight derivatives in the aliphatic and thiols region were also detected. For fraction SOw_s, different monosaccharides and related glycosides were the major compounds detected. The isolation of compounds 1-3 was performed by size exclusion chromatography of the fraction SOw_s followed by semi-preparative HPLC of fractions VII and VIII (see experimental). They were identified as adenine, adenosine, and guanosine, by $^1$H and $^{13}$C NMR analyses and comparison with pure standards. Saccharides were detected especially in fractions III-V.

The crude extract and its methanol soluble (SOw_s) and insoluble (SOw_p) fractions were tested for their topical anti-inflammatory effect in the classic in vivo model of Croton oil-induced ear oedema in mice (Tubaro, et al., 1985). The results are shown in Table 1. The extract induced only 25 % oedema inhibition at very high concentrations, similarly to its fraction SOw_s and SOw_p. These results did not validate the putative anti-inflammatory activity claimed for S. officinale, but we cannot rule out a better topical or systemic action of the infusion on the throat mucosa. Different NMR experiments allowing the detection of the binding activity between plant metabolites and biological targets, e.g. protein-receptors, can be used for the screening of crude plant extracts (Politi, et al. 2005). Toll Like Receptor 4 is a receptor involved in phagocytosis activation and therefore a key protein on the inflammatory process (Roeder, et al., 2004). The commercial lectin from Lens culinaris has been used in order to mimic its binding activity. Glucose and, in particular, mannose derivatives are the natural agonists (Schwarz et al., 1993). In our analyses, non-covalent interaction with this protein was detected only for fraction SOw_s (Figure 4). NMR analysis of fraction IV (Figure 5) that is rich in the unknown ligand, allowed the identification of a glucose moiety as part of this compound. Further HMBC analysis (data not showed) detected a quaternary carbon at 103.6 ppm correlated with the glucose anomeric proton at 5.37 ppm; by comparison with pure reference standard, saccharose was finally identified as the interacting constituents. We do not think that saccharose could be the active principle of S. officinale and, again, we could not validate the claimed bioactivity of this unique medicinal plant.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dose (µg/cm$^2$)</th>
<th>n.</th>
<th>Edema (mg ± E.S.)</th>
<th>%I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>--</td>
<td>10</td>
<td>6.4 ± 0.2</td>
<td>--</td>
</tr>
<tr>
<td>S. officinale (Sow)</td>
<td>2000</td>
<td>10</td>
<td>4.8 ± 0.2*</td>
<td>25</td>
</tr>
<tr>
<td>SOw_s</td>
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<td>10</td>
<td>4.7 ± 0.3*</td>
<td>26</td>
</tr>
<tr>
<td>SOw_p</td>
<td>1000</td>
<td>10</td>
<td>5.5 ± 0.3*</td>
<td>14</td>
</tr>
</tbody>
</table>

*p<0.05 "t" of Student test.

CONCLUSIONS

Despite the efforts described in this work including the use of different chemical and pharmacological approaches, the unusual therapeutic activities for which S. officinale is traditionally recommended remains unproven using our models, and further pharmacological assays are needed. However, our results show the importance in following the traditional manufacturing processes if we want to validate ethnopharmacological knowledge. In the case of Hierba del canto the composition of the infusions is characterised by the presence of the adenine, adenosine and guanine that are not present in the alcoholic extracts. In fact adenosine has been described as the anxiolytic-like principle of the arillus of Euphoria longana (Okuyama et al., 1999; Tuominen et al., 1992) and it might contribute to the popular use of S. officinale for the loss of voice in singers, a very multifactor condition where nervousness plays a big role. This highlights the importance of following the traditional recipes, in this particular case the infusion of semi-fresh material. Further studies must be undertaken in order to solve the pharmacological and chemical mysteries of “Hierba del canto”.

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REFERENCES


