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Full Length Research Paper

Antinociceptive properties of *Trigonella foenumgreacum* seeds extracts

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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 conditions. In the present study, we attempted to verify the possible antinociceptive action of different extracts obtained from the seeds of this plant. Three experimental models were used (acetic acid, formalin, and hot-plate tests) in order to characterize the analgesic effect. The extracts significantly, and in a dose-dependent manner, reduced the pain induced by intraperitoneal injection of acetic acid. In the formalin test, the extracts, except ethyl acetate extract (Tfge), significantly reduced the painful stimulus but only in the early phase of the test. On the contrary, these extracts, except Tfge, were ineffective to increase 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 effect.**

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

INTRODUCTION

Trigonella foenum-graecum [(Tfg), Fenugreek] (leguminosae), locally known by its Arabic name "Helba", is one of the oldest medicinal plants, originating in India and Northern Africa. It is extensively cultivated in most regions of the world (Bellakhdar, 1997). The applications of Tfg were documented in ancient Egypt, where it was used in incense and to embalm mummies (Basch et al., 2003). In 2003). In Chinese traditional medicine, the seeds of this plant have been prescribed as a tonic for stomach disorders, and the whole aerial part of the plant is used as a folk medicine for the treatment of renal diseases in the Northern-east region of China and Morocco (Laroubi et al., 2007). The seeds of Tfg which are commonly used as a condiment in Moroccan eating are reported to have nutritive properties and to stimulate digestive process. Its leaves are used internally and externally to reduce swelling, prevent falling of hair and in the treatment of burns (Bellakhdar, 1997). Tfg is known to have several pharmacological effects such as hypoglycaemia (Abdel-Barry et al., 2000), hypocholestrolemia (Kholsa et al., 1995), antioxidation (Dixit et al., 2005), and laxation (Dirk et al., 1999).

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 reac-

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Non-standard Abbreviations

ASA, acetylsalicylic acid; Tfga, aqueous extract; Tfgb, butanolic extract; COX I, cycloxygenase I; COX II, cycloxygenase II; Tfgd, dichloromethane extract; Tfge, ethyl acetate extract; Tfgh, hexane extract; I.P, intraperitoneal; I.C.V, intra-cerebroventricular; NSAIDs, nonsteroidal anti-inflammatory drugs; Tfg, *Trigonella foenum-graecum*; s.c, subcutaneously; S.S., saline solution; SN, nervous system.

tion resulting from a non selective inhibition of cycloxygenase I (COX I) and cycloxygenase II (COX II) (Tjolsen et al., 1992; Vane and Botting, 2003). Opioids induce nausea, constipation, confusion, respiratory depression, and possibly dependence (Dray and Urban, 1996). Therefore, searching for less harmful compounds is still an out-standing 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 (Bellakhdar, 1997). This plant is known to contain alkaloids, saponins, flavonoides, Sali-cylate, and nicotinic acid (Saxena and Shalem, 2004; Yingmei et al., 2001). The present study was designed to investigate if the Tfg seeds extract has antinociceptive effect.

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 kept in a room maintained on a 12h/12h light/dark cycle, on 25°C constant temperature and on 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. All experiments were carried out in accordance with the European community guidelines (EEC Directive of 24 November 1986; 86/609/EEC). All efforts were made to minimise animal suffering and to reduce the number of animals.

Plant materials

T. foenum-graecum seeds were collected in the Chaouia region of Morocco, it was identified and stored by Pr A.Ouhamou in the Herbarium of Faculty of Science Semlalia Marrekech (voucher number 4228).

Preparation of extracts

The seeds were dried and coarsely powdered. A 210 g powder was extracted (24 h) in a Soxhlet apparatus using methanol and concentrated on a rotaevaporator. The methanolic extract (51.71 g) was successively separated with water, hexane, dichloromethane, ethyl acetate and n-butanol according to the method of Shaheen et al. (2000). 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 intraperitoneal (I.P) injections and two doses (50 and 90 µg/3 µl/rat) were selected for intra-cerebro-ventricular (I.C.V) injections. Control animals were treated with saline solution (S.S.)

The dose employed in present research is based on that used in the traditional medicine (Bellakhdar, 1997), and the precedents researches (Laroubi et al., 2007).

Writhing test

The anti-nociceptive effect was evaluated in mice by the writhing test induced by 0.6% acetic acid (0.1 ml/10 g; I.P). Each dose of the extracts was administered 30 min before the acetic acid injection. 5 min 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) (De Miranda et al., 2001).

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 (s.c) to the plantar region of its right hind paw. The doses of the extracts and ASA were injected I.P 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 (Tjolsen et al., 1992).

Hot plate test

The heated surface of a hot plate analgesia meter (Ugo Basil,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 damage to the animal paw the cut-off time for latency of response was taken as 20 s (Shaheen et al., 2000).

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 mean of dental cement. 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 a polyethylene tube type PE-10 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 S.S were injected into the lateral ventricle (volume of injection: 3 µl) through the injection cannulae (0.15 mm 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 and Watson (1986).

Phytochemical screening

Phytochemical screening of the tested extracts was performed to detect the eventual presence of different classes of constituents, such as: alkaloids with H_2SO_4 and Dragendorff's reagents, flavonoids with the use of Mg and HCl, tannin with $Fecl_3$ solution, anthocyanes with HCl, sterols and/or terpenes with acetic anhy-

Table 1. The effect of IP administration of *T. foenum-graecum* seeds extracts on abdominal constriction test of mice.

Extracts	Dose (mg/kg)	Number of abdominal constriction	Inhibition %
Control	……..	80.50 ± 14,74	…….
ASA	200	40.75 ± 10,43***	49.38
	200	71.50 ± 17.27	11.18
Dichloromethane	350	59.13 ± 14.66*	26.55
	500	52.88 ± 12.56**	34.31
	200	74.38 ± 11.58	7.6
Ethyl Acetate	350	57.75 ± 15.16**	28.26
	500	48.13 ± 8.04***	40.21
	200	73.12 ± 20.61	9.16
Hexane	350	76.00 ± 18.75	5.59
	500	67.62 ± 9.55	16
	200	76.62 ± 9.66	4.81
Aqueous	350	69.25 ± 7.92	13.97
	500	64.63 ± 9.41*	19.71
	200	75.62 ± 14.90	6.05
Butanolic	350	72.37 ± 13.54	10.09
	500	63.75 ± 13.69*	20.81

*Denotes significant difference from the corresponding values obtained from control rats.
*P < 0.05; ** P < 0.01; *** P < 0.001.

dride and H_2SO_4, quinons with HCl and ammoniac, and saponin with ability to produce suds (Farouk et al., 2008).

Drugs

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

Statistical analysis

The results were presented as means ± S.E.M and the comparesons between the experimental groups were made using Student's t-test and ANOVA. *: 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 = (1-Vt/Vc) x 100,

Where Vt and Vc represent the number of writhes or the licking paw time of the treated and control groups respectively.

RESULTS

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 Table 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 (Table 1). ASA 200 mg/kg was effective in reducing the writhing response by 49.38%.

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 s, whereas for the late phase (15 - 30 min) it was 49.98 ± 24.54 (control group, Table 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 (Table 2) but weaker doses have 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 (Table 2). ASA was significantly more active in the second phase (61.1%; P< 0.01). As shown in Table 2, a pre-treatment with different doses of hexane extract (Tfgh) has no significant effect on the duration of licking in both phases.

In the hot-plate test, I.P 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 (Figure 1A 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 (Figure 1C

Table 2. Effects of *T. foenum graecum* seeds extracts on the nociceptive responses in the formalin test.

Extracts	Dose mg/kg	Licking response (Sec)		Inhibition %	
		Early phase	Late phase	Early phase	Late phase
Control	……..	84.25 ± 9.52	49.98 ± 24.54	……..	……..
ASA	200	75.28 ± 7.87*	19.44 ± 12.03**	10.65	61.1
	200	81.38 ± 12.90	40.89 ± 25.10	3.4	18.19
Dichloromethane	350	78.34 ± 9.00	28.47 ± 14.19*	7.01	43.04
	500	68.36 ± 14.89*	24.86 ± 10.45*	18.86	50.26
	200	87.50 ± 8.93	46.14 ± 25.40	…….	7.68
Ethyl acetate	350	73.16 ± 7.38*	29.00 ± 12.71*	13.16	41.98
	500	71.53 ± 7.99**	20.75 ± 14.19**	15.1	58.48
	200	86.32 ± 7.37	42.89 ± 15.58	……..	14.18
Hexane	350	81.96 ± 5.76	46.93 ± 15.03	2.72	6.1
	500	83.63 ± 5.11	45.00 ± 21.91	0.73	9.96

*denote the significance levels as compared with control groups (Saline solution).
*P < 0.05; ** P < 0.01; *** P < 0.001.

and D). Figure 1A and E 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 500 mg/kg (P<0.05) (Figure 1B). Acetylsalicylic acid (200 mg/kg) induced a weak protection against heat-induced pain (Figure 1).

Intra-ventricular injection of Tfge (50 and 90 µg/rat) significantly increased the pain reaction latency (Figure 2B). Injection of the other Tfg extracts was ineffective (Figure 2).

Phytochemical screening indicated the presence of tannins at high concentration in the aqueous extract while high flavonoids content were found in the ethyl acetate, aqueous and butanolic extracts. Quinons were not detected and Alkaloids were found in low concentrations in the dichloromethane extract. Positive reactions to Saponins were found in the butanolic and aqueous extracts.

DISCUSSION

The results of this study indicate that Tfg seeds extract has potent analgesic effect. 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 (Paulino et al., 2003). The formalin test is used to investigate both peripheral and central mechanisms (Tjolsen et al*.,* 1992). Besides, our results bring scientific evidence for the use, in Moroccan traditional medicine, of Tfg as antinociceptive (Bellakhdar, 1997).

The analgesic effects observed were dose dependants which indicate that the compounds presents in the extracts exert their effects by activation of specific recaptors. Tfg seeds contain saponins, alkaloids and flavonoids that have been shown to possess analgesic activity in other plant extracts (Golshani et al., 2004). The different degree of effectiveness between the extracts 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 recaptors that could be activated. Some hypothesis on this will be suggested as the results obtained in the three pain models are discussed.

Assessment of the abdominal constrictions elicited by acetic acid revealed that the extract of Tfg seeds, when given IP, produces significant dose-related analgesic effect. It has been suggested that acetic acid acts by releasing endogenous mediators that stimulate the nociceptive neurons (Collier et al., 1968). It was postulated that the abdominal constriction response is induced by local peritoneal receptors activation (Bentley et al*.,* 1983) and involved prostanoids mediators. As a matter of fact, increased levels of PGE2 and PGF2 in peritoneal fluids (Derardt et al., 1980) as well as lipooxygenase production were reported (Dahara et al., 2000). The results of the present study showed that ASA, which inhibit cycloxygenase, cause 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) (Biswal et al., 2003; Vane and Botting*,* 2003). Therefore, the analgesic and anti-inflammatory actions of Tfg extracts seems 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

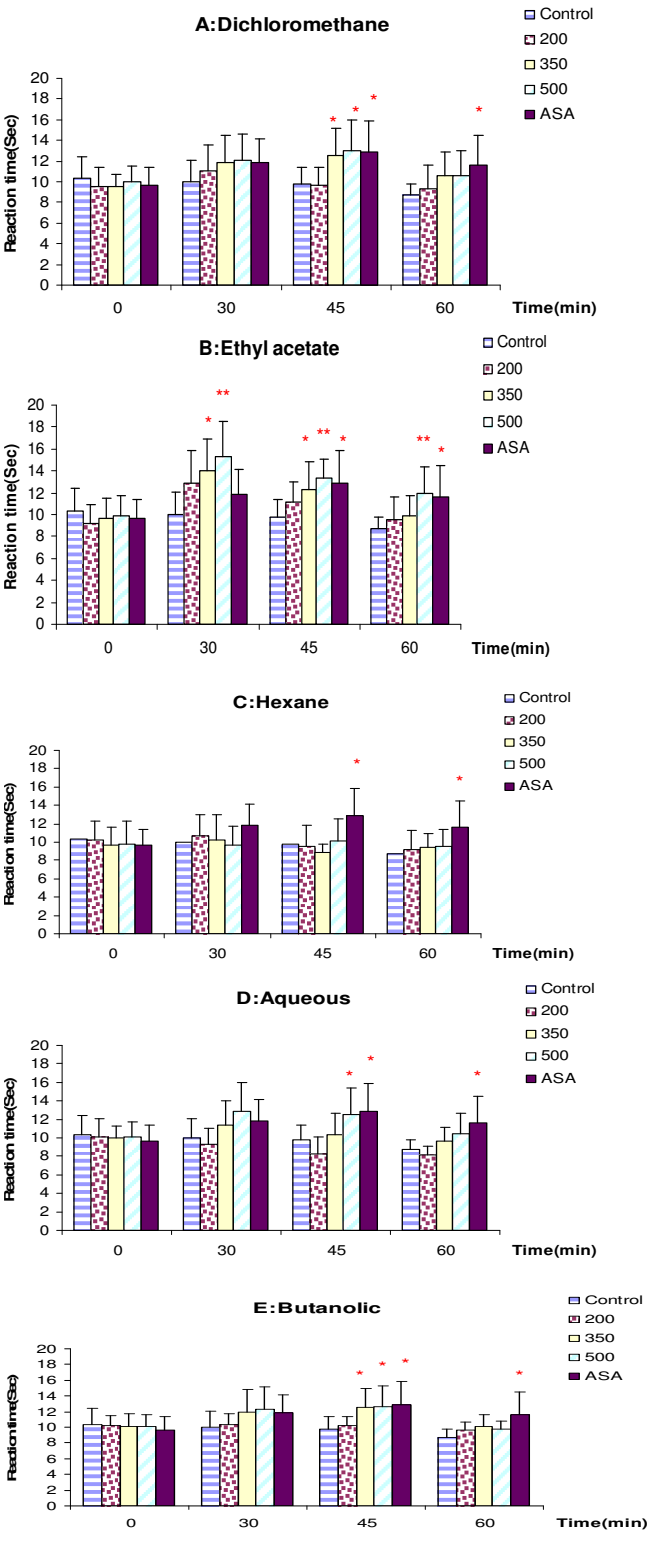

Figure 1. The effect of dichloromethane (A), ethyl acetate (B), hexane (C), aqueous (D) and butanolic (E) extracts of *T. foenum graecum* seeds on the hot plate test. Each column and vertical bar represents mean ± S.E.M. of six to eight mice. The extracts were administered intraperitoneally at doses of 200, 350 and 500 mg/kg.
"*" Denotes significant differences (P< 0.05) from the corresponding values from control.

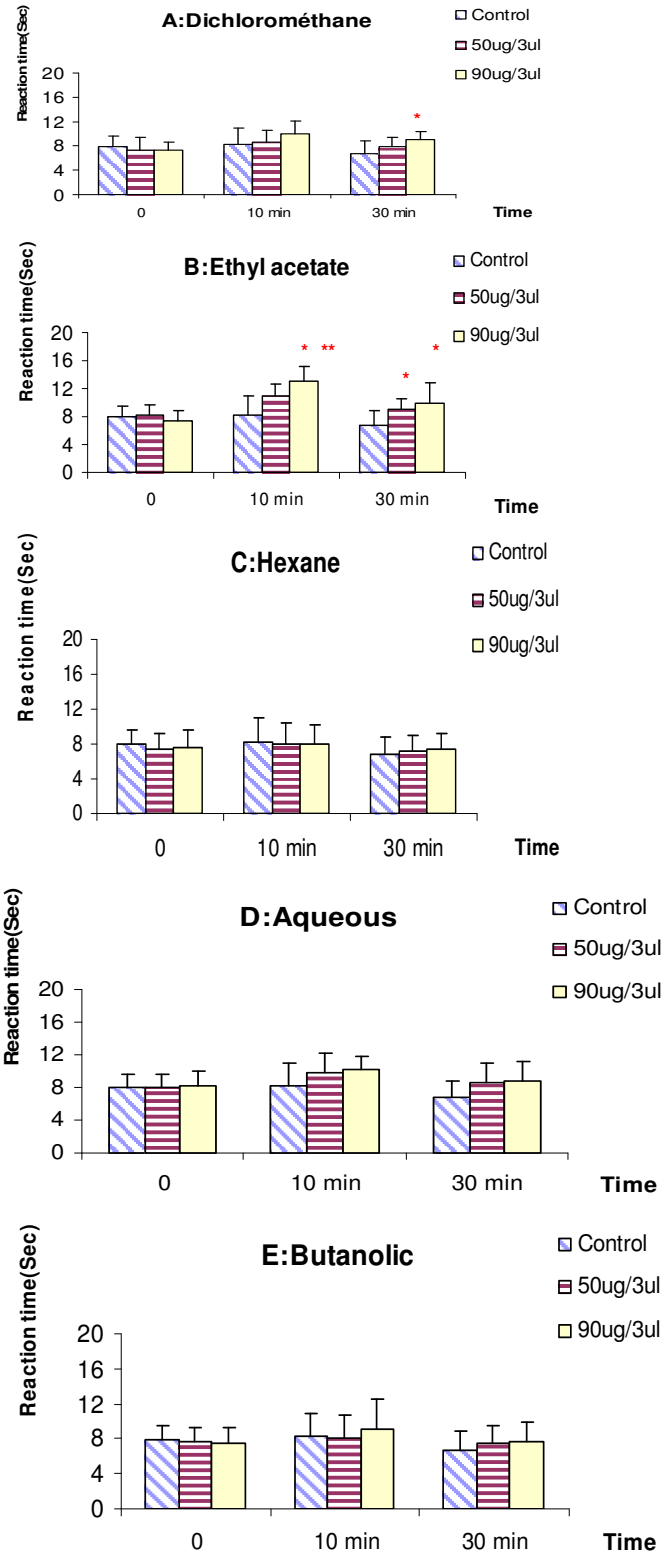

Figure 2. The effect of dichloromethane (A), ethyl acetate (B), hexane (C), aqueous (D) and butanolic (E) extracts of *T. foenum graecum* seeds on the hot plate test. Each column and vertical bar represents mean ± S.E.M. of six rats. The extracts were administered intra-cerebro-ventricularly at doses of 50 and 90 ug/3ul.
"*" Denotes significant differences (P< 0.05) from the corresponding values from control.

and mast cells, as shown by Reibero et al. (2000), or by both mechanisms.

In our experiment using the formalin test, the ethyl acetate and dichloromethane extracts suppressed both phases suggesting that both extracts contains molecular products active on the SN centrally and peripherally. Indeed, in this test there is a distinctive biphasic nociceptive response termed early and late phases (Hunskaar and Hole, 1987). Drugs that act primarily on the central nervous system inhibit both phases equally, whereas peripherally acting drugs inhibit 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 (Tjolsen et al., 1992). But also could be due, to a lesser degree, to the activation of central nociceptive neurons (Tjolsen et al., 1992; Parvizpur et al., 2006). 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 increase 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 (Araujo et al., 2005) and narcotic analgesia (Asongalem et al., 2004). 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 naloxones to reverse such analgesic effects are needed to support this assumption (Parvizpur et al., 2004). 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.

In conclusion, our results support the traditional use of Tfg in some painful 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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