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Evaluation of the analgesic effect of *Thymus kotschyanus* hydroalcoholic extract in male mice by formalin and tail flick tests

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ABSTRACT

Background and Objective: Considering the wide side effects of synthetic pain killers, use of herbal drugs in folk medicine as analgesic agents has increased. In the present work, we tried to uncover the pharmacological potential of ethanolic extracts by using various animal models and also to explore the potent antinociceptive effect of the plant *Thymus kotschyanus*.

Materials and Methods: The ethanolic extract from the aerial part of *Thymus kotschyanus* after intraperitoneal administration at doses of 50, 100 and 200 mg/kg was evaluated against pain using formalin test and tail flick test.

Results: *Thymus kotschyanus* extract at doses of 100 mg/kg ($p<0.05$) and 200 mg/kg ($p<0.001$) induced analgesia in the early and late phases of the formalin test. Maximum analgesia in acute and late phases was experienced at a dose of 200 mg/kg, while at a dose of 50 mg/kg was ineffective. The extract at a dose of 200 mg/kg increased the antinociceptive activity in minutes 30 ($p<0.01$), 45, 60 and 75 ($p<0.05$) after formalin injection and also in tail-flick test in comparison to control. The significant effect for doses of 100 and 200 mg/kg after injection of *Thymus kotschyanus* was observed at minute 60.

Conclusion: The results obtained in this study highlight that aerial part extract of *Thymus kotschyanus* possesses analgesic properties in both acute and chronic inflammatory pain.

1. Introduction

Pain is an unpleasant feeling and an experience of tissue damage outcome, caused by numerous diseases, troubling millions of people worldwide (1, 2). Although there are applicable conventional medicines used to relieve this symptom (3), however, folk medicine practitioners in developing countries use herbal medications to cure most of the diseases including pain (4). Plants possess a huge natural resource of beneficial chemical constituents that might provide a raw material for developing new drugs (5).

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Testifying the effectiveness of plant-based drugs used in the folk medicine has attracted huge attention because of inexpensiveness, least side effects, and still about 80% of world population trust chiefly on plant-based drugs (6). One of such plants used in traditional medicine is *Thymus kotschyanus* (7). Thyme (*Thymus kotschyanus*), a small subshrub native to the western Mediterranean region of Europe, has a prolonged history of use and is a chemically diverse species (7, 8). *Thymus kotschyanus* is an aromatic and medicinal plant that grows widely in Iran (Azerbaijan, Gilan, Mazandaran, Qazvin and Tehran provinces) too (9). In traditional medicine, Thymus species are used as antihelminthic, expectorant, antiseptic, antispasmodic, antimicrobial, antifungal, antioxidant, antibiotic, carminative, sedative, and diaphoretic purposes (8). They are frequently administered by infusion or are used externally in baths to treatment rheumatic and skin diseases (10, 11). Reports showed that the volatile oils from thyme are among the main essential oils used as preservative and antioxidant in the manufacturing of food stuff and cosmetics (12).

Analgesic drugs are one of the numerous products used in disease for alleviating the consequences of pain. Most of the analgesic drugs available in market exhibit an extensive range of difficulties such as efficacy, side effects including GIT disorders and other unwanted effects. Therefore, it highlights the need for the development of safe, novel and effective analgesic compounds with least adverse effects. Thus, we tried to investigate the aerial part extract of this plant for its analgesic activity. In the present work, we tried to uncover the pharmacological potential of ethanolic extracts by using various animal models and also to explore the potent antinociceptive effect of plant.

2. Materials and Methods

2.1. Handling and housing of animals

The animals were handled in accordance to the criteria outlined in the Guideline for the Care and Use of Laboratory Animals (NIH US publication 86-23 revised 1985). Male NMRI mice at 6-8 weeks of age weighting 20-25 g were purchased from Pasteur Institute (Tehran, Iran) and were kept under controlled environment (23 ± 2°C, 50 ± 5% humidity) having 12 hour light/dark cycle (light on 08:00-20:00) and free access to a standard pellet chow and tap water throughout the study except for short period of experimentation during which they were removed from cage. All experiments were conducted in accordance to the recommendations of the research and ethics committee of Tehran University of Medical Sciences for animal welfare.

2.2 Preparation of the aerial part ethanolic extract

One hundred gram of aerial parts of plant were placed in a flask followed by the addition of one liter of 96% ethanol. After 24 hours, when the solution had become clear, it was transferred into another flask. Again, one liter of 70% ethanol was then added to the solid residue and after 12 hours the supernatant was again decanted into another flask. Both solutions were then combined and concentrated by vacuum distillation at a temperature of 50°C and 70 rpm rotation speed, until the volume decreased to 1/3 of the original volume. In order to isolate proteins, fat and chlorophyll, chloroform was added to clear solution. This process was done in two steps, in each stage, chloroform phase was removed and the alcoholic phase was retained for subsequent stage. The solution was poured into a petri dish and dried in the autoclave at temperatures below 50°C in sterile conditions.

2.3. Nociceptive behavioral tests

2.3.1. Formalin test

The formalin test is an acceptable and reliable model of nociception that generates two separate phases of increased licking activity that can be referred by different mechanisms. After an injection of formalin, the initial licking stage lasts for the first 5 minutes and a late phase takes from 15 to 45 minutes. As described in previous literature, formalin (20 µl of a 2.5% solution) was injected subcutaneously into the dorsal surface of the right hind paw. The animals were then placed under a glass funnel on a glass surface, under which; a mirror was angled at 45 degrees (1). The pain response time (licking time) was calculated in two periods: 0 to 5 min-the first phase (neurogenic pain caused by direct stimulation of the nociceptors), and 15 to 30 min-the second phase (inflammatory pain which is produced by

release of inflammatory mediators) (13). Animals were randomly divided into five groups (n=6). Animals in the negative control group received 0.5 ml of normal saline. Morphine at dose of 10 mg/kg (Temad Co., Iran) was injected into animals in the positive control group. The other groups received different doses of the extract of *Thymus kotschyanus* (50, 100, and 200 mg/kg). All injections were given via intraperitoneal route. The experimenter was blind to treatment and dose.

2.3.2. Tail flick test

The tail flick examination was used to calculate the analgesic activity by a method defined previously by Amour & Smith in 1941 (14) with minor modifications in the procedure. The tail flick method was utilized to study the antinociceptive activity in mice. A radiant heat automatic tail flick analgesiometer was applied to measure reaction latencies. Basal reaction time of animals to radiant heat was recorded by locating the tip (last 1-2 cm) of the tail on radiant heat source. The tail removal from the radiant warmth was taken as an end point. The cutoff time of 12 s was required to avoid tail injury by heat. The animal who fails to withdraw its tail within 12 s was withdrawn from experiment. Mice were divided into 5 groups (n=6). Mice were treated with, morphine (10 mg/kg), normal saline and *Thymus kotschyanus* (50, 100 and 200 mg/kg). The latent period of the tail-flick response was determined at 30, 45, 60, 75 and 90 min after the administration of compounds. The experimenter was blind to treatment and dose. The maximum possible analgesia (MPA) was calculated as:

$$\text{MPA} = \frac{Test\ reaction\ time\ -\ Saline\ reaction\ time}{15 - Salin\ reaction\ time}$$

2.4. Statistical analysis

Data are expressed as mean ± SEM. Statistical evaluation was carried out by one-way analysis of variance followed Student-Newman-Keuls for formalin test and two-way repeated measures analysis of variance followed Bonferroni for tail flick were used to determine significant differences between groups and $p < 0.05$ was considered significant.

3. Results

3.1. Formalin test

Figure 1 shows the results obtained from the formalin test. The treatment of mice with *Thymus kotschyanus* (50, 100, and 200 mg/kg, i.p.) resulted in an inhibition of the formalin-induced licking in the neurogenic (first phase) pain and inflammatory (second phase) pain of the formalin test. The treatment of animals with *Thymus kotschyanus* at a dose of 200 mg/kg inhibited the formalin-induced licking in the neurogenic and inflammatory pain efficiently as by morphine (10 mg/kg, i.p.). Different doses of *Thymus kotschyanus* including 100 ($p<0.05$) and 200 mg/kg ($p<0.001$) induced analgesia in the formalin test dose-dependently. Maximum analgesia, in acute and late phases was observed at a dose of 200 mg/kg and it seems that the plant at a dose of 50 mg/kg was ineffective.

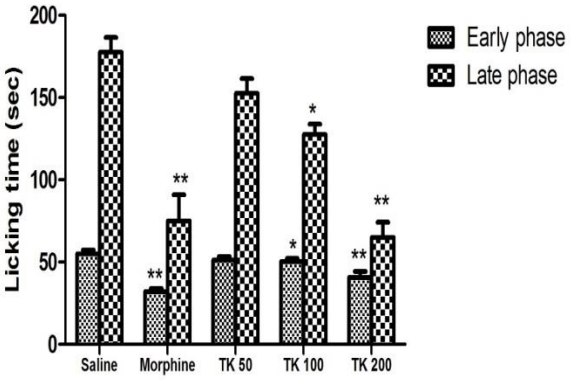

Figure 1. Effects of the ethanolic extract of *Thymus kotschyanus* (TK) on the results of the formalin-induced paw licking test in mice. First phase: 0-5 min; second phase: 15-30 min. Vertical bars represent mean ± S.E.M. * statistically different from the correspondence phase of the control group. * $p<0.05$, ** $p<0.001$.

3.2. Tail-flick test

Pre-treatment with *Thymus kotschyanus* at a dose of 200 mg/kg demonstrated a significant antinociceptive activity in the tail flick test (Figure 2). The extract at a dose of 200 mg/kg increased antinociceptive activity in 30 ($p<0.01$), 45, 60 and 75 ($p<0.05$) minutes after injection as compared to normal saline. This effect was significant at 60 minute after injection for doses of 100 and 200 mg/kg of *Thymus kotschyanus.* Under similar conditions, treatment with

morphine (10 mg/kg) significantly increased latency to thermal stimulation 30 min after administration and the antinociceptive effect was maintained during the entire period of evaluation.

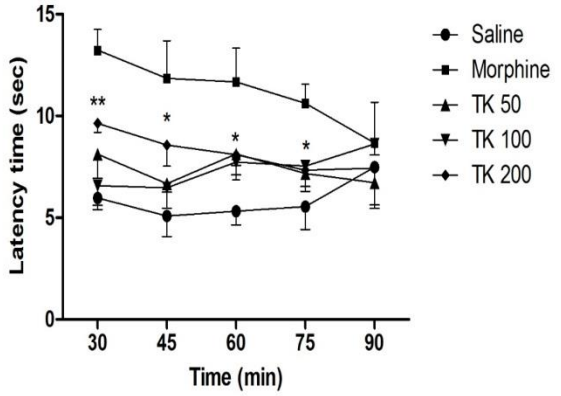

Figure 2. Effects of the ethanolic extract from *Thymus kotschyanus* (TK) in latency time of tail flick test in mice. Vertical bars represent mean ± S.E.M. * statistically different from the correspondence time of the control group. * p<0. 05, ** p<0. 01.

4. Discussion

In this study, we aimed to investigate the potential antinociceptive effect of aerial part ethanolic extract from *Thymus kotschyanus* by chemical and thermal models of nociception. The results described above confirmed that *Thymus kotschyanus* exerts a significant effect against pain in the antinociceptive models of pain in mice including formalin and tail flick tests.

The first test to assess the antinociceptive property of *Thymus kotschyanus* was the formalin test. Formalin is a noxious stimulus frequently used in animal behavioral experimentations. The formalin test initially described by Dubuisson and Dennis (13) which is categorized into first phase (neurogenic), which is induced by direct formalin stimulation of the sensorial C-fibers followed by substance P release (15), and second phase (inflammatory) principally due inflammatory reaction in the peripheral tissue mediated by the release of various inflammatory mediators, attributed by augmented level of PG, stimulation of COX and release of nitric oxide (NO) (16,17). The biphasic nature of pain response in this test, which presents diverse pathological procedures, can be used to clarify the probable mechanism involved in analgesia (18). Centrally acting drugs, such as opioids, prevent both phases of pain, whereas peripheral-acting drugs such as acetylsalicylic acid, that inhibit COX activity, only inhibit the second phase (15, 19). In the formalin test which is sensitive for numerous classes of analgesic drugs (19), our results showed that the time spent in licking the injured paw was significantly decreased by intraperitoneal administration of the ethanolic extract in both phases. In fact, the effect of the ethanolic extract on both phases showed that it contains active analgesic principles acting both centrally and peripherally.

The essential oils obtained from the aerial part of Thymus are rich in monoterpene phenols, particularly thymol (38.6%) and carvacrol (33.9%) in *Thymus kotschyanus* (20). Carvacrol has antinociceptive activity which can be associated to its antioxidant property (21). These effects are definitely related to the inhibition of prostaglandin synthesis by carvacrol (22), since this compound is a strong suppressor of cyclooxygenase (COX)-2 expression and an stimulator of the peroxisome proliferator-activated receptors (PPAR) α and γ (23).

For studying the spinal antinociceptive action, we performed the tail flick test. This model, is similar to hot plate test (14, 24), that measures animal nociceptive response latencies to thermal stimulus. Tail flick is principally a spinal response (25-27). Treating the animals with *Thymus kotschyanus*, at a dose of 200 mg/kg alters mouse latency to painful thermal stimulus in the tail flick test. These findings suggest that peripheral mechanisms are involved in the antinociceptive activity of the extract.

The peripheral antinociceptive outcome could be credited by tannins and flavonoids because of their existence in the extracts. Since the flavonoids are recognized for their antinociceptive and/or anti-inflammatory activity (28-30), so it might have action on the peripheral antinociceptive property of thyme extracts. Furthermore, previous study had stated the anti-inflammatory action of *Thymus broussonetii* which is due to the two compounds ursolic and oleanolic acids (31).

The aqueous and butanolic extracts of *T. broussonetii* showed an antinociceptive activity in two phases of formalin (50-300 mg/kg), tail immersion and writhing tests; while the ethyl

acetate extract diminished the nociceptive response only in the second phase of formalin (100–300 mg/kg) and writhing tests; so the alcoholic extract contains active analgesic principles acting both centrally and peripherally (32).

Our results showed that the *Thymus kotschyanus* extract at a dose of 100 mg/kg and 200 mg/kg was more effective in the first and second phases, as like as morphine at a dose of 10 mg/kg, suggesting possible peripheral and central antinociceptive mechanism. This extract was possibly able to inhibit this inflammation, so it can be deduced that peripheral mechanisms might also be involved in antinociceptive effects. In this study it was shown that the administration of ethanolic extract of aerial part exerts antinociceptive effects on tail flick and also on both phases of formalin test.

Conclusion

The results obtained in this study indicate that the extract possesses analgesic properties, which are mediated via peripheral inhibitory mechanisms. This could provide a rationale for the use of this plant in pain and inflammatory disorders in folk medicine. Further studies will be performed for the isolation of new chemical constituents of the plant as well as for a better understanding of the mechanism of antinociceptive activity presented by the extract.

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Conflict of interests

The authors declare that they have no conflict of interests.

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