In vivo topical anti-inflammatory and in vitro antioxidant activities of two extracts of *Thymus satureioides* leaves

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Abstract

Four extracts at increasing polarity were prepared from the leaves of *Thymus satureioides* Coss. (Labiatae) and assayed for the in vivo topical anti-inflammatory effect using the croton oil ear test in mice, and for in vitro both antioxidant (DPPH◦ test) and anti-bacterial (broth microdilution method) activities. The chloroform extract showed a topical anti-inflammatory activity (ID50 = 282 H9262 gc m−2 ), only three times lower than that of the reference drug indomethacin (ID50 = 93 H9262 gc m−2 ) and its active components were identified as ursolic and oleanolic acids. The methanol extract, showing a significant radical-scavenging effect (SC50 = 14.54 H9262 g), was characterized by the isolation and identification of some flavonoids. On the contrary, the extracts did not show any anti-bacterial effect against four standard aerobial bacteria strains.

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1. Introduction

Various *Thymus* species are aromatic plants of the Mediterranean flora, commonly used as spices and as traditional medicine remedies. They are also reported to possess some biological effects such as antispasmodic (Meister et al., 1999), antibacterial (Essawi and Srour, 2000), antiviral, expectorating (Brassier, 1983) and antioxidant activities (Mura and Nakatami, 1989). Recently, we found that the chloroform extracts of two related *Thymus* species, endemic to Morocco, *Thymus willdenowii* Boiss and *Thymus broussonetti* Boiss (Ismaili et al., 2001, 2002), were responsible for the topical anti-inflammatory activity of the plants. *Thymus satureioides* Coss. (Labiatae), trivial name “azukni,” is a North African species typical of arid habitats used in the Moroccan folk medicine in form of infuse and decoctions to treat whooping cough, bronchitis and rheumatism (Bellakhadar et al., 1991) and, generally, for its anti-inflammatory properties after topical or oral administration. Previous investigations on this species have been limited to the study of the composition and anti-microbial activity of the essential oil (Lattaoui et al., 1993; Lattaoui and Tantaoui-Elaraki, 1994) and to the survey of the presence of some methoxylated flavonoids (Voirin et al., 1985), whereas no pharmacological studies on the non-volatile extracts are reported. In this paper the in vivo topical anti-inflammatory effect (croton oil ear test in mice), and the in vitro antioxidant (DPPH◦ test) and anti-bacterial (broth microdilution assay) activities were studied. While no anti-microbial activity was observed against four standard aerobial bacteria strains, the bioassay-oriented fractionation (Tubaro et al., 1985) revealed that the highest anti-inflammatory activity is due to the chloroform extract and its components, ursolic and oleanolic acids. A significant radical-scavenging activity was shown by the methanolic extract containing flavonoids.

2. Materials and methods

2.1. General

A Bruker DRX-600 spectrometer operating at 599.2 MHz for 1H and 150.9 for 13C, using the UXNMR software
package, was used for NMR measurements in CD$_3$OD solutions. 1D and 2D NMR spectra were obtained by employing the conventional pulse sequences, as previously described (Saturnino et al., 1997). ESI-MS, in the negative ion mode, was performed using a Finnigan LCQ Deca Ion Trap instrument from Thermo Finnigan (San José, CA, USA), equipped with Xcalibur software. HPLC separations were performed with a Waters model 600A pump equipped with a U6K injector and a Model 401 refractive index detector (Waters Corporation, Milford, USA).

2.2. Chemicals

Croton oil and indomethacin were Sigma products (St. Louis, MO, USA). The standards of ursolic and oleanolic acids were supplied by Indena S.p.A. (Milano, Italy) and Roth (Karlsruhe, Germany). Ketamine hydrochloride and Croton oil were Sigma products (St. Louis, MO, USA). The standards of ursolic and oleanolic acids were supplied by Indena S.p.A. (Milano, Italy) and Roth (Karlsruhe, Germany). Ketamine hydrochloride and Croton oil were Sigma products (St. Louis, MO, USA). The standards of ursolic and oleanolic acids were supplied by Indena S.p.A. (Milano, Italy) and Roth (Karlsruhe, Germany). Ketamine hydrochloride and Croton oil were Sigma products (St. Louis, MO, USA).

2.3. Plant material

Thymus satureioides Coss. was collected in April 1998 in the Marrakesh region, Morocco. The plant was identified by Dr. Ben Tahou and a voucher specimen (T.S. 1) was deposited at the herbarium of the Scientific Institutes, Université Mohammed V, Rabat, Morocco.

2.4. Extraction and fractionation procedure

The air dried leaves of Thymus satureioides (644.3 g) were chopped into small pieces, defatted with n-hexane, and then consecutively extracted by maceration with CHCl$_3$, CHCl$_3$, MeOH (9:1) and MeOH.

Part of the methanol extract (7 g) was purified by the same procedure previously reported for Thymus wilde-novii and Thymus broussonetii (Ismali et al., 2001, 2002) to gave rosmarinic acid, luteolin-3′-O-glucuronide, luteolin-7-O-glucoside, eriodictyol-7-O-glucoside, luteosin, eriodictyol and thymolin. They were identified and the purity checked by comparison of their NMR, MS and UV spectra against literature data (Hernandez et al., 1987; Agrawal, 1989; Mahmood et al., 1993; Harborne, 1994; Okamura et al., 1994; Corticchiato et al., 1995; Encarnacion et al., 1999).

Part of the chloroform extract (1 g) was separated, as already reported (Ismali et al., 2001, 2002), by successive partitions between Et$_2$O/1% NaOH (100 ml:250 ml, 5×) to give fraction I (293 mg) and fraction II (627 mg). Part of fraction I (30 mg) separated by RP-HPLC gave oleanoic acid and ursolic acid, which identity and purity was determined by NMR spectra in comparison with literature data (Doddrell et al., 1974; Seo et al., 1975).

2.5. Quantitative determination of total phenols

The Thymus satureioides methanol extract, dissolved in MeOH, was analysed for its total phenolic content according to the Folin-Ciocalteu colorimetric method (Aquino et al., 2001). The result (total phenols) was expressed as luteolin equivalents (µg/g extract, mean ± S.D. of three determinations).

2.6. Topical anti-inflammatory activity


Male CD-1 mice (28–32 g: Harlan-Italy, Udine, Italy) were anaesthetized with ketamine hydrochloride (145 mg kg$^{-1}$, intraperitoneally). Cutaneous inflammation was induced on the inner surface of the right ear (surface: about 1 cm$^2$) of anaesthetized mice by application of 80 µg of croton oil dissolved in an appropriate vehicle. Control animals received only the irritant solution, whereas the other animals received both the irritant and the test substances. The following vehicles were used: acetone (for hexane, chloroform and chloroform-methanol extracts, fractions I and II, pure compounds and the relevant controls), 42% aqueous EtOH (v/v, for methanol extract and its controls), acetone–EtOH 1:1 (v/v, for total extract and its controls). At the maximum of the edematous response, 6 h later, mice were sacrificed and a plug (6 mm $^2$) was removed from both the treated (right) and the untreated (left) ears. The edematous response was measured as the weight difference between the two plugs. The anti-inflammatory activity was expressed as percentage of the edema reduction in treated mice in comparison to control mice. As a reference, the non-steroidal anti-inflammatory drug (NSAID) indomethacin was used.

2.7. Bleaching of the free-radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) test

The antiradical activities of the Thymus satureioides four extracts and positive controls (luteolin and n-tocopherol) were determined using the stable 1,1-diphenyl-2-picrylhydrazyl radical (DPPH$^+$) and the procedures described by Aquino et al. (2001). DPPH$^+$ has an absorption band at 515 nm, which disappears upon reduction by an antiradical compound. An aliquot (37.5 µl) of the MeOH solution containing different amounts of the extracts or controls was added to 1.5 ml of freshly prepared DPPH$^+$ solution (0.025 g/l in MeOH), the maximum concentration employed was 100 µg ml$^{-1}$. An equal volume (37.5 µl) of MeOH was added to control tubes. Absorbance at 515 nm was measured on a Shimadzu UV-1601 UV-Vis spectrophotometer 20 min
after starting the reaction. The DPPH\(^\cdot\) concentration in the reaction medium was calculated by linear regression from a calibration curve. The percentage of remaining DPPH\(^\cdot\) (% DPPH\(\text{Residue}\)) was calculated as follows:

\[
\% \text{DPPH}_{\text{Residue}} = \frac{[\text{DPPH}^\cdot]_r}{[\text{DPPH}^\cdot]_0} \times 100
\]

where \(T\) is the experimental duration time (10 min). All experiments were carried out in triplicate and the mean effective scavenging concentrations (SC50) were calculated.

### 2.8 Anti-bacterial activity

The anti-bacterial activity was tested against four aerobic reference bacterial strains (Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, Staphylococcus aureus ATCC 25923 and Enterococcus faecalis ATCC 29212), using a common broth microdilution method in 96-multiwell microtiter plates, as reported by Koneman (1995) and Camporese (1997), and recommended by the National Committee for Clinical Laboratory Standard (NCCLS, 2001). For susceptibility testing, in a first step 50 µl of Mueller Hinton Broth were distributed from the second to the 12th test tubes. Dry extracts were initially dissolved in 100 µl of dimethyl sulfoxide (DMSO) and then in Mueller Hinton Broth, to reach a final concentration of 40 mg ml\(^{-1}\); 100 µl of these suspensions were added to the first test well of each microtiter line, and then 50 µl of scalar dilution were transferred from the second to the 11th well. The 12th tube was considered as growth control, since no extracts solutions were added. Then, 50 µl of a bacterial suspension (10\(^5\) colony forming units—CFU/ml\(^{-1}\)) were added to each well. The final concentration of the extracts was included from 5 mg ml\(^{-1}\) (3rd well) to 0.019 mg ml\(^{-1}\) (11th well). Plates were incubated for 18 h at 37°C and then examined from below with a reflective viewer and the lowest concentration of each extract showing growth was taken as its Minimal Inhibitory Concentration (MIC). A blank control was taken using DMSO alone (100 µg ml\(^{-1}\)) added to a series of tubes and the MIC was evaluated as described above. No growth inhibition was observed at DMSO concentrations lower or equal to 25 µg ml\(^{-1}\).

### 2.9 Statistical analysis

Edema was expressed as mean ± standard error of the mean. Edema values were analysed by one-way analysis of variance followed by the Dunnett’s test for multiple comparison of unpaired data. A probability level lower than 0.05 was considered as being significant. ID\(_50\) values (dose giving 50% edema inhibition) were calculated by graphic interpolation of the dose-effect curves. The mean effective scavenging concentrations (SC50) were calculated by the Litchfield & Wilcoxon test (Tallarida and Murray, 1984).

### 3. Results

#### 3.1 Bioassay-oriented fractionation and phytochemical analysis of the extracts

The leaves of Thymus satureoides (644.3 g) were sequentially extracted with solvents of increasing polarity, giving hexane, chloroform, chloroform–methanol (9:1) and methanol extracts in the amounts of 25.7, 47.3, 12.0 and 40.7 g (3.9, 7.3, 1.9 and 6.3 wt/wt % with respect to dry plant material), respectively. Each of the four extracts was submitted to preliminary screenings of its in vivo anti-inflammatory effects, as well as of in vitro radical-scavenging and anti-bacterial activities. TLC analysis of the chloroform extract revealed the presence of triterpenic acids as its major constituents. In order to obtain a triterpenic acid-enriched fraction, part of the extract (1 g) was submitted to repeated partitions between EtOAc/1% NaOH to obtain fractions I (293 mg, 29.3% of the parent extract) and II (627 mg, 62.7% of the parent extract). Separation of fraction II by reverse-phase HPLC and \(^1\)H NMR analysis (Doddrell et al., 1974; Seo et al., 1975) revealed ursolic acid and oleanolic acids as its only components in the ratio 1:2.5. TLC analysis of the methanol extract revealed the presence of flavonoids as its major constituents. Fractionation of the extract (7 g) by a combination of chromatographic methods (see Section 2) gave rosmarinic acid (\(R_t = 12\) min, isolated amount: 2 mg; Mahmood et al., 1993), the flavonoid glycosides luteolin-7′-O-glucuronic acid (\(R_t = 15\) min, isolated amount: 5 mg; Harborne, 1994; Okamura et al., 1994), luteolin-7-O-glucoside (\(R_t = 19\) min, isolated amount: 11 mg; Hernandez et al., 1987), and eriodictyol-7-O-glucoside (\(R_t = 25\) min, isolated amount: 2.2 mg; Harborne, 1994; Okamura et al., 1994), as well as the aglicones luteolin (\(R_t = 15.5\) min, isolated amount: 1.7 mg; Hernandez et al., 1987; Corticchiato et al., 1995), eriodictyol (\(R_t = 20.1\) min, isolated amount: 1.5 mg; Encarnacion et al., 1999) and thymonin (\(R_t = 30\) min, isolated amount: 1.1 mg; Hernandez et al., 1987; Agrawal, 1989). The same triterpenic and flavone derivatives have been isolated in different concentrations from Thymus wildenedowii and Thymus broussetti (Ismaili et al., 2001, 2002).

#### 3.2 Topical anti-inflammatory activity

Each extract from Thymus satureoides was submitted to the croton oil ear test, at the dose of 300 µg cm\(^{-2}\), to evaluate the topical anti-inflammatory effect. As reported in Table 1, all the less polar extracts exerted some anti-inflammatory activity, inducing 24% (hexane), 29% (chloroform–methanol) and 65% (chloroform) edema inhibition, while methanol extract was inactive. The dose–activity relationship of the most active extract (chloroform) was further investigated in comparison to that of the reference NSAID indomethacin. This extract induced...
contribution to the anti-inflammatory activity of
Thymus satureioides leaves.

Fractions I and II, obtained from the chloroform extract, were evaluated for their anti-inflammatory activity at the respective doses of 88 and 188 µg cm\(^{-2}\), calculated on the basis of the fractionation yield, and corresponding to 300 µg of the parent extract. While fraction I provoked only 18% edema inhibition, fraction II reduced the edematous response by 63%. Therefore, fraction II, representing 62.7% of the chloroform extract, accounted from the effect of the parent chloroform extract (55% edema reduction) (Table 2) as well as of the whole plant extract.

Separation of fraction II by HPLC gave ursolic and oleanolic acids as the only constituents in the ratio 1:2.5. As already observed (Ismaili et al., 2001, 2002), both the compounds induced a significant dose-dependent edema inhibition. Ursolic acid was more potent than oleanolic acid (ID\(_{50}\) = 56 and 132 µg cm\(^{-2}\), corresponding to 0.12 and 0.29 µmol cm\(^{-2}\), respectively), as well as than indomethacin (ID\(_{50}\) = 93 µg cm\(^{-2}\), corresponding to 0.26 µmol cm\(^{-2}\)).

### 3.3. Free radical-scavenging activity

As to the bleaching of the free-radical 1,1-diphenyl-2-picrylhydrazyl (DPPH\(^*\) test, Aquino et al., 2001), this test is carried out in methanol solution and thus is useful to determine the free radical-scavenging effect of complex mixtures such as plant extracts. Only the methanol extract of Thymus satureioides elicited a significant free radical-scavenging effect at 10 min; the effect was concentration-dependent, so the SC\(_{50}\) value of the extract was calculated as 1.46 µg (12.4–16.8 µg, 95% confidence limits) with respect to pure compounds α-tocopherol (SC\(_{50}\) = 10.1 µg; 8.8–11.4 µg) and luteolin (SC\(_{50}\) = 8.7 µg; 7.6–9.8 µg), used as positive controls. The antioxidant activity of luteolin and eriodictyol derivatives as well as methoxy flavones have been reported in the literature (Miura and Nakatami, 1989; Haraguchi et al., 1996; Wang et al., 1998). The total phenolic content of the methanol extract, determined by the Folin–Cóccuel method and expressed as luteolin equivalents, was 355.07 ± 4.16 µg/mg.

### 3.4. Screening of the anti-bacterial activity

The extracts of Thymus satureioides, tested at different concentrations ranging from 5 to 0.019 mg ml\(^{-1}\), did not
inhibit the growth of *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212 (data not shown).

4. Discussion

*Thymus satureoides* is a well-known aromatic plant producing an essential oil, rich in bornool, with high antimicrobial effect (Lattaoui et al., 1993). Its leaves are traditionally used in the Moroccan folk medicine, after topical or oral administration, as anti-inflammatory herbal drug against whooping cough, bronchitis and rheumatism. In this study, we report the in vivo topical anti-inflammatory properties of *Thymus satureoides* extracts, as well as in vitro free radical-scavenging activity, together with the chemical profile of the extracts.

While Lattaoui et al. (1993) revealed a high antimicrobial activity of *Thymus satureoides* essential oil, the non-volatile extracts of this species did not possess anti-bacterial activity, at least against *Escherichia coli* ATCC 29222, *Pseudomonas aeruginosa* ATCC 25923 and *Enterococcus faecalis* ATCC 29212. On the contrary, a topical anti-inflammatory activity was observed for the chloroform extract (ID$_{50}$ = 282 μg/cm$^2$), which antiphlogistic potency was only three times lower than that of the non-steroidal anti-inflammatory drug indomethacin (ID$_{50}$ = 93 μg/cm$^2$). The bioassay-oriented fractionation pointed out that the activity of this extract is related to its triterpene constituents since a triterpene-enriched fraction (fraction II) showed an antiphlogistic potency similar to that of the parent extract, and ursolic and oleanolic acids were identified as the anti-inflammatory principles. In fact, the strong activity showed by ursolic and oleanolic acids (ID$_{50}$ = 0.12 and 0.29 μmol/cm$^2$), higher than or comparable to that of indomethacin (ID$_{50}$ = 0.26 μmol/cm$^2$), seems to account for the effects both of the chloroform extract and of the triterpene fraction II of *Thymus satureoides*. Similar activity was showed by different species of thyme, *Thymus wildeonii* and *Thymus broussonetii* (Ismaili et al., 2001, 2002), growing in the same habitat. Ursolic and oleanolic acids have been previously found to be the main topological anti-inflammatory principles of *Salvia officinalis* L. (Baricic et al., 2001). They are reported to exert an anti-inflammatory effect also after oral or intraperitoneal administration in carrageenan-induced rat paw edema (Singh et al., 1992; Kapil and Sharma, 1995; Recio et al., 1995). Moreover, their anti-inflammatory activity seems to be due to different mechanisms of action, such as inhibition of histamine release, inducible cyclooxygenase, 5-lipooxygenase and complement and elastase activities (Kapil and Sharma, 1995; Liu, 1995; Ringbom et al., 1998; Díaz et al., 2000; Ryu et al., 2000).

To investigate if the anti-inflammatory effect could be also related to antioxidant activity, all the extracts were evaluated by the DPPH$^+$ test. However, no free radical-scavenging activity was exerted by the chloroform extract, responsible for the anti-inflammatory activity (data not shown). Only the methanol extract of *Thymus satureoides* possesses antioxidant/free radical-scavenging activity (SC$_{50}$ = 14.6 μg), comparable to that of α-tocopherol (SC$_{50}$ = 10.1 μg), and correlated to its high level of total phenols (355.07 μg/mg). The extract contains, as major components, rosmarinic acid and flavonoids. Among the isolated aglycones, thymone has been already identified in *Thymus satureoides* (Voirin et al., 1985), while among flavonoid glycosides luteolin-3′-O-glucuronide and eriodictyol-7-O-glucoside are rare in Labiatae family, and have been previously isolated only from *Rosmarinus officinalis* (Okamura et al., 1994), Mentha spicata (Nair and Gunasegaran, 1981) and recently found in *Thymus wildeonii* (Ismaili et al., 2002) and *Thymus broussonetii* (Ismaili et al., 2002). The antioxidant effects, evaluated by different methods, of eriodictyol (Harajuchi et al., 1996), eriodictyol-7-O-rutinoside and luteolin-7-O-glucoside (Wang et al., 1998) isolated from *Thymus vulgaris* have been reported. Also methoxyflavones from *Thymus vulgaris* were found to be active as antioxidant agents in the ferric thiocyanate and thiobarbituric acid (TBA) tests (Miura and Nakatami, 1989). Therefore, the antioxidant activity of *Thymus satureoides* methanol extract seems to be due to the flavonoid constituents, whose effectiveness as free radical-scavenging agent is well known.

In conclusion, even though the plant is generally considered for its content in essential oil, it can also represent a useful source of anti-inflammatory triterpenic acids as well as of phenolic antioxidants. Both these active constituents can be considered of therapeutic relevance for *Thymus satureoides* based preparations, which efficacy in the treatment of some pathological conditions can be the consequence of a combined anti-inflammatory and antioxidant effect.

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