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Alkaloid and flavonoid rich fractions of fenugreek seeds (*Trigonella foenum-graecum* L.) with antinociceptive and anti-inflammatory effects

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ABSTRACT

The seeds of fenugreek (*Trigonella foenum-graecum* L.) have medicinal uses as hypoglycemic, antinociceptive and anti-inflammatory agents. We aimed to evaluate the antinociceptive and anti-inflammatory effects of the major fractions of fenugreek seeds. The methanolic extract of the plant seeds was partitioned using a liquid–liquid extraction procedure to give six major fractions. Following phytochemical screening of isolated fractions, the total extract and each fraction were evaluated for their antinociception and anti-inflammatory effects using formalin and carrageenan-induced paw edema tests respectively. The methanolic extract exhibited both antinociceptive and anti-inflammatory effects at a dose of 100 mg/kg. Among the tested fractions, alkaline chloroform fraction (AKC), which was alkaloid positive in screening tests, showed the most anti-nociceptive effect in a dose-dependent manner. AKC fraction was as effective as morphine (5 mg/kg) in this regard. Both aqueous and acidified chloroform fractions (ACC) could significantly inhibit paw edema at a different dose. The latter fraction dose-dependently inhibited carrageenan-induced paw edema. The results of phytochemical screening tests confirmed the presence of flavonoids in both ACC and aqueous fractions. It can be concluded that the alkaloid and flavonoid content of fenugreek seeds can be responsible for antinociception and anti-inflammatory effects of the plant respectively.

1. Introduction

Inflammation and pain are two kinds of defense reactions of living systems in reply to any invasive factor. Considering the frequent occurrence of adverse effects of current drugs, the research for new antinociceptive and anti-inflammatory drugs with minimal toxicity is of particular interest in phytochemistry (Darland et al., 1998). Traditional medicines and particularly medicinal plants are worthy sources that can fulfill these objectives. One of the major approaches in developing new drugs from plant sources is to examine the uses claimed for a traditional preparation. Following confirmation and isolation of active ingredient(s) of medicinal plants and elucidation of their structure(s), pharmaceutical industries may utilize them as leading compounds to produce new classes of medicines.

Trigonella foenum-graecum L. belongs to Fabaceae family and is a foodstuff plant with many beneficial medicinal uses. Some of its effects such as hypoglycemic, hypocholesteremic, anticancer, and gastroprotective effects have been studied (Sharma et al., 1990; Ajabnoor and Tilmisany, 1998; Pandian et al., 2002). Phytochemical analysis has shown that the fenugreek seeds contain a variety of alkaloids, saponins, flavonoids and carbohydrates (Chauhan et al., 2010). There are several reports citing the antinociceptive and anti-inflammatory effects of this plant that suggest it is a promising candidate for further studies to find new therapeutic compounds (Biswal et al., 2003; Vyas et al., 2008; Bhalke et al., 2009; Sharififar et al., 2009; Malviya et al., 2010; Kawabata et al., 2011; Sharififar et al., 2012). However, all of the reports describe the antinociceptive and anti-inflammatory effect of total extract of the plant and no constituents of the plant have identified responsible for these activities and to the best of our knowledge, there was no study on the evaluation of antinociceptive and anti-inflammatory effects of the major fractions of fenugreek seeds so far. Therefore in the present study we will focus on the fractionation of methanolic extract of fenugreek seeds and assessment the antinociceptive and anti-inflammatory effects of its major fractions using formalin and carrageenan-induced paw edema tests.

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2. Methods and materials

The seeds of *T. foenum graecum* L. were purchased from local market; and it was authenticated by Dr. Sharififar, Department of Pharmacognosy, Faculty of Pharmacy, Kerman University of Medical Sciences, Kerman, Iran.

Ibuprofen powder (Iran, Hakim Pharmaceutical Co. Ltd., Iran), morphine (Darupakhsh, Iran), carboxymethylcellulose-sodium (CMC-Na, Iran), and carrageenan (type I, Sigma Co., UK) were of a pharmaceutical grade; and the other chemicals and reagents were from an analytical grade.

An amount of 5 kg of dried plant seeds was extracted with methanol applying percolation method. The obtained extract was evaporated under vacuum to give a viscose mass. Then, an amount of 200 g of the extract was suspended in 600 mL of distilled water and was partitioned sequentially with *n*-hexane (5 × 300 mL), carbon tetrachloride (5 × 300 mL), dichloromethane (DCL) (5 × 300 mL), acidified chloroform (pH 3) (5 × 300 mL) and basified chloroform (pH 9) (5 × 300 mL) at room temperature. Totally, six major fractions were collected and concentrated under vacuum and stored at −20 °C until pharmacological tests.

2.1. Phytochemical screening

The total extract of the fenugreek seeds and each of the fractions were screened to investigate the presence of alkaloids, flavonoids, terpenoids and saponins (Trease and Evans, 1983).

2.2. Animals

Male NMRI mice, weighing 25–30 g were used for the antinociceptive and anti-inflammatory tests. The animals were obtained from the Neuroscience Research Center, Kerman University of Medical Sciences. They were housed at a room temperature of 22 ± 2 °C, at 12-h light:12-h dark cycle, with free access to food and water. In all pharmacological tests, the animals were divided in groups of six members and acclimatized to the laboratory condition for at least 1 h before testing. Each animal was used just for one test. The ethical approval of the study was received from local authorities, which was in compliance with UK current ethical regulations for using animals in scientific procedures (Act 1986, 86/609/EEC); and also all animals used for pharmacological tests received human care (NO, EC/KNRC/85-2).

2.3. Formalin test

The antinociception was determined using formalin test as described by Saddi et al., with some modifications (Saddi and Abbott, 2000a). The dose of 5 mg/kg of six major fraction and total extract (100 mg/kg) were administrated intraperitoneally (i.p.) to animals and after 30 min, a volume of 20 μl of 1% (w/v) formalin and an equal volume of normal saline were subscutaneously (s.c.) injected into plantar surface of right and left hind paws of the animals respectively. Morphine (2.5 and 5 mg/kg) and ibuprofen (200 mg/kg) were used as positive controls. The behavioral categories and their weights, as described by Dubuisson and Dennis (1977) and Saddi and Abbott (2000b), were as follows:

0 = No pain, the injected paw is pressed firmly on the floor, 1 = favoring, resting the injected paw (right paw) on floor or limping, 2 = lifting, the injected paw is elevated and is not in contact with any surface, 3 = licking, the animal licks, bites, or shakes the affected paw.

The number of each repetitive response, and the total number of behavior responses were recorded at intervals of 10, 20, 30 and 40 min after injection. The mean of behavior observations was calculated as follows:

$$\text{Mean of behavior observation/min} = \frac{(N_1 * 1) + (N_2 * 2) + (N_3 * 3)}{T}$$

where N_1, N_2 and N_3 are the number of repetitive responses and T is the total time in min. The potent fraction (AKC) was studied at doses of 5, 10, 15 and 20 mg/kg.

The analgesic effect of AKC at different doses was calculated using the following formula: percentage of pain inhibition = $[(M_{cont} - M_{sam})/M_{con}] \times 100$, where M_{con} and M_{sam} are means of behavior observations for control and sample, respectively.

2.4. Carrageenan-induced mouse paw edema

Edema was induced by s.c. injection of 25 μl of 1% carrageenan and an equal volume of normal saline into the sub-plantar region of the left and right hind paw of the animals respectively. Ibuprofen (200 mg/kg as positive control), normal saline (0.1 ml/10 g as negative control), the total extract (100, and 200 mg/kg) and each fraction (20 mg/kg) were administered i.p. to the animals 30 min prior to induction of edema by s.c. injection of 25 μl of 1% carrageenan into the sub-plantar region of the right hind paw. Similarly, equal volume of normal saline was injected into the left paw as reference. All drugs were immediately administered after carrageenan injection ($T = 0$). The volume of both hind paws was measured with a plethysmometer at zero time (immediately after carrageenan injection), 1, 2 and 3 h after carrageenan injection. The difference of volumes of two paws of each animal was measured and considered as carrageenan-induced edema (Vasudevan et al., 2007). The effective fraction (AKC) was further studied at doses of 10, 15 and 20 mg/kg.

The increase in paw volume in Tn was calculated by subtracting the initial paw volume (basal) to the paw volume measured at each time point as follow: Δ edema: $(V_r - V_l)\ t_n - (V_r - V_l)\ t_0$ where V_r: volume of right paw, V_l: volume of left paw, t_n: the time n after carrageenan injection, t_0: the time just after carrageenan injection (Posadas et al., 2004).

2.5. Statistical analysis

The results are expressed as mean ± SEM. The differences between the mean of the control and treated groups in each pharmacological test were evaluated using a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. The differences with $p < 0.05$ were considered significant.

3. Results

3.1. Fractionation and phytochemical screening

The yield of extraction was measured about 37.5% from which six major fractions were separated. As shown in Table 1, in phytochemical screening, the fractions of AKC and ACC exhibited strong positive reaction for alkaloids and flavonoids respectively.

3.2. Formalin test

As indicated in Fig. 1, treatment with total extract (100 mg/kg), AKC (5 mg/kg) and morphine (2.5 and 5 mg/kg) significantly decreased the number of painful behaviors at early phase (T_{10}) of formalin test ($p < 0.05$). At the later phases of experiment (T_{20}, T_{30}, T40), the most inhibition of pain was observed for morphine (5 mg/kg) and AKC (5 mg/kg) which significantly decreased the pain score in animals. In addition, ibuprofen (200 mg/kg), morphine (2.5 mg/kg) and total extract (100 mg/kg) could significantly inhibit the nociception responses in mice in a decreasing order ($p < 0.05$). Aqueous fraction, ACC and DCM at dose 10 mg/kg, exhibited inhibition of pain at both early and late phases of formalin test in order; however, this effect was not significantly different from control. The results of percentage of pain inhibition of different does of AKC show that this fraction dose-dependently decreased the pain in animal (Fig. 2). As shown in Fig. 2, the percentage of pain inhibition was more than ibuprofen at both early and late phases (T_{10}, T_{20} and T_{30}).

3.3. Carrageenan-induced paw edema test

In carrageenan-induced paw edema experiment, as shown in Table 2, the examined samples demonstrated a significant anti-inflammatory activity at all tested doses in comparison with control group 3 h after carrageenan administration ($p < 0.05$). Amongst

Table 1
The result of phytochemical screening of total extract and separated fractions from fenugreek seeds.

Sample	Saponin	Flavonoid	Alkaloid	Terpenoids
Total extract	+++	++	+++	+++
n-Hexane	+	–	_	+++
CCl_4	–	–	++	++
DCM[a]	–	–	+	++
ACC[b]	–	+++	–	–
AKC[c]	–	–	+++	–
Aqueous fraction	–	+++	–	–

[a] Dichloromethane.
[b] Acidified chlorofom fraction.
[c] Alkaline chloroform fraction; +++: high content; ++: medium content; +: low content; –: no content (content was evaluated as the sediment or the intensity of color).

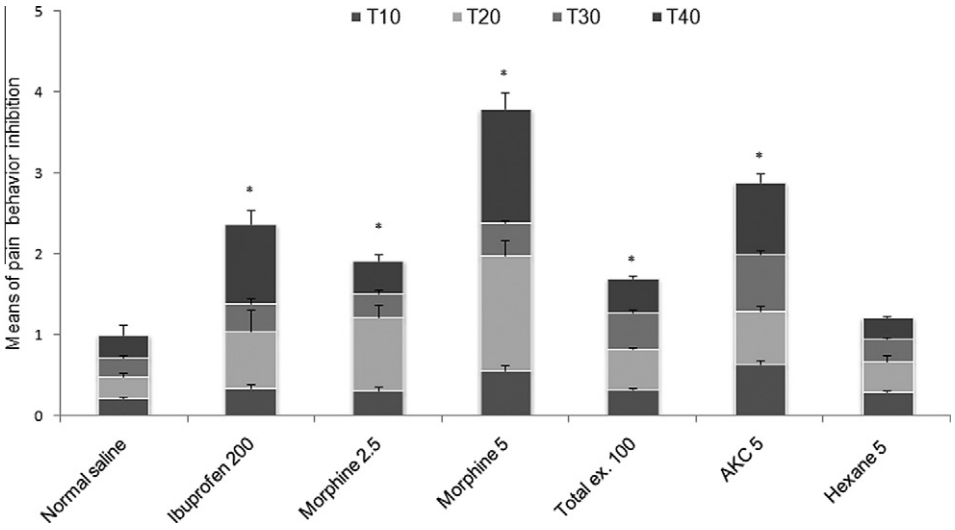

Fig. 1. The anti nociceptive effect of total extract and major fractions separated from fenugreek seeds in formalin test in comparison to ibuprofen and morphine. NS: Normal saline; AKC: Alkaline chloroform fraction; Hex: Hexane extract. The animal behavioral rating was recorded using the following scale: N_1: resting the injected paw (right paw) on floor or limping, N_2: Completely up warding or lifting the right paw, N_3: the number of licking or biting the right paw. Each point is the mean ± SEM of six animals. $^{*}p < 0.05$, significantly different from control group.

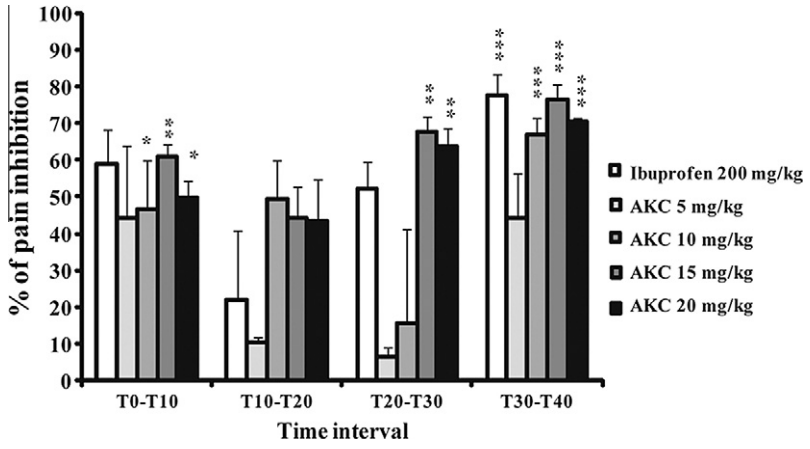

Fig. 2. The antinociceptive effect of different doses of alkaline chloroform fraction (AKC) form fenugreek seeds in comparison to ibuprofen. The effect has been calculated on the basis of percent of pain inhibition. Each point is the mean ± SEM of six animals. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ in comparison to normal saline group.

Table 2
The anti-inflammatory effect of total extract and major fractions separated from fenugreek seeds in carrageenan-induced paw edema method.

Treatment group	Percent of inhibition of carrageenan-induced paw edema in definite intervals in mice			
	0 h.	1 h.	2 h.	3 h.
Total extract (100 mg/kg)	9.02 ± 5.30	22.40 ± 6.09	70.00* ± 5.48	85.33* ± 6.24
Total extract (200 mg/kg)	18.00 ± 9.80	78.33* ± 8.60	78.33* ± 8.68	93.80* ± 4.99
Hexane (20 mg/kg)	4.21 ± 5.2	28.5 ± 7.97	27.00 ± 8.71	43.92** ± 8.30
CCl_4 (20 mg/kg)	9.020 ± 6.80	25.76 ± 6.34	27.43 ± 3.22	42.93** ± 9.40
DCM[a] (20 mg/kg)	4.03 ± 6.68	28.50 ± 7.97	27.00 ± 8.71	44.93** ± 8.68
ACC[b](20 mg/kg)	13.12 ± 8.27	61.33*** ± 8.52	79.83* ± 3.51	93.87* ± 3.72
AKC[c] (20 mg/kg)	9.3 ± 4.28	32.50 ± 8.43	33.00 ± 8.43	45.73** ± 6.74
Aqueous fraction (20 mg/kg)	20.21 ± 9.32	64.63*** ± 8.24	85.33* ± 5.74	94.80* ± 3.83
Ibuprofen (200 mg/kg)	8.00 ± 13.6	59.63** ± 7.65	75.83*** ± 7.87	96.87* ± 2.81

[a] Dichloromethane.
[b] Acidified chlorofom fraction.
[c] Alkaline chloroform fraction.
* $p < 0.001$ significantly different from control.
** $p < 0.05$ significantly different from control.
‡ $p < 0.01$ significantly different from control.

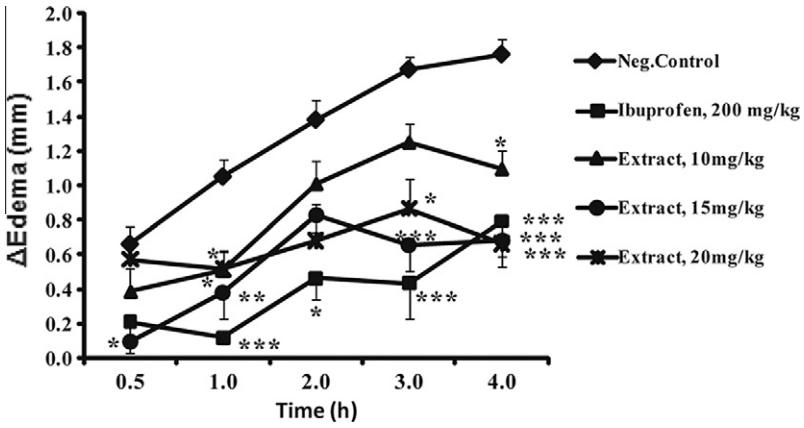

Fig. 3. The anti-inflammatory effect of different doses of ACC fraction from fenugreek seeds using carrageenan-induced paw edema. Δ Edema in the time of T_n has been calculated using formula: Δ edema: $(V_r - V_l)\, t_n - (V_r - V_l)\, t_0$. Each point is the mean ± SEM of six animals. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ in comparison to normal saline group.

the examined samples, the aqueous fraction (20 mg/kg), ibuprofen (200 mg/kg), and ACC could significantly induce a reduction in paw edema through the whole time of experiment in animal ($p < 0.05$–0.001). The total extract exhibited the highest inhibition of paw edema at 1 h in comparison with the control group ($p < 0.001$). As shown in Fig 3, the ACC fraction reduced edema in all doses (5, 10, 15 and 20 mg/kg) in 1, 2, 3 and 4 h after carrageenan administration.

4. Discussion

The fractionation of methanolic extract of fenugreek seeds provided six major fractions which were evaluated for their antinociceptive and anti-inflammatory effects. Previous studies have proven these activities for the total extract of the plant seeds (Sharififar et al., 2009; Malviya et al., 2010). Amongst the tested samples, alkaline chloroform fraction (AKC) at different doses, total extract (100 mg/kg) and morphine (5 mg/kg) have significantly reduced the pain score in both early and late phases of formalin test (Figs. 1 and 2). In formalin test, the central antinociceptive agents can inhibit both phases of formalin-induced pain while peripherally active ones inhibit the late period of pain (Miguel et al., 2002). An important feature of formalin test is its biphasic identity (Tjolsen et al., 1992). The early phase is modulated by those CNS mechanisms which can be inhibited by the opioid receptor antagonists. This is in accordance with our results showing that morphine could prevent pain in the first phase but not in the late stage. The results also show the effectiveness of the total extract, AKC, and ACC fractions in inhibition of pain in the first phase of the test. In accordance to the results of the present study, Biswal and Bhalke have also claimed a central antinociceptive effect for fenugreek seeds using hot plate and tail flick methods (Biswal et al., 2003; Bhalke et al., 2009). The results of preliminary phytochemical screening confirmed the presence of alkaloids, saponins and flavonoids in total extract while the AKC fraction exhibited a positive reaction just for alkaloids (Table 1); therefore, the antinociceptive effect of both AKC and total extract might be mostly due to the presence of the active alkaloid content. The antinociceptive effect of certain alkaloids has been investigated and cited in the literature (Garcia-Pastor et al., 1999; Matsumoto et al., 2005; Reanmongkol et al., 2005). Taking all these findings into consideration, one can attribute the antinociceptive effect of AKC fraction and total extract to their alkaloid and opioid-like compounds.

The AKC fraction dose-dependently inhibited both phases of pain in the applied model (Fig. 2). Since the late phase of formalin test is an inflammatory stage, which is prevented by cyclooxygenase inhibitors like NSAIDs, it might be suggested that the antinociceptive effect of AKC fraction is mediated via the inhibition of cyclo-oxygenases and/or lipoxygenases (and/or inflammatory mediators). This is entirely consistent with our results that show the effectiveness of ibuprofen on inflammation in the late phase of formalin test.

In carrageenan method, the findings show that the greatest significant inhibition of paw-edema was due to aqueous fraction (20 mg/kg), ACC fraction especially at different doses and the total extract ($p < 0.001$). The most inhibition was obtained for aqueous fraction and ACC, 1 and 4 h after the injection of carrageenan (Table 2). The fraction of ACC inhibited carrageenan-induced paw edema in a dose-dependent manner (Fig. 3).

The other fractions of the fenugreek seeds could also significantly inhibit paw-edema ($p < 0.001$), indicating their possible prominent mechanism of inhibitory effects on prostaglandins and bradykinin which are responsible for the second phase of edema (Yoshimoto et al., 1983). The results of phytochemical screening indicated the presence of flavonoids in both aqueous and ACC fractions of the plant, while the test for alkaloid and saponin detection were negative. Therefore, the anti-inflammatory effect of these fractions might be attributed mostly to their contents of flavonoids. Flavonoids inhibit cyclooxygenase and lipoxygenase which are involved in initiation stage of inflammation reactions (Damas et al., 1985). The precise mechanism of flavonoids in inhibition of these enzymes is not known. Flavonoids also can inhibit the biosynthesis of eicosanoids like prostaglandins. In addition, flavonoids are putative antioxidant with high activity of free radical scavenging. Free radicals can attract various inflammatory mediators, contributing to a general inflammatory response (Moroney et al., 1988; Formica and Regelson, 1995; Nijveldt et al., 2001). The other fractions like DCM, CCl_4, *n*-hexane and AKC which contained saponins, terpenoids and alkaloids could significantly reduce the paw edema (Table 2). The total extract exhibited a potent anti-inflammatory effect in the experiment. Different components of the plant might be responsible for this effect.

Although previous studies have reported the antinociceptive and anti-inflammatory effects of fenugreek seeds, it is for the first time that the major fractions of the plant have been separated and studied for these activities. More investigations for separation of active agents and toxicological studies are being carried out.

Conflict of Interest

The authors declared that there are no conflicts of interest.

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