Antinociceptive and anti-inflammatory activities of *Trigonella foenum-greacum* seeds

AMINE LAROUBI 1, LOUBNA FAROUK 1, RACHIDA ABOUFATIMA1, ABDELFETTAAH BENSALAH2, ABDELM AJID ZYAD4, AHMED BENHARREF2, ABDELLAH BAGRI3, ABDERRAHMAN CHAIT1*

1Laboratory of Pharmacology, Neurobiology and Behaviour
Faculty of Sciences
Cadi-Ayyad University
Semlalia Marrakech, Morocco

2Chemical Laboratory of the Natural Substances and the Heterocycle
Faculty of Sciences
Cadi-Ayyad University
Semlalia Marrakech, Morocco

3Laboratory of Biochemistry and Neurosciences
Faculty of Science and Technics
Universiy Hassan 1
B.P. 577, Settat 26000, Morocco

4Natural Substances and Pharmacologic Team
Faculty of Science and Technics
Beni Mellal, Morocco

*Corresponding author: fax: +212 5 24 43 74 12, e-mail: chait@ucam.ac.ma

Summary

*Trigonella foenum-graecum* L. (Leguminosae), known in Morocco as “Helba”, is used in folk medicine for its anti-ulcer, anti-inflammatory, cicatrizing activities and to treat various pain-related physiological states. In present study, we attempted to verify the possible antinociceptive and anti-inflammatory activities of different extracts obtained from the seeds of this plant. Three experimental models were used (i.e. acetic acid, formalin, and hot-plate tests) in order to characterize both the analgesic and the anti inflammatory effects. Dichloromethane (350 and 500 mg/kg), ethyl acetate [(EAE) (350 and 500 mg/kg)],
aqueous (500 mg/kg) and butanolic (500 mg/kg) extracts significantly and in a dose-dependent manner reduced the pain induced by IP injection of acetic acid. In the formalin test, the extracts, except EAE, significantly reduced the painful stimulus but only in the early phase of the test. On the contrary, these extracts, except EAE, were ineffective in increasing the latency of licking or jumping in the hot-plate test. These results suggest that the compounds present in the extracts activated both central and peripheral mechanisms to elicit the analgesic and anti-inflammatory effects. However, pharmacological and chemical studies are continued in order to characterize these mechanisms and also to identify the active principles present in each extract.

**Key words:** *Trigonella foenum-graecum* seeds, writhing test, formalin test, hot-plate test, nociception, inflammation, mice, rats

**INTRODUCTION**

*Trigonella foenum-graecum* [(Tfg), fenugreek], a leguminosae from the family Fabaceae, locally known by its Arabic name “Helba”, is one of the oldest medicinal plants originating from India and Northern Africa. Nowadays, it is extensively cultivated in most regions of the world [1]. The applications of Tfg were documented in ancient Egypt, where it was used in incense and to embalm mummies [2]. In Chinese traditional medicine, the seeds of this plant have been prescribed as a tonic for stomach disorders and whole aerial part of the plant is used as a folk medicine for the treatment of renal diseases in North-East regions of China [3]. Recently, we have shown that the Tfg aqueous extract is endowed with antiurolithiatic activity [4]. The seeds of Tfg which are commonly used as a condiment, in Moroccan eating, are reported to have nutritive properties and stimulate digestive process. Its leaves are used internally and externally to reduce swelling, prevent falling of hair and in the treatment of burns [1]. Tfg has several pharmacological effects such as hypoglycaemia [5, 6], hypocholesterolemia [7, 8], antioxidation [9], and laxation [10, 11].

Most protocols for the control of pain rely on using nonsteroidal anti-inflammatory drugs (NSAIDs) and opioid analgesics. However, both of them produce several side effects. NSAIDs produce gastrointestinal disturbances and ulceration, renal damage and hypersensitivity reactions resulting from a non selective inhibition of cyclooxygenase I (COX-I) and cyclooxygenase II (COX II) [12, 13]. Opioids induce nausea, constipation, confusion, respiratory depression and possibly dependence [14]. Therefore, searching for less harmful compounds is still an outstanding domain of investigation. Some research focused on plant medicines used in traditional medicine as they could be good sources for natural analgesic agents. There are several reports concerning the antinociceptive and anti-inflammatory effects of Tfg seeds in Moroccan traditional medicine [1]. This plant is known to contain alkaloids, saponins, flavonoids, salicylate, and nicotinic acid [15-17] like those that some plant extracts with analgesic effects contains. Therefore, in the present study, the antinociceptive and anti-inflammatory effects of the Tfg seeds have been evaluated.
MATERIAL AND METHODS

Animals

Male Swiss mice weighing 20–30 g and male Sprague-Dawley rats weighing 180–280 g were used. The animals were bred in a room, maintained in a 12 h/12 h light/dark cycle in 25°C constant temperature and 55% relative humidity. They had free access to food and water. Before testing, they were allowed to adapt in the test room for at least 12 h. Each rat was used in a single experiment.

Plant material

Trigonella foenum-graecum seeds were collected in the Chaouia region of Morocco and botanically authenticated during June 2004 period. It was identified and stored by a taxonomist in the Herbarium of Faculty of Science Semlalia Marrakech (voucher No. 4228).

Preparation of extracts

The seeds were dried and coarsely powdered. A 210 g of powder was extracted in a Soxhlet apparatus using methanol and concentrated on a rotavapor. The methanolic extract (51.71 g) was successively separated with water, hexane, dichloromethane, ethyl acetate and butanol according to the method of Shaheen et al. [18]. The extraction has given 18.06 g of aqueous extract (Tfga), 7.51 g of hexane extract (Tfgh), 12.57 g of dichloromethane extract (Tfgd), 4.34 g of ethyl acetate extract (Tfge) and 8.52 g of butanolic extract (Tfgb).

The extracts were prepared just before use. A preliminary experiment was made to check effective doses. Three doses (200, 350 and 500 mg/kg) of each extract were selected for intra-peritoneal (IP) injections and two doses (50 and 90 μg/3 ul/rat) were selected for intra-cerebro-ventricular (ICV) injections. Control animals were treated with saline solution (SS).

Writhing test

The anti-nociceptive effect was evaluated in mice by the writhing test induced by acetic acid 0.6% (0.1 ml/10g; IP). Each dose of the extracts was administered 30 min before the acetic acid injection. Five minutes after the administration of the acid, the number of writhes and stretching movements (contraction of the abdominal musculature and extension of hind limbs) was counted over a 5 min for a period of 30 min. The strength of the elicited analgesic effect was compared to that of an effective dose of acetylsalicylic acid (ASA, 200 mg/kg).
Formalin test

Each mouse was placed 5 min before formalin injection in a transparent plastic cage for habituation to the new environment. A dose of 20 μl of 2% formalin was injected subcutaneously (SC) to the plantar region of its right hind paw. The doses of the extracts and ASA were injected IP 30 min before the formalin injection. The time spent licking the injected paw was recorded every 5 min using a chronometer. Observations were carried out for 30 min.

Hot plate test

The heated surface of a hot plate analgesia meter (Ugo Basile, Italy; Socrel DS-37) was maintained at 55±0.2°C. Each animal was placed into a glass cylinder (diameter 20 cm) on the heated surface of the plate. The latency to exhibit nociceptive reaction was determined before and 30, 45 and 60 min after IP injections and also before and 10 and 30 min after ICV injection. Licking of paws and jumping were the parameters evaluated as the thermal reactions. In order to minimise the damage of the animal paw, the cut-off time for latency of response was taken as 20 sec.

Surgical preparation and technique of intra-cerebro-ventricular injection

The rats were anaesthetized with ketamine (60 to 80 mg/kg, I.P) and were implanted stereotaxically with a cannula that descended into the lateral ventricle (coordinates: 1.3 mm posterior to the bregma, lateral 1.6 mm from midline, deep 3.2 mm from the dura). The cannula was fixed to the skull by means of dental ciment. Animals were allowed to recover for 7 days during which they were handled daily.

On the day of the experiment, an injection cannula connected by type PE-10 polyethylene tube to an inhalation syringe of 10 μl, was introduced into the fixed cannula. A volume of 50 and 90 μg/rat of every extract of Tfg seeds, or SS were injected into the lateral ventricle (volume of injection: 3 μl) through the injection cannulae (0.15 mm of inner diameter).

At the end of the experiments, the rats were anaesthetized and perfused intracardially with 0.9 saline followed by a 10% formalin solution. The brain were extracted, fixed in 10% formalin for 2 days and cut at 80 μm. Localization of the cannulae tips was determined according to the Atlas of Paxinos an Watson [19].

Drugs

Drug solutions were prepared just before the start of the experiments. Intraperitoneal (IP) injections were performed using a volume of 10 ml/kg body weigh; whereas intra cerebro-ventricular (ICV) injections were performed using a volume of 3μl/rat. Each drug was dissolved in appropriate solvents as follows: acetic acid (0.6 %) and formalin (2 and 10%) in water [20], extracts of plant and acetylsalicylic
acid in saline solution [21]. The chemicals used in the extractions were: methanol, hexane, dichloromethane, ethyl acetate and butanol.

In this study, all animal experiments were performed according to the national rules which are comparable to the accepted principles for laboratory animal use and care as found in the European community guidelines. The study mentioned in this paper was approved by the committee of Cadi Ayyad University (approval number: L01. B08).

**Statistical analysis**

The results were presented as means ±SEM and the comparisons between the experimental groups were made using Student’s t-test. *: p<0.05; **: p<0.01; ***: p<0.001) were considered as indicative of significance. The inhibition percents were calculated by the following formula: Inhibition percent equals \((1-V_t/V_c)\times100\), where \(V_t\) and \(V_c\) represent the number of writhes or the licking paw time of the treated and control groups, respectively.

**RESULTS AND DISCUSSION**

Tfg seeds extracts, dichloromethane (Tfgd) and ethyl acetate (Tfge), significantly (p<0.01 for most doses and p<0.001 for 500 mg/kg of Tfge) reduced the writhing and the stretching reactions induced by 0.6% acetic acid. As shown in figure 1, there was a dose-dependent effect. The percent of reduction were 26.55% and 28.26% for 350 mg/kg, whereas it was 34.31% and 40.21% for 500 mg/kg for Tfgd and Tfge, respectively. The aqueous (Tfga) and the butanolic (Tfgb) extracts at 500 mg/kg induced a percent of reduction of the writhing response of 19.71% and 20.81% respectively (fig. 1D and 1E). ASA in a dose of 200 mg/kg was effective in reducing the writhing response by 49.38%.
Figure 1. The effect of IP administration of dichloromethane (A), ethyl acetate (B), hexane (C), aqueous (D) and butanolic (E) extracts of *Trigonella foenum-graecum* seeds on abdominal constriction test of mice. Each column and vertical bar represents mean ± SEM of 8 animals. Acetyl salicylic acid (ASA): 200 mg/kg. Vehicle (control): saline solution.

* Denotes significant difference with the corresponding values obtained from control rats.
Intraplantar injection of 2% formalin evoked a characteristic biphasic licking response. The duration of licking for the early phase (0–5 min) was 84.25±9.52 sec, whereas for the late phase (15–30 min) it was 49.98±24.54 (control group, fig. 2). The doses of 500 mg/kg Tfgd produced a marked reduction of 18.86% and 50.26% of the licking time in the early and late phase, respectively (fig. 2A) but lower doses had no significant effect. The Tfge inhibited significantly the two phases of the formalin response but higher inhibition (58.48% at 500 mg/kg) was seen in the second phase (fig. 2B). ASA was significantly more active in the second phase (61.1%; p<0.01). As shown in figure 2C, a pre-treatment with different doses of hexane extract (Tfgh) has no significant effect on the duration of licking in both phases.

**Figure 2.** Effects of dichloromethane (A), ethyl acetate (B) and hexane (C) extracts of Trigonella foenum-graecum seeds on the nociceptive responses in the formalin test. Vehicle, acetylsalicylic acid or the extracts of TFG seeds was administered IP 30 min before the injection of 2% formalin solution into the plantar area of the hindlimb. The total time (mean ±SEM) spent licking the hindpaw was measured in the first phase (0–5 min, early) and against the second phase (15–30 min, late) after intraplantar injection of formalin in the hindpaw. Each column represents the mean of six to eight animals and the vertical lines indicate the SEM. The asterisks “*” denote the significance levels as compared with control groups (Saline solution). * p<0.05; ** p<0.01; *** p<0.001.
In the hot-plate test, IP administration of 500 mg/kg of Tfgh or of Tfga produced a significant (p < 0.05) increase in the latency 45 min after the administration of extract (fig. 3A and E). However, Tfgh (200, 350 and 500 mg/kg) and Tfga (200, 350 and 500 mg/kg) showed no significant anti-nociceptive effect in this test (fig. 3C and D). Figure 3 also shows that neither the Tfgd doses 200 and 350 mg/kg nor the Tfgb doses 200 and 350 mg/kg exerted a significant analgesic effect. Tfge (200, 350 and 500 mg/kg) produced an analgesic effect that was most pronounced with the dose 500mg/kg (p < 0.05, fig. 3B). Acetylsalicylic acid (200 mg/kg) induced a weak protection against heat-induced pain (fig. 3).

Figure 3. The effect of dichloromethane (A), ethyl acetate (B), hexane (C), aqueous (D) and butanolic (E) extracts of *Trigonella foenum graecum* seeds on the hot plate test measured 0 min, 30 min, 45 min and 60 min after treatment. Each column and vertical bar represents mean ±SEM of six to eight mice. The extracts were administered intraperitoneally at doses of 200, 350 and 500 mg/kg. * Denotes significant differences (p < 0.05) from the corresponding values from control.

Intra-ventricular injection of Tfge (50 and 90 μg/rat) significantly increased the pain reaction latency (fig. 4B). Injection of the other Tfg extracts was ineffective.
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Figure 4. The effect of dichloromethane (A), ethyl acetate (B), hexane (C), aqueous (D) and butanolic (E) extracts of *Trigonella foenum-greacum* seeds on the hot plate test measured 0 min, 15 min and 30 min after treatment. Each column and vertical bar represents mean ± SEM of six rats. The extracts were administered intra-cerebro-ventricularly at doses of 50 and 90 μg/3μl.

* Denotes significant differences (p<0.05) from the corresponding values from control.
The results of this study indicate that Tfg seeds extract has potent analgesic and anti-inflammatory effects. The extracts were shown to possess anti-nociceptive effects evident in three pain models thus indicating that the observed effects may involve both central and peripheral mechanisms. Indeed, the acetic acid-induced abdominal constriction is believed to show the involvement of peripheral mechanisms, whereas the hot plate test is believed to show that of central mechanisms [22]. The formalin test is used to investigate both peripheral and central mechanisms [12]. Besides, our results bring scientific evidence for the use, in Moroccan traditional medicine, of Tfg as antinociceptive and anti-inflammatory [1]. It also confirms and extends previous data.

The analgesic effects were dose-dependant which indicate that the compounds presents in the extracts exert their effects by activation of specific receptors. Tfg seeds contain saponins, alkaloids and flavonoids that have been shown to possess analgesic activity in other plant extracts. The differences of the extracts effectiveness may probably depend on their compounds concentrations and on some physical factors such as the polarity which is related to the nature of the solvent used. Besides, the effectiveness may also depend on the nature of the receptors that could be activated. Some hypothesis on the results similar to those obtained in the three pain models are discussed.

The assessment of the abdominal constrictions elicited by acetic acid revealed that the extracts of Tfg seeds, when given IP, produces significant dose-related analgesic and anti-inflammatory effects. It has been suggested that acetic acid acts by releasing endogenous mediators that stimulate the nociceptive neurons [23]. It was postulated that the abdominal constriction response is induced by local peritoneal receptors activation [24] and involved prostanoïds mediators. As a matter of fact, increased levels of PGE2 and PGF2 in peritoneal fluids [25] as well as lipoxygenase production were reported [26]. The results of the present study showed that ASA, which inhibit a cyclooxygenase, causes significant inhibition of acetic acid-induced pain. This is in accordance with previous reports indicating that this test is sensitive to non-steroidal anti-inflammatory drugs (NSAIDs) [13-27]. Therefore, the analgesic and anti-inflammatory actions of Tfg extracts seem to be mediated by inhibition of lipoxygenase and/or cyclo-oxygenase activity or by release of cytokines such as TNF-α, interleukin-1β and interleukin-8; by resident peritoneal macrophages and mast cells, as shown by Ribeiro et al. [28], or by both mechanisms.

In our experiment with the use of the formalin test, the ethyl acetate and dichloromethane extracts suppressed both phases suggesting they contain molecular products active in the central nervous system (CNS), centrally and peripherally. Indeed, in this test there is a distinctive biphasic nociceptive response that termed early and late phases [29]. Drugs that act primarily on the central nervous system inhibit both phases equally, whereas peripherally acting drugs inhibit only the late phase. The early phase is probably a direct result of stimulation of nociceptors in the paw. The late phase is due to the release of serotonin, histamine,
bradykinin and prostaglandins during the inflammatory process [12], but also could be due, to a lesser degree, to the activation of central nociceptive neurons [30, 12]. The Tfg extracts anti-nociceptive and anti-inflammatory properties reported in our study resemble the NSAIDs properties, specifically the salicylates and their derivatives.

In the hot plate test, only the ethyl acetate extract significantly increases the latency. It could be suggested that ethyl acetate Tfg extract contains products that may exert analgesic effect through activation of central mechanisms. Indeed, the hot-plate test is commonly used to assess opioidergic analgesic mechanisms [31] and narcotic analgesia [32]. Our hypothesis is further confirmed by the observed analgesic effect elicited by ICV injections of this extract. The nature of the neurochemical substrate of such effect is not known but it could be suggested that probably an activation of the opioidergic system may occur. However, pharmacological experiment using naloxone to reverse such analgesic effects are needed to support this assumption. The remaining Tfg extracts were ineffective in the hot plate test suggesting that the compounds they contains have no central action. They have a similar profile as ASA which exerted little or no influence on the response in tests with phasic stimuli such as the hot-plate and early phase of formalin test. This suggests that the compounds of these extracts may have similar properties as NSAIDs as it was suggested from the results obtained in the formalin test.

CONCLUSION

In conclusion, our results support the traditional use of Tfg in some painful and inflammatory conditions. However, further investigations are needed to elucidate the mechanisms related to the actions of the Tfg seeds extracts. As a next step, studies in our laboratory are currently under way to isolate and characterize the active principles of each extracts.

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REFERENCES


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PRZECIWBÓLOWE I PRZECIWZAPALNE DZIAŁANIE NASION *TRIGONELLA FOENUM-GREACUM*

AMINE LAROUBI 1, LOUBNA FAROUK 1, RACHIDA ABOUFATIMA 1, ABDELFETTAH BENSALAH 1, ABDELMJID ZYAD 1, AHMED BENHARREF 2, ABDELLAH BAGRI 1, ABDERRAHMAN CHAIT 1

1 Laboratorium Farmakologii, Neurobiologii i Behawioru
Wydział Nauk Ścisłych
Uniwersytet Cadi-Ayyad
Semlalia Marakesz, Maroko

2 Laboratorium Chemiczne Substancji Naturalnych i Heterocyklicznych
Wydział Nauk Ścisłych
Uniwersytet Cadi-Ayyad
Semlalia Marakesz, Maroko

3 Laboratorium Biochemii i Nauk Medycznych
Wydział Nauki i Techniki
Uniwersytet Hassana 1
B.P. 577, Settat 26000, Maroko

4 Zespół Surowców Naturalnych i Farmakologii
Wydział Nauki i Techniki
Beni Mellal, Maroko

*autor, do którego należy kierować korespondencję: faks: +212 524 43 74 12, e-mail: chait@ucam.ac.ma
Streszczenie

Trigonella foenum-graecum L. (Leguminosae) znana w Maroku pod nazwą Helba, jest używana w medycynie tradycyjnej jako środek przeciwrzodowy, przeciwpalny, wspomagający gojenie ran i w leczeniu wielu stanów związanych z odczuwaniem bólu. W niniejszej pracy podjęto próbę sprawdzenia możliwego działania przeciwbólowego i przeciwpalnego różnych wyciągów z nasion tej rośliny. Zastosowano trzy modele eksperymentalne (tj. test z użyciem kwasu octowego, formaliny i hot-plate) w celu określenia działania zarówno przeciwbólowego, jak i przeciwpalnego. Ekstrakty sporządzone za pomocą dichlorometyanu (350 i 500 mg/kg), octanu etylu [EAE) (350 i 500 mg/kg)], wody (500 mg/kg) i butanolu (500 mg/kg) wyraźnie i dawkozależnie zmniejszały odczuwanie bólu powodowanego przez dootrzewnowe podanie kwasu octowego. W teście formalinowym, za wyjątkiem EAE, badane ekstrakty wyraźnie redukowały odczuwanie bodźca bólowego, ale tylko we wcześniejszej fazie działania danego testu. Natomiast ekstrakty, z wyjątkiem EAE, nie były efektywne w zmianie parametrów charakteryzujących test hot-plate (opóźnienie lizania czy czasu letencji skoku). Na podstawie otrzymanych wyników można sugerować, że związki obecne w wyciągach działały przeciwbólowo i przeciwpalnie zarówno poprzez mechanizmy centralne, jak i obwodowe. Trwają dalsze prace, zarówno farmakologiczne, jak i chemiczne w celu określenie dokładnych mechanizmów, jak i zidentyfikowania aktywnych związków w każdym z badanych wyciągów.

Słowa kluczowe: nasiona Trigonella foenum-graecum, test wicia, test formalinowy, test hot-plate, nocycspeja, zapalenie, myszy i szczury