JPP Journal of Pharmacy and Pharmacology

JPP 2002, 54: 1137–1140

Received February 13, 2002
Accepted May 9, 2002
ISSN 0022-3573

Topical anti-inflammatory activity of extracts and compounds from *Thymus broussonettii*

H. Ismaili, S. Sosa, D. Brkic, S. Fkih-Tetouani, A. Ilidrissi, D. Touati, R. P. Aquino and A. Tubaro

Abstract

The topical anti-inflammatory activity of four extracts from *Thymus broussonetii* Boiss (Labiatae) leaves, a herbal drug used in Moroccan traditional medicine, has been studied using the croton oil ear test in mice. A bioassay-oriented fractionation revealed that the pharmacological activity is mainly in the chloroform extract. Fractionation and analysis of this extract allowed the identification of ursolic acid and oleanolic acid as the main anti-inflammatory principles. Some flavonoids (luteolin, eriodictyol, thymonin) and glycosides (luteolin-7-*O*-glucoside, luteolin-3′-*O*-glucuronide, eriodictyol-7-*O*-glucoside) were also isolated from the methanol extract.

Faculté de Mèdecine et Pharmacie, Université Mohammed V, B.P. 1014 Rabat, Morocco

H. Ismaili

Dipartimento di Economia e Merceologia, Università di Trieste, via A. Valerio 6, 34127 Trieste, Italy

S. Sosa, D. Brkic, A. Tubaro

Faculte des Sciences, Université Mohammed V, Laboratoire de Chimie des Plantes et de Synthese Organique et Biorganique, Faculte des Sciences, B.P. 1014 Rabat, Morocco

S. Fkih-Tetouani, A. Ilidrissi, D. Touati

Dipartimento di Scienze Farmaceutiche, Università di Salerno, 84084 Fisciano-Salerno, Italy

R. P. Aquino

Correspondence: A. Tubaro, Dipartimento di Economia e Merceologia, Università di Trieste, Via A. Valerio 6, 34127 Trieste, Italy. E-mail: tubaro@univ.trieste.it

Funding: This work was supported by a grant of the Italian Ministry for University and Scientific Research (Project ''Piante medicinali ed alimentari di Paesi in via di sviluppo: studio chimico e biologico'' and Project ''Fitoterapici: ottimizzazione delle caratteristiche tecnologiche e biofarmaceutiche'').

Introduction

Continuing our studies on *Thymus* species endemic to Morocco (Ismaili et al 2001), we investigated *Thymus broussonettii* Boiss (Labiatae), known by the trivial name of z'itra. A decoction of its leaves is traditionally used as a remedy against inflammatory diseases after both oral and topical application (Bellakhadar et al 1991). While the composition and antimicrobial activity of its essential oil have been already studied (Lattaoui & Tantaoui-Elaraki 1994; Lattaoui et al 1993), the effects and the composition of its non-volatile components have not been investigated. Therefore, a bioassay-oriented fractionation of *T. broussonettii* leaves was carried out using the croton oil ear test in mice as a model of acute inflammation (Tubaro et al 1985). This in-vivo inflammatory model allows the testing of very small amounts of extracts or pure compounds and, consequently, is particularly suitable for bioassay-oriented fractionation studies. This procedure showed that the anti-inflammatory activity was present in the chloroform extract, and, particularly, in the triterpenic fraction which contains ursolic acid and oleanolic acid as the active anti-inflammatory principles.

Materials and Methods

Chemicals

Croton oil and indometacin were Sigma products (St Louis, MO). Ketamine hydrochloride and TLC plates were purchased from Virbac S.r.l. (Milano, Italy) and Merck (Darmstadt, Germany).

Plant material and fractionation

T. broussonettii Boiss was collected in April 1998 in the Rabat region of Morocco. A voucher specimen (N. TB1) was deposited at the herbarium of the Scientific Institutes, Université Mohammed V, Rabat, Morocco. The air-dried leaves of *T. broussonettii* (644.3 g) were consecutively extracted by maceration with n-hexane, chloroform, chloroform–methanol (9:1) and methanol giving, after filtration and concentration in vacuum, the hexane extract (15.1 g), the chloroform extract (14.0 g), the chloro-

form–methanol extract (9.5 g) and the methanol extract (28.0 g), respectively (yields = 2.3, 2.2, 1.5 and 4.3% w/w of dry plant material, respectively).

Part of the chloroform extract (1.015 g) was separated by consecutive partitions between diethyl ether–1% NaOH (100:250 mL, 12 times). The combined organic layers, dried in vacuum, gave fraction I (385.7 mg, 38% w/w of the parent extract). The combined alkaline aqueous layers were acidified with acetic acid until pH 5 and then re-extracted with diethyl ether 12 times; the combined diethyl ether layers were dried in vacuum to give fraction II (629.3 mg; 62% w/w), following the procedure previously reported (Ismaili et al 2001). Fraction II was chromatographed by RP-HPLC on a μ-Bondapack C-18 column (Waters Corporation, Milford, USA), using methanol–water (9:1) as a solvent system, giving ursolic acid and oleanolic acid, identified by their NMR spectra in comparison with literature data (Doddrell et al 1974; Seo et al 1975).

Part of the methanol extract (7 g) was chromatographed on Sephadex LH-20 column (100 × 5 cm; Amersham Pharmacia Biotech, Uppsala, Sweden) and RP-HPLC to isolate the flavonoid aglycones luteolin, eriodictyol and thymonin, and the flavonoid glycosides luteolin-7-*O*-glucoside, luteolin-3′-*O*-glucuronide and eriodictyol-7-*O*-glucoside (for experimental details see Ismaili et al 2001). These compounds were identified by NMR, MS and UV spectra in comparison with literature data (Agrawal 1989; Harborne 1994; Okamura et al 1994; Saturnino et al 1997).

Anti-inflammatory activity

Topical anti-inflammatory activity was evaluated as inhibition of the croton oil-induced ear oedema in mice (10 mice per treatment group) (Tubaro et al 1985). Inflammation was induced on the right ear (about 1 cm^2) of male CD-1 mice (28–32 g; Harlan-Italy, Udine, Italy) anaesthetized with ketamine hydrochloride (145 mg kg^{-1}, i.p.) by application of 80 μg of croton oil dissolved in an appropriate vehicle. Control mice received only the irritant solution, whereas the others received both the irritant and the substances being tested. The following vehicles were used: acetone (for hexane, chloroform and chloroform–methanol extracts, fractions I and II and the relevant controls), 42% aqueous ethanol (v/v) (for methanol extract and its controls). Six hours later, mice were sacrificed and a plug (6 mm Ø) was excised from both the treated and untreated ears. Oedema was quantified by the difference in weight between the two plugs. The anti-inflammatory activity was expressed as percent reduction of the oedematous response in treated mice compared to the control mice treated only with the inflammatory agent. As a reference, the non-steroidal anti-inflammatory drug indometacin was used. 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).

Statistical analysis

Oedema was expressed as mean ± standard deviation of the mean. Oedema values were analysed by one-way analysis of variance followed by the Dunnett's test for multiple comparisons of unpaired data. A probability level lower than 0.05 was considered as being significant. ID50 values (dose giving 50% oedema inhibition) were calculated by graphic interpolation of the dose–effect curves.

Results and Discussion

The leaves of *T. broussonettii* were successively extracted with solvents of increasing polarity. The anti-oedematous effects of the hexane, chloroform, chloroform–methanol and methanol extracts of *T. broussonettii*, at a dose of 300 μg cm^{-2}, are reported in Table 1. The chloroform extract exerted the highest activity, provoking 47% oedema reduction, while the hexane and methanol extracts did not show any significant effect. The dose–activity relationship of the most active extract (chloroform) was carefully inves-

Table 1 Anti-inflammatory activity of *T. broussonettii* extracts and fractions after 6 h induction of croton oil mouse ear oedema.

Substance	Dose (μg cm^{-2})	Oedema (mg)	Oedema reduction (%)
Control	–	7.5 ± 1.0	–
Hexane extract	300	6.8 ± 1.4	9
Chloroform extract	300	4.0 ± 1.8*	47
Chloroform–methanol extract	300	6.3 ± 1.1*	16
Control	–	6.6 ± 1.1	–
Methanol extract	300	6.9 ± 0.8	−5
Control	–	7.4 ± 1.0	–
Fraction I	114[a]	6.6 ± 1.0	11
Fraction II	186[a]	3.2 ± 1.2*	57

Oedema values are expressed as mean ± s.d. *$P < 0.05$, at the analysis of variance, as compared with controls. [a]Dose of the fraction equivalent to 300 μg of the chloroform extract by fractionation yield.

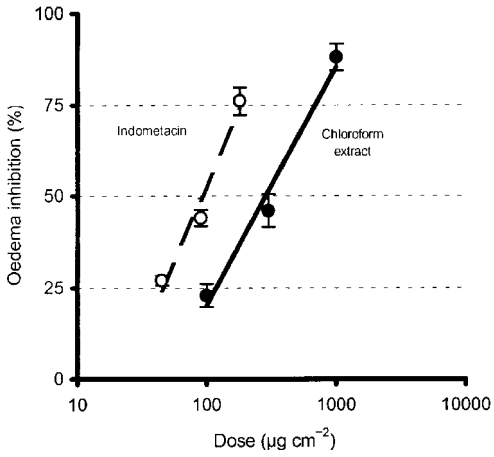

Figure 1 Dose-dependent inhibition, by *T. broussonettii* chloroform extract and indometacin, of oedema induced by croton oil in mouse ear (data represent the mean±s.d.).

tigated in comparison with that of indometacin. The reference drug showed a potency three times higher than that of the chloroform extract in reducing the oedematous response: the ID50 values were 286 (chloroform extract) and 93 (indometacin) μg cm^{-2} (Figure 1).

TLC analysis of the chloroform extract revealed the presence of triterpenic acids as its major constituents. To obtain a triterpenic acid-enriched fraction, the extract was submitted to repeated partitions between diethyl ether–water containing 1% NaOH, to obtain fractions I (38%) and II (62%), which were evaluated for their anti-inflammatory activity at doses of 114 and 186 μg cm^{-2}, respectively, corresponding to 300 μg of the parent chloroform extract. Fraction II showed the highest activity and reduced the oedematous response by 57%, while fraction I provoked a non-statistically significant oedema reduction (11%) (Table 1). Separation of fraction II by reverse-phase HPLC gave two main compounds, identified by ^{1}H NMR analysis as ursolic and oleanolic acids (Doddrell et al 1974; Seo et al 1975), in a ratio of 1:3. As previously reported (Ismaili et al 2001), both the compounds revealed a significant dose-dependent anti-oedema activity: ursolic acid was more potent than oleanolic acid (ID50 = 56 and 132 μg cm^{-2}, corresponding to 0.12 and 0.29 μmol cm^{-2}, respectively) and indomethacin (ID50 = 93 μg cm^{-2}, corresponding to 0.26 μmol cm^{-2}). The activity showed by these triterpenic acids justifies the oedema inhibition observed for fraction II and for the parent chloroform extract of *T. broussonettii*.

In the methanol extract of *T. broussonettii* leaves, never studied before, flavonoid aglycones (luteolin, eriodictyol and thymonin) and glycosides (luteolin-7-*O*-glucoside) were present, besides luteolin-3′-*O*-glucuronide and eriodictyol-7-*O*-glucoside, rare in Labiatae family. They have been previously isolated from *Mentha spicata* (Nair & Gunasegaran 1981), *Rosmarinus officinalis* (Okamura et al 1994) and recently found in *Thymus willdenowii* (Ismaili et al 2001).

Although decoctions of the leaves of *T. broussonettii* are used as an anti-inflammatory remedy in Moroccan traditional medicine, this is the first controlled study of its anti-inflammatory properties and composition. Similarly to recent results reported for *Thymus willdenowii* (Ismaili et al 2001), *T. broussonettii* can be considered not only an aromatic plant but also a medicinal plant with topical anti-inflammatory properties. Particularly, the chloroform extract of *T. broussonettii* possesses a topical antiphlogistic effect ascribable to its triterpenic acid content, while no antiphlogistic effects seem to be exerted by the flavonoid fraction. Since oleanolic acid and ursolic acid are reported to exert an anti-inflammatory effect in the carrageenan rat paw oedema model after oral administration (Kapil & Sharma 1995; Recio et al 1995a, b) and carvacrol found in the essential oil of this plant (Lattaoui et al 1993) is known as an inhibitor of prostaglandin biosynthesis (Wagner & Wiessenauer 1995), a direct involvement of these, and other, constituents of the plant in the systemic effect of the leaf decoction may be hypothesized.

In conclusion, these results give a rational support to the traditional use of *T. broussonettii* leaf decoction for internal use, in countries where synthetic drugs are scantily employed because of their high costs. It is noteworthy that a genus usually considered only for its essential oil content revealed a therapeutically exploitable activity in the non-volatile fraction too. Besides its direct therapeutic use, this aromatic species can also represent a useful source of potent anti-inflammatory compounds.

References

Agrawal, P. K. (ed.) (1989) *Carbon-13 NMR of flavonoids*. Elsevier, London

Bellakhadar, J., Claisse, R., Fleurentin, J., Younos, C. (1991) Repertory of standard herbal drugs in the Moroccan pharmacopoea. *J. Ethnopharmacol.* **35**: 123–143

Doddrell, D. M., Khong, P. W., Lewis, K. G. (1974) The stereochemical dependence of ^{13}C chemical shifts in olean-12-enes and urs-12-enes as an aid to structural assignments. *Tetrahedron Lett.* **15**: 2381–2384

Harborne, J. B. (ed.) (1994) *The flavonoids: advances in research since 1986*. Chapman & Hall, New York

Ismaili, H., Tortora, S., Sosa, S., Fkih-Tetouani, S., Ilidrissi, A., Della Loggia, R., Tubaro, A., Aquino, R. (2001) Topical anti-inflammatory activity of *Thymus willdenowii*. *J. Pharm. Pharmacol.* **53**: 1645–1652

Kapil, A., Sharma, S. (1995) Effect of oleanolic acid on complement in adjuvant- and carrageenan-induced inflammation in rats. *J. Pharm. Pharmacol.* **47**: 585–587

Lattaoui, N., Tantaoui-Elaraki, A. (1994) Comparative kinetics of microbial destruction by the essential oils of *Thymus broussonettii*, *T. zygis* and *T. satureioides*. *J. Ess. Oil Res.* **6**: 165–171

Lattaoui, N., Tantaoui-Elaraki, A., Errifi, A. (1993) Composition and antimicrobial activity of the essential oils of *Thymus broussonettii*, *T. zygis* and *T. satureioides*. *J. Ess. Oil Res.* **5**: 45–53

Nair, A. G. R., Gunasegaran, R. (1981) Rare flavone glucuronides from *Mentha spicata*. *Current Sciences* **50**: 181

Okamura, N., Haraguchi, H., Hashimoto, K., Yagi. A. (1994) Flavonoids in *Rosmarinus officinalis* leaves. *Phytochemistry* **37**: 1463–1466

Recio, M. C., Giner, R. M., Máñez, S., Gueho, J., Julien, H. R., Hostettmann, K., Ríos, J. L. (1995a) Investigation of the sterodial anti-inflammatory activity of triterpenoids from *Diospyros leucomelas*. *Planta Med.* **61**: 9–12

Recio, M. C., Giner, R. M., Máñez, S., Ríos, J. L. (1995b) Structural requirements for the anti-inflammatory activity of natural triterpenoids. *Planta Med.* **61**: 182–185

Saturnino, P., Capasso, A., Saturnino, C., De Martino, G., Lozano Reyes, N., Aquino, R. (1997) Flavonol glycosides from *Aristeguetia discolor* and their inhibitory activity on electrically-stimulated guinea pig ileum. *Int. J. Pharmacogn.* **35**: 305–312

Seo, S., Tomita, S., Tori, K. (1975) Carbon-13 NMR spectra of urs-12-enes and application to structural assignments of components of *Isodon japonicus* Hara tissue cultures. *Tetrahedron Lett.* **16**: 7–10

Tubaro, A., Dri, P., Delbello, G., Zilli, C., Della Loggia, R. (1985) The Croton oil ear test revisited. *Agents Actions* **17**: 347–349

Wagner, H., Wiessenauer, M. (1995) *Phytotherapie*. G. Fischer Verlag, Stuttgart, p. 236