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Full Length Research Paper

Analgesic activity of three thyme species, *Thymus satureioides, Thymus maroccanus* and *Thymus leptobotrys*

K. Elhabazi[1*], A. Ouacherif[1], A. Laroubi[1], R. Aboufatima[1], A. Abbad[1], A. Benharref [2], A. Zyad[1], A. Chait[1] and A. Dalal[1].

[1]Département de Biologie. Faculté des Sciences Semlalia. B. P. 2390, Morocco.
[2]Département de Chimie. Faculté des Sciences Semlalia. B. P. 2390, Morocco.

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In our previous work, we have demonstrated that *Thymus broussonetii* exerts a significant analgesic activity, which was more potent than the reference analgesic drug (acetyl salicylic acid; ASA). In this study, we examined the antinociceptive effect of three Moroccan Thyme species, i.e. *Thymus satureioides*, *Thymus maroccanus* and *Thymus leptobotrys*. The effect of aqueous, butanolic and ethyl acetate extracts of each species were tested on the nociceptive response in mice using a formalin test as a model of nociception. The results obtained showed that the treatment with aqueous and butanolic extracts (50, 100, 200 and 300 mg/kg, i.p.) of the three species induced a marked inhibition of the nociceptive response in both neurogenic and inflammatory phases of formalin test. The ethyl acetate extracts had a weak effect on the neurogenic phase, but it had a significant effect on the inflammatory phase. These results suggest that the aqueous and butanolic extracts act both peripherally and centrally to inhibit the nociceptive response, while the ethyl acetate extracts act rather peripherally. According to ID_{50} values and the maximal inhibition of the nociceptive behaviour, we could conclude that *T. satureioides* was more potent and efficacious in inhibiting the formalin nociceptive response. Phytochemical screening carried out on these species showed the presence of quinons, saponins, tannins, terpenes and flavonoids. Our results provide evidence that *T. satureioides, T. maroccanus* and *T. leptobotrys* possess active principles that exhibit marked analgesic effect, thus confirming and justifying the popular uses of these plants to relieve some pains.

Key words: Analgesic effect, formalin test, mice, *Thymus satureioides, Thymus maroccanus, Thymus leptobotrys*.

INTRODUCTION

Thymus species (Family: Labiatae) are widely distributed and found in several areas in Morocco (Jahandiez and Maire, 1934). These species are used in folk medicine as a powder, decoction or infusion to relieve some pains and to treat several disturbances such as gastro-intestinal infections, whooping coughs, bronchitis, flue and infections of throat and mouth (Bellakhdar, 1997). Pharmacological studies conducted with the Moroccan species have confirmed and extended the medicinal properties of thyme in folk medicine. These studies have confirmed the antimicrobial effect of *Thymus broussonetii, Thymus zygis* and *Thymus satureioides* (Lattaoui et al., 1993; Lattaoui and Tantaoui-Elaraki, 1994). Van Den Brouck and Lemli (1981) have demonstrated the antispasmodic effect of *T. satureioides*, which is due to its flavonoids. In addition, Ismaili et al. (2001; 2002; 2004) have reported the anti-inflammatory effect of *T. satureioides, T. broussonetii* and *Thymus willdenowii*. This effect was attributed to two triterpenes, i.e. ursolic and oleanolic acids. Recently, we have reported the immunological and behavioural effects of *T. broussonetii* Boiss., an endemic species in Morocco (Elhabazi et al., 2006b). Furthermore, we have shown in our previous work that aqueous, butanolic and ethyl acetate extracts displayed a potent analgesic effect in four antinociceptive models: hot plate and tail immersion tests in rats, and formalin and writhing tests in mice (Elhabazi et al., 2006a). Also, these previous data

*Corresponding author. E-mail: kelhabazi@ucam.ac.ma. Phone: 212 4 43 46 49. Phax: 212 4 43 67 69.

have shown that the aqueous extract, which is the most effective and more potent than acetyl salicylic acid (ASA), acts partly through an opioid-mediated mechanism.

Considering the interesting results obtained with *T. broussonetii* and as suggested by their traditional use, the analgesic profile of three other Moroccan thyme species was investigated in this work. In this context, we tested the effect of ethyl acetate, butanolic and aqueous extracts of *T. satureioides* Coss., *Thymus maroccanus* Boiss. and *Thymus leptobotrys* Murbeck on the mice nociceptive response, using the formalin-induced pain model. This test was chosen because it provides a more valid model for clinical pain than tests with mechanical or thermal stimuli (Alreja et al., 1984). Moreover, the two distinct phases of the test response may be used to address different aspects of nociception since the first phase seems to be due to direct chemical stimulation of nociceptors, whereas the second phase is dependent on the peripheral inflammation and changes in central processing (Tjolsen et al., 1992).

MATERIAL AND METHODS

Animal model

Male Swiss mice, weighing 25 - 30 g, were used. They were housed in groups of 6 mice per standard makrolon cage, on a 12 h light/dark cycle, and the air temperature was maintained at 22 ± 2 °C. The mice were offered free food and water *ad libitum*. Experiments reported in this study were carried out in accordance with the ethical guidelines for investigation of experimental pain in conscious animals (Zimmermann, 1983).

Plant material

The plants were collected in March 2003, in two regions in Morocco. *T. satureioides* and *T. maroccanus* were collected in Asni Moulay Brahim, while *T. leptobotrys* was collected in Tiznite. The species were identified and classified by Prof. Ouyahya Aicha from the Scientific Institute (Rabat). A voucher specimen of each species (*T. satureioides*: 65888; *T. maroccanus*: 65885; *T. leptobotrys*: 65887) was deposited at the herbarium of the Scientific Institute, Mohamed V University, Rabat, Morocco.

Preparation of plant extracts

Extracts of the thyme species were obtained, as previously described by Elhabazi et al. (2006a). Briefly, leaves and their stem barks were dried at 40°C and enough triturated in order to obtain a powder. Then, 1 liter of ethanol was added to this powder (200 g) to obtain an ethanolic extract using a soxhlet apparatus. This ethanolic extract was concentrated under reduced pressure using a rotatory evaporator, made up with hot water and then successively partitioned with solvents of increasing polarity (hexane, chloroform, ethyl acetate and butanol). The three fractions (ethyl acetate, butanolic and aqueous) were concentrated, dissolved and made up to an appropriate volume with 0.9% NaCl just before use.

Formalin test

The formalin test was performed to assess the way that the animal responds to moderate and continuous pain (tonic) generated by injured tissue. This test provides a valid and reliable model for clinical pain (Dubuisson and Dennis, 1977; Abbot et al., 1981; Alreja et al., 1984). The method used was similar to that described previously by de Miranda et al. (2001) with slight modifications. It consists of injecting subcutaneously 20 µl of 2% formalin into the right posterior paw of mice placed in a transparent enclosure. Throughout 5 min prior to this procedure, each mouse was allowed to adapt to the testing box and left to freely move and explore (habituation) the testing box. The formalin-induced licking of the paw was considered as indicative of nociceptive behaviour. Using a chronometer, the total time spent in licking and biting the injected paw was recorded, thus quantifying the nociceptive behaviour. However, as the formalin test in rodent consists of two successive phases (Hunskaar and Hole, 1987), the amount of time spent licking the injected paw was subsequently noted during the two relevant periods: the first one over the 10 min after the formalin injection and the second during the following 50 min. Thyme extracts were administered intraperitoneally (i.p.) at 50, 100, 200 and 300 mg/kg of the animal body weight. As a control, acetyl salicylic acid (ASA) was administered intramuscularly at 200 mg/kg.

Phytochemical screening

Phytochemical screening of the tested extracts was performed to detect the eventual presence of different classes of constituents such as alkaloids, flavonoids, quinons, saponins, sterols and tannins using specific reagents (Okpo et al., 2001, Hosseinzadeh and Younesi, 2002).

Acute toxicity

Groups of ten mice received orally doses of 1, 2, 3, 4 and 5 g/kg of aqueous, butanolic and ethyl acetate extracts of the three thyme species. The control group received orally distilled water. The groups were observed for 48 h to note any signs of toxicity or mortality and during the following 7 days.

Data analysis

Data obtained were expressed as mean per group (±SEM). Differences between groups in any test were statistically analysed by one-way analysis of variance (ANOVA), followed by Student-Neuman-Keuls as the post-hoc test. Significance was defined at the 0.05 level. The ID_{50} values (dose giving 50% of the nociceptive response inhibition) were determined using ID_{50} V1.0.

RESULTS

Effect of *T. satureioides* extracts on the nociceptive response

The results represented in Table 1 showed that during the first phase, aqueous and butanolic extracts decreased significantly the time spent licking the injured paw; this effect began at 100 mg/kg ($p < 0.05$). The ethyl acetate extract reduced the formalin nociceptive response only at 100 mg/kg. In the second phase, the three extracts attenuated significantly the formalin nociceptive response. Compared with the butanolic and ethyl acetate extract's effect, the aqueous extract was the most effective in the two phases (ID_{50} 192 ± 69 mg/kg in the first

Table 1. Effect of *T. satureioides* extracts on the nociceptive response in the formalin test.

Treatment	Time spent licking in the first phase (s)	Time spent licking in the second phase (s)
Control	155±8.40	287±17.83
Aqueous extract (9%)		
50	105.2± 20.88 ns	115± 15.84 **
100	107.25± 15.64 *	138± 20.35 **
200	84.37± 17.63 ***	71± 14.33 ***
300	85.5± 28.85 **	76±22.25 **
ID 50 (mg/kg)	192±69	115±55
Butanolic extract (6%)		
50	128.75± 23.02 ns	207± 14 ns
100	93± 21.93 *	93.83±21 **
200	85.45± 14 **	116.81±27 **
300	71.25± 27.06 *	57.75±16.39 ***
ID 50 (mg/kg)	235±99	149±31
Ethyle acetate extract (2.3%)		
50	125±12 ns	109±11**
100	109.83± 12.10 *	138.33± 24.5 *
200	121.81± 16.24 ns	102.27± 16.32 ***
300	134±9 ns	98±12***
ID 50 (mg/kg)	-	157±72
ASA (200 mg/kg)	132 ± 9 ns	40± 12 ***

Extracts (with the [w/w] extraction yield) were administered (i.p.) at doses of 50, 100, 200 and 300 mg/kg, 30 min before the start of the test. ASA was administered intramuscularly at 200 mg/kg, 30 min before the start of the test. Each point represents the mean ± SEM for 6 mice. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$ compared to control, while ns indicates no significant difference compared to the control. The ID_{50} values were determined using ID50 V1.0.

phase and 115 ± 55 mg/kg in the second phase).

Effect of *T. maroccanus* extracts on the formalin nociceptive response

As reported in Table 2, during the first phase the aqueous extract attenuated the nociceptive response at 100 ($p < 0.01$) and 200 mg/kg ($p < 0.01$). The butanolic extract reduced significantly the time spent licking the injured paw at doses of 100 ($p < 0.01$), 200 ($p < 0.01$) and 300 mg/kg ($p < 0.05$). The ethyl acetate extract reduced the time spent licking the injured paw only at 300 mg/kg ($p < 0.05$). In the late phase, the three tested extracts at the four doses attenuated significantly the nociceptive response in this test. The comparison of the ID_{50} values indicated that the butanolic extract was the most effective in the first phase of the formalin test (ID_{50} of 203 ± 69).

Effect of *T. leptobotrys* extracts on the formalin nociceptive response

As indicated in Table 3, during the first phase, the aqueous extract reduced significantly the formalin nociceptive response at the three doses: 100 ($p < 0.01$), 200 ($p < 0.001$) and 300 mg/kg ($p < 0.05$). The butanolic and ethyl acetate extracts attenuated the nociceptive response only at 200 ($p < 0.05$) and 300 mg/kg ($p < 0.05$). In the second phase, the aqueous and butanolic extracts reduced significantly the nociceptive response at the four tested doses. The ethyl acetate extract exerted its effect only at three doses, i.e. 100, 200 and 300 mg/kg. Comparison of the ID_{50} values indicated that the aqueous extract was the most effective (ID_{50}: 262 ± 38 mg/kg in the early phase and 219 ± 89 mg/kg in the late phase).

Phytochemical screening

Preliminary phytochemical analysis indicated the presence of flavonoids, quinons, saponins, tannins and terpenes in the ethyl acetate extract of the three species. The butanolic extract contained flavonoids, quinons and tannins, while the aqueous extract contained tannins and flavonoids.

Acute toxicity essay

During this essay, no signs of toxicity and mortality have

Table 2. Effect of *T. maroccanus* extracts on the nociceptive response in the formalin test.

Treatment	Time spent licking in the first phase (s)	Time spent licking in the second phase (s)
Control	156.45 ±9	275±36
Aqueous extract (5.3%)		
50	133.4± 14.3 ns	132±24 **
100	96.2± 13 **	73± 13 **
200	78.8±8.47 **	49.2± 18 ***
300	145± 15 ns	134±17.21**
ID 50 (mg/kg)	-	279±80
Butanolic extract (1.6%)		
50	121.75± 26 ns	217± 58 ns
100	105± 6.7 **	83± 20 ***
200	93.5± 10 **	96.5± 15 **
300	89± 25 *	119± 31 *
ID 50 (mg/kg)	-	203±69
Ethyl acetate extract (1.2%)		
50	135±13.2 ns	155±13 *
100	128±17 ns	143±22 *
200	99±12 *	126±12 **
300	121± 17 ns	95.23±19 **
ID 50 (mg/kg)	-	-
ASA (200 mg/kg)	132 ±9 ns	40 ±12 ***

Extracts (with the [w/w] extraction yield) were administered (i.p.) at doses of 50, 100, 200 and 300 mg/kg, 30 min before the start of the test. ASA was administered intramuscularly at 200 mg/kg, 30 min before the start of the test. Each point represents the mean ± SEM for 6 mice. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$ compared to control, while ns indicates no significant difference compared to the control. The ID_{50} values were determined using ID50 V1.0.

been observed at the tested doses indicating that the three thyme species present low toxicity.

DISCUSSION

In the current paper, we aimed to study the analgesic effect of three thyme Moroccan species from Morocco. Our results revealed that the three species showed marked inhibition on the formalin nociceptive response. The nociceptive response in the formalin test is biphasic with an acute (first) phase and a tonic (second) phase, separated by a quiescent period (Le Bars et al., 2001). In this study, the results showed that the aqueous and butanolic fractions were effective in both phases of the model, however, the ethyl acetate fraction and ASA failed to inhibit the first phase nociceptive response. The two phases of nociceptive behaviour seem to involve two distinct mechanisms. The first phase response is probably due to direct chemical stimulation of nociceptors predominantly in C nociceptive afferent fibers (Heapy et al., 1987). The pain in the second phase is less well understood, activation of NMDA and NK-1, nitric oxide modulation, intracellular Ca^{++} increase, histamine release and prostaglandin production have all been reported (Tjolsen et al., 1992). It has also been reported that drugs acting primarily on the central nervous system inhibit both phases equally, while peripherally acting drugs inhibit only the late phase (Tjolsen et al., 1992). In fact, the ability of the aqueous and butanolic extracts on both phases showed that they contain active analgesic princeples acting both centrally and peripherally, whereas the ethyl acetate extracts act rather peripherally. These data suggests that several compounds of distinct nature are acting as antinociceptive in these plants. We also concluded that polar and non polar compounds are acting against the inflammatory phase, but only polar compounds are effective against neurogenic phase of the formalin model.

Phytochemical screening carried out on these species showed the presence of numerous constituents such as quinons, saponins, tannins, terpenes and flavonoids. The antinociceptive effect could be attributed to one of these constituents or several different constituents that may act synergistically. Since the flavonoids are known for their antinociceptive and/or anti-inflammatory activity (Bittar et al., 2000; Pathak et al., 1991; Meyre-Silva et al., 1999), they may have action on the antinociceptive effect of the thyme species. Indeed, it was reported that thyme species contain luteolin (Ismaili et al., 2004), which is known

Table 3. Effect of *T. leptobotrys* extracts on the nociceptive response in the formalin test.

Treatment	Time spent licking in the first phase (s)	Time spent licking in the second phase (s)
Control	156.4± 10.06	301.6 ±14
Aqueous extract (5.1%)		
50	154±19 ns	235±22 *
100	58.66± 15.8 **	102.2± 25 ***
200	76.16± 9.28 ***	74.5± 29 ***
300	92.5±17 *	134±35 **
ID 50 (mg/kg)	262±38	219±89
Butanolic extract (2%)		
50	140.4± 21 ns	172.6± 4.3 *
100	132.25± 9.9 ns	184± 28.9 *
200	97.87± 19.9 *	82.5± 15.7 ***
300	81.5± 26 *	117.25± 38.6 *
ID 50 (mg/kg)	-	-
Ethyle acetate extract (1.7%)		
50	119±9.6 ns	227±29.45 ns
100	112.14± 20 ns	187.8± 33 *
200	123.27± 9.3 *	155± 15.2 ***
300	96±23 *	176±24 *
ID 50 (mg/kg)	-	-
ASA (200 mg/kg)	132 ±9 ns	40±12 ***

Extracts (with the [w/w] extraction yield) were administered (i.p.) at doses of 50, 100, 200 and 300 mg/kg, 30 min before the start of the test. ASA was administered intramuscularly at 200 mg/kg, 30 min before the start of the test. Each point represents the mean ± SEM for 6 mice. ***: $p < 0.001$; **: $p < 0.01$; *: $p < 0.05$ compared to control, while ns indicates no significant difference compared to the control. The ID_{50} values were determined using ID50 V1.0.

for its antinociceptive effect (Block et al., 1998). There are, however, few reports in the literature about the anti-nociceptive effect of tannins and quinons. Also, it has been reported that thyme species displayed significant anti-inflammatory activity attributed to two triterpens, i.e.ursolic and oleanolic acids (Ismaili et al., 2001; 2002; 2004). Thus, thyme species could exert their antinoci-ceptive effect especially in the second phase, at least in part, by interacting with the inflammatory process. How-ever, as preliminary phytochemical results indicated that the aqueous extract, which is the most effective in inhi-biting both the neurogenic and the inflammatory phases of the formalin model, contained only tannins and a low proportion of flavonoids. This extract, which had also the most yielding in the dry plant material, may contain other polar constituents such as glycosides. Indeed, Ismaili et al. (2001) have isolated from the polar fraction of metha-nolic extract of *T. satureioides* three flavonoid glycosides, that is luteolin-3'-O-glucuronide, luteolin-7-O-glucoside and eriodictyol-7-O-glucoside, and rosmarinic acid. These compounds may therefore be in part responsible for the antinociceptive activity of the aqueous extract. Moreover, in the toxicity assay the results revealed that the thyme species presented low toxicity up to an oral dose of 5 mg/kg.

Conclusion

In conclusion, our results provide evidence that *T. satureioides, T. maroccanus* and *T. leptobotrys* possess active principles that exhibit marked analgesic effect, confirming and justifying the popular uses of these plants to relieve some pains. Comparison of their effect showed that *T. satureioides* is more effective than *T. maroccanus* and *T. leptobotrys*.

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