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Antinociceptive and anti-inflammatory mechanisms of fenugreek (Trigonella foenum- graecum L.) fractions in a murine model: involvement of the L-arginine/nitric oxide pathway and pro-inflammatory cytokine suppression

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Abstract

Background

The adverse effects associated with conventional non-steroidal anti-inflammatory drugs and opioids have intensified the search for safer phytotherapeutic alternatives. Although Trigonella foenum-graecum L. (fenugreek) is traditionally used for various ailments, its precise antinociceptive and anti-inflammatory mechanisms remain incompletely understood. This study aimed to evaluate the bioactive fractions of fenugreek seeds and elucidate their underlying molecular pathways in a murine model.

Methods

A methanolic extract of fenugreek seeds was prepared and subjected to bioassay-guided fractionation using flash chromatography to obtain three sub-fractions (Fa, Fb, and Fc). Antinociceptive activity was assessed in male mice using the formalin-induced paw-licking test. Anti-inflammatory potential was evaluated by measuring lipopolysaccharide (LPS)-induced secretion of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β). Mechanistic involvement of the nitric oxide (NO) pathway was investigated using L-NAME (a non-specific NOS inhibitor) and L-arginine (an NO precursor), while glutamatergic involvement was tested via glutamate-induced nociception. Phytochemical profiling of the most active fraction was performed using thin-layer chromatography (TLC).

Results

The total extract and all three fractions significantly reduced formalin-induced pain responses, with fraction Fb demonstrating the highest potency. 2.5 mg/kg dose of Fb exhibited comparable antinociceptive efficacy to the higher doses (5 and 10 mg/kg) in both phases, establishing 2.5 mg/kg as the optimally effective dose for all subsequent mechanistic studies. Mechanistically, the antinociceptive effect of Fb (2.5 mg/kg) was significantly potentiated by L-NAME in the early phase ($p = 0.002$; $F_{3,20} = 37.00$) but not in the late phase ($p > 0.05$; $F_{3,20} = 61.49$), whereas pretreatment with L-arginine significantly reversed the effect of Fb in both phases (early: $p < 0.001$, $F_{3,20} = 19.7$; late: $p < 0.001$, $F_{3,20} = 38.52$), confirming nitrergic pathway involvement. The opioid receptor antagonist naloxone failed to reverse Fb-induced analgesia, while effectively reversing morphine-induced analgesia ($p < 0.01$), ruling out opioid system engagement. Furthermore, Fb significantly inhibited glutamate-induced nociception in both phases ($p = 0.0014$ and $p = 0.0034$, respectively; $F_{2,15} = 22.47$ and $F_{2,15} = 30.02$). In the LPS-induced inflammation model, Fb significantly suppressed TNF-α secretion at all tested concentrations ($p < 0.001$) and reduced IL-1β levels at 5 mg/kg ($p < 0.001$). Importantly, Fb did not alter spontaneous locomotor activity in the open field test at any dose, confirming that its antinociceptive effects were not attributable to non-specific motor impairment.

Conclusion

Our findings demonstrate that fenugreek, particularly the flavonoid-rich Fb fraction, exerts significant antinociceptive and anti-inflammatory effects via suppression of the NO pathway and inhibition of key

pro-inflammatory cytokines. These results position fenugreek as a promising natural candidate for pain and inflammatory management, warranting further purification and characterization of its active flavonoid constituents.

Introduction

Pain, an unpleasant sensory and emotional experience linked to actual or potential tissue damage, significantly impacts human activity [1]. Physiologically crucial for survival and adaptation [2]. Moreover, pain often follows tissue injury, prompting responses to alleviate the stimulus [3–6]. When tissue injury occurs, both the central and peripheral nervous systems undergo modifications to manifest increased sensitivity and pain resulting from tissue inflammation [7]. Inflammation, a complex response to various factors, such as injury and infection, involves increased blood flow, vascular permeability, and immune cell infiltration [8, 9]. Pain, a typical symptom of inflammation, is a defensive process initiated by noxious stimuli from damaged tissue [10]. Inflammatory pain can arise from excessive inflammatory mediator secretion; immune cells release cytokines such as TNF-α and IL-1β, which interact with pain-sensing nerves to contribute to central sensitization and pain [11, 12].

The primary objective of pain management is to diminish or eradicate pain while minimizing any adverse consequences [13]. Common treatments involve anti-inflammatory and analgesic drugs. Current options include opioids such as morphine and pentazocine and NSAIDs such as diclofenac and indomethacin. However, these drugs often have side effects such as respiratory depression, gastrointestinal problems, kidney damage, and liver toxicity [14, 15]. There is increasing interest in new pain management drugs that have fewer side effects and serve as alternatives to traditional pharmaceuticals, with a focus on exploring natural substances for pain relief without adverse effects [16].

Trigonella foenum-graecum L., commonly known as fenugreek, belongs to the Fabaceae family. These countries originated from regions such as India, Egypt, China, and various Middle Eastern countries such as Iran [17]. Several key phytochemicals, including amino acids such as 4-hydroxy isoleucine; alkaloids such as trigonelline; flavonoids; complex carbohydrates such as galactomannan; and steroidal sapogenins such as diosgenin, are found in fenugreek seeds (and, to a lesser extent, in their leaves). These compounds are widely recognized for their therapeutic properties [18, 19]. Fenugreek has demonstrated a broad spectrum of therapeutic effects, including hypoglycemic [20], lactation-stimulating [21], and hypochlostremic effects [22], as well as immune modulation [23], antitumor effects [24, 25], and antioxidant effects [26]. Additionally, fenugreek has been found to protect the stomach and liver [27, 28], aid in weight reduction [29], and alleviate inflammation [30].

Numerous studies have highlighted the pain-relieving properties of fenugreek extract [31–34]. For example, the fenugreek dermal patch was effective in alleviating postsurgical pain in patients who underwent inguinal hernia operation [32]. Moreover, fenugreek seed powder reportedly relieves the pain associated with dysmenorrhea [33].

A 2012 study using liquid–liquid extraction to fractionate fenugreek seed extract revealed that alkaloids are responsible for analgesic effects and that flavonoids are responsible for anti-inflammatory properties. [35] However, the limitations of this method in the isolation of specific metabolites prompted our study, where we used flash chromatography to obtain three main fractions and confirmed the presence of flavonoids via TLC. Our aim was to determine the precise mechanisms behind the analgesic and anti-inflammatory effects of fenugreek.

This study aimed to elucidate the exact mechanisms underlying the analgesic and anti-inflammatory effects of fenugreek. Despite the existing knowledge, the fundamental mechanisms behind the analgesic and anti-inflammatory effects of fenugreek extract remain largely unexplored. Therefore, this study investigated these effects and their mechanisms of action in mice via a formalin-induced pain model and a lipopolysaccharide-induced inflammation model.

Materials and Methods

Drugs and chemicals

Tween 20, absolute ethanol, formalin, L-arginine, NG-nitro-L-arginine methyl ester (L-NAME), and glutamate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ibuprofen powder was sourced from Hakim Pharmaceutical Co. Ltd., Iran, whereas morphine, naloxone, and dexamethasone were obtained from Darupakhsh, Iran. All chemicals were of pharmaceutical grade. All drugs and extracts were dissolved in 3% dimethyl sulfoxide (DMSO). The solvent vehicle was the only treatment given to the respective controls, which did not affect nociceptive responses. All the chemicals, medications, and extraction solutions were fresh.

Plant material

T. foenum graecum L. seeds were obtained from a local market and validated by Dr. Fariba Sharififar from the Department of Pharmacognosy, Faculty of Pharmacognosy, Kerman University of Medical Sciences (Voucher number: KF1523).

Quality Control of the Plant

The water content, total ash value, and acid-soluble ash content of the plant seeds were determined as previously described [36]. A qualitative phytochemistry test was conducted to determine the presence of alkaloids, flavonoids, saponins, tannins, and terpenoids via conventional methods [37].

Extraction and fractionation of Fenugreek seeds

A total extract was obtained from 3 kg of dried, milled seeds via maceration with methanol for three days at room temperature [38]. The dried methanol extract (10 g) was then subjected to flash chromatography on silica gel (> 400 mesh, 50 × 2 cm) via the solvent system detailed in Table 1. On the basis of TLC patterns, seventy-eight fractions were combined, concentrated under vacuum, and stored at -20°C until pharmacological testing.

Table 1
The mobile phase system used for washing the flash chromatography, column loaded with fenugreek seed extract

Ratio of mobile phase solvents		Mobile phase volume	Sample number
Petroleum ether	Ethyl acetate		
100:0		100 ml	1 & 2
98:2		100 ml	3 & 4
95:5		200 ml	5 to 8
90:10		200 ml	9 to 12
80:20		200 ml	13 to 17
7:1		160 ml	18 to 21
6:1		140 ml	22 to 25
5:1		180 ml	26 to 30
4:1		300 ml	31 to 36
3:1		200 ml	37 to 41
2:1		200 ml	42 to 46
1:1		400 ml	47 to 55
0:100		200 ml	56 to 60
Ethyl acetate	Methanol		
95:5		100 ml	61 & 62
9:1		100 ml	63 to 65
6:1		70 ml	66 & 67
1:1		100 ml	68
Methanol	Water		
97:3		100 ml	70 & 71
9:1		100 ml	72
8:1		63 ml	73
6:1		217 ml	74 to 78

Experimental animals

Male mice (25–30 g) obtained from the Neuroscience Research Center, Kerman University of Medical Sciences, were used for the antinociceptive tests. The animals were housed at 22 ± 2°C under a 12-hour light/dark cycle with free access to food and water. Mice were allocated to groups of six and acclimatized to the laboratory conditions for at least 1 hour prior to testing. Each mouse was used only once.

As this study involved behavioral nociceptive assessments, no anesthesia was administered during the experimental procedures to prevent interference with the pain-related endpoints. At the conclusion of the experiments, all mice were humanely euthanized via gradual filling of carbon dioxide (CO_2) into the euthanasia chamber at a rate of 20% chamber volume displacement per minute. Following confirmation of deep anesthesia, all animals were subjected to cervical dislocation as a secondary method to ensure death.

All experimental protocols were reviewed and approved by the Kerman University of Medical Sciences ethics committee (Ethical codes: IR.KMU.AEC.1402.009, IR.KMU.AEC.1402.063, and IR.KMU.AEC.1402.095).

Evaluation of Antinociceptive Activity

Formalin-Induced Paw Licking Test

The analgesic effects of a total extract and three primary fractions (Fa, Fb, and Fc) were evaluated via the formalin test. The mice received vehicle (3% DMSO, 10 mL/kg; i.p.), morphine (5 mg/kg, s.c.), ibuprofen (200 mg/kg), total extract, Fa, Fb, or Fc (5 mg/kg, i.p.). After 30 minutes (i.p.) or 60 minutes (s.c.), 20 µL of 2.5% formalin was injected into the right hind paw (i.pl.). Paw licking/biting duration was measured for 35 minutes postinjection as an indicator of nociception. Nociceptive scores were assessed during the early (0–5 min) and late (15–35 min) phases. A 2.5 mg/kg dose was used to evaluate neuromodulation and anti-inflammatory mechanisms

Investigation of the Mechanisms of Action

Involvement of the nitric oxide (NO) pathway

To determine whether the NO pathway plays a role in the antinociceptive effect of F_b, different groups of mice were given a substrate of nitric oxide synthase (NOS), i.e., L-arginine (200 mg/kg, i.p.) or the NOS inhibitor L-NAME (30 mg/kg, i.p.) 30 minutes before F_b injection (2.5 mg/kg, i.p.). After 30 min, the mice were subjected to the formalin-induced paw licking test [39].

Involvement of Opioid Receptors

To explore the possible involvement of the opioid system in the antinociceptive activity of F_b, separate groups of mice were pretreated with the nonselective opioid receptor antagonist naloxone (5 mg/kg;

i.p.), 30 minutes prior to the administration of the vehicle (3% DMSO, 10 mL/kg; i.p.), morphine (5 mg/kg; s.c.), or F_b (2.5 mg/kg; i.p.). After 30 minutes (for i.p. administration) and 60 minutes (for s.c. administration), the formalin test was conducted [40].

Involvement of the glutamate receptor

The participation of the glutamate receptor in the antinociceptive activity of F_b was investigated through a glutamate-induced paw-licking model. The experiment was conducted as previously described [41]. The mice were pretreated with F_b (2.5 mg/kg, i.p.), morphine (2.5 mg/kg), ibuprofen (200 mg/kg), or vehicle (3% DMSO, 10 mL/kg; i.p.). After 30 minutes, 20 µL of glutamate (10 µmol/paw; i.pl.) was injected into the ventral surface of the right hind paw of each mouse. The mice were then observed individually for 15 minutes after glutamate injection. The time spent licking the glutamate-injected paw was recorded as an indicator of pain behavior.

Evaluation of the Anti-inflammatory Activity

To assess the anti-inflammatory activity of the F_b fraction, the animals were administered ibuprofen (10 mg/kg body weight; i.p.), dexamethasone (10 mg/kg body weight; i.p.), or the F_b fraction (1, 2.5, 5, or 10 mg/kg; i.p.). After 30 min, 20 µL of lipopolysaccharide (LPS, 10 ng/mL) was injected into the ventral surface of the right hind paw of each mouse, and the same amount of normal saline was injected into the ventral surface of the left foot. The animals were subsequently euthanized and their paws were separated and stored at -80°C until further analyses. The levels of the inflammatory cytokines TNF-α and IL-1β were measured via enzyme-linked immunosorbent assay (ELISA) kits (Karmania Pars Gene Co., Iran) according to the manufacturer's instructions.

Assessment of locomotor activity

The mice (n = 6/group) were given vehicle (3% DMSO, 10 mL/kg, i.p.) or F_b (1, 2.5, 5, or 10 mg/kg; i.p.). Thirty minutes after treatment, the animals were placed in the center of an open field arena (40 × 40 × 40 cm), and the total number of line crossings was recorded over a 5-minute period.

Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM) obtained from six animals per group. GraphPad Prism 10 software was used for statistical analysis. The Kolmogorov–Smirnov test was used to determine the normal distribution of the data. To compare the means of the variables between the groups, one-way ANOVA was employed, followed by Tukey's post hoc test. *P values* < 0.05 were considered significant.

Results

Quality Control Results

The plant water content was determined to be 0.44% (w/w), and the total ash and acid-insoluble ash contents of the seeds were 3.12% and 0.41% (w/w), respectively.

Fractionation and Phytochemical Screening

The extraction yielded a dry plant material of 7.46% (w/w), with a water content of ???%, and total ash of 3.12% (w/w). Phytochemical screening revealed the presence of saponins, alkaloids, terpenoids, and flavonoids (Table 2). The three main fractions (F_a, F_b, and F_c) were selected from flash chromatography because of their values and flavonoid patterns on TLC. The weights of F_a, F_b, and F_c were 104, 5.9, and 110.3 (mg), respectively.

Table 2
Qualitative phytochemical analysis of the plants (methanol extract)

Phytochemical types	Result
Saponins	+++
Alkaloids	+++
terpenoids	+++
flavonoids	+++

The analgesic effects of the total extract and three primary fractions in the formalin test in rats

As indicated in Fig. 1, the intraplantar injection of formalin (2%, 25 µL) elicited a nociceptive response characterized by a biphasic time course, consisting of the first and second phases. All tested samples (total extract, F_a, F_b, and F_c fractions; 5 mg/kg) significantly reduced the paw licking time in the early phase ($F_{5, 30} = 34.9$, $p = 0.008$, $p = 0.002$, $p < 0.0001$, $p = 0.0007$, respectively; Fig. 1A) as well as the late phase ($F_{5, 30} = 24.68$, $p = 0.007$, $p = 0.002$, $p < 0.0001$, $p < 0.0001$; Fig. 1B). In particular, F_b displayed the strongest antinociceptive effect. Further dose–response analysis of F_b (1, 2.5, 5, and 10 mg/kg) confirmed its ability to decrease paw licking in both the early phase ($F_{5, 30} = 49.36$, $p = 0.01$, $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively; Fig. 2A) and the late phase ($F_{5, 30} = 44.96$, $p = 0.002$, $p < 0.001$, $p < 0.001$, and $p < 0.001$, respectively; Fig. 2B). Interestingly, F_b at a dose of 2.5 mg/kg had an effect similar to that of higher doses (5 and 10 mg/kg) in both phases. Therefore, a dose of 2.5 mg/kg was selected for further studies.

Investigation of the Mechanisms of Action

The role of the NO pathway in the antinociceptive effect of F_b

The antinociceptive effect of F_b (2.5 mg/kg) on formalin-induced paw licking in mice was significantly potentiated by L-NAME in the early phase ($F_{3, 20} = 37.00$, $p = 0.002$; Fig. 3A) but not in the late phase ($F_{3, 20} = 61.49$, $p > 0.05$; Fig. 3B). In addition, pretreatment of the mice with L-arginine significantly reduced the antinociceptive activity of F_b (2.5 mg/kg) in both the early phase ($F_{3, 20} = 19.7$, $p < 0.001$; Fig. 4A) and the late phase ($F_{3, 20} = 38.52$, $p < 0.001$; Fig. 4B). The administration of L-NAME (30 mg/kg) or L-arginine (200 mg/kg) alone did not significantly affect the nociceptive response of the mice (Figs. 3 and 4).

The involvement of the opioid system in the antinociceptive activity of F_b

Figure 5 shows that pretreatment of the mice with the nonspecific opioid receptor antagonist naloxone (5 mg/kg; i.p.) did not reverse the antinociceptive effect of F_b (2.5 mg/kg; i.p.). In contrast, naloxone significantly ($p < 0.01$) reversed the effect of morphine (5 mg/kg; s.c.) during the early phase ($p > 0.05$). Furthermore, significant antinociceptive effects were observed in the group treated with both F_b and naloxone compared with the naloxone-only group ($p < 0.05$). These findings suggest that the opioid system is not involved in the antinociceptive activity of F_b.

The involvement of the glutamate receptor in the antinociceptive activity of F_b

In a study on glutamate-induced nociception, pretreatment with F_b (2.5 mg/kg; i.p.) was observed to reduce the degree of paw licking induced by glutamate, with a significant antinociceptive effect in both phases ($F_{2,15} = 22.47$, $p = 0.0014$; Fig. 6A and $F_{2,15} = 30.02$, $p = 0.0034$; Fig. 6B).

Anti-inflammatory activity of F_b

The administration of LPS (10 ng/mL; i.pl.) led to an inflammatory response in mice, as evidenced by the secretion of the inflammatory cytokines TNF-α and IL-1β. As shown in Fig. 7A, pretreatment with the F_b fraction significantly reduced TNF-α levels ($p < 0.001$ for all concentrations). Similarly, lower IL-1β levels were observed in F_b-treated mice following LPS injection, and this reduction was significant at the F_b concentration of 5 mg/kg ($p < 0.001$; Fig. 7B).

Effects of the total extract and three primary fractions on locomotor activity

F_b (1, 2.5, 5, or 10 mg/kg) did not alter the locomotor activity of the animals in the open field test (Fig. 8).

Discussion

Mandegari et al. investigated the analgesic and anti-inflammatory effects of fractions from fenugreek seed extract and reported that the total extract and its alkaline (AKC) and acidified (ACC) chloroform fractions effectively reduced pain and inflammation. The AKC fraction, which is rich in alkaloids, significantly relieved pain during formalin testing, whereas the ACC fraction reduced paw swelling, indicating potential inhibition of inflammatory mediators. Flavonoids in the extract were identified as key contributors to its anti-inflammatory properties [35].

To identify the precise mechanism underlying the analgesic and anti-inflammatory effects, we separated the complete fenugreek seed extract via flash chromatography, resulting in three main fractions. Additionally, the presence of flavonoids was confirmed via thin layer chromatography (TLC).

In this study, quality control parameters for fenugreek seeds, including water content, total ash, and acid-insoluble matter, were found to align with the literature values [42]. Phytochemical screening revealed the presence of alkaloids, flavonoids, saponins, and terpenoids, whereas tannins were absent.

The methanolic extract of fenugreek seeds was fractionated into three main fractions (Fa, Fb, and Fc). TLC analysis revealed that flavonoids were the predominant compounds in all three fractions, showing a yellow color under UV light at 366 nm after treatment with a natural product. These fractions were then evaluated for their analgesic and anti-inflammatory activities. All the tested fractions (including the whole extract, Fa, Fb, and Fc at 5 mg/kg) significantly reduced the duration of paw licking in both phases of the formalin assay (Fig. 1A and 1B), highlighting the biphasic nature of this test. [43]. The first phase of licking is associated with the direct action of formalin injection on nociceptors, whereas the second phase seems to be caused by central sensitization and subsequent peripheral inflammation [44]. In line with our results, in Bhalke's study, the central and peripheral analgesic effects of fenugreek seeds were reported via hot plate and acetic acid-induced burning methods [45]. Treatment of diabetic mice with *T. foenum* graecum seeds and quercetin significantly increased the latency time in the tail flick test (hot dip test) and significantly reduced the paw flick response (formalin test) in type II diabetes, attenuating diabetes-induced axonal degeneration [46]. In a double-blind, placebo-controlled trial, a 10% fenugreek transdermal patch (FDP) was shown to effectively manage postoperative pain in inguinal hernia patients. When applied twice daily after surgery, the patch significantly reduced pain and lowered the need for morphine and diclofenac suppositories [32]. Malviya et al. investigated the analgesic effects of aqueous and alcoholic extracts of fenugreek seeds in rats via the tail flick method and compared them to those of oral doses of diclofenac potassium (50, 100, and 200 mg/kg). This study highlights the role of various neurotransmitter systems—such as opioid, oxidative, and cholinergic systems—in pain transmission, suggesting that intervening in these pathways can lead to complex outcomes [47]. In this study, we investigated the interaction of the active analgesic fraction of fenugreek seeds with various neurotransmitter systems involved in pain modulation. Prior to the administration of this fraction, the animals were pretreated with different modulators. A series of comprehensive studies have been conducted to elucidate the mechanism of action of the analgesic fraction, particularly focusing on its effects on glutamate and NO [48]. NO is a gaseous molecule produced by the NOS enzyme, which has three isoforms: inducible NOS (iNOS), neuronal NOS (nNOS), and endothelial NOS (eNOS) [49]. The

generation of NO can promote either inflammation or anti-inflammatory reactions, depending on the location and type of cells involved. Low concentrations of NO produced by eNOS activate proinflammatory cytokines, including nuclear factor kappa B (NF-κB) and cyclooxygenase 2 (COX-2) [50]. However, NO generated in excess by iNOS and nNOS is involved in biological reactions such as inflammation and pain [51]. Several molecules, such as L-NAME, can pharmacologically suppress NOS [52]. Our results indicate that pretreatment with L-NAME enhanced the antinociceptive effects of the fenugreek seed fraction (Fb) during the first phase, but this effect was not observed in the second phase. In contrast, L-arginine, a substrate for nitric oxide synthase (NOS), diminished the pain-relieving effects of Fb in both phases. These findings suggest that the nitric oxide (NO) pathway plays a significant role in mediating the analgesic effects of Fb. On the other hand, the opioid system is one of the key players in pain regulation, influencing both the perception and modulation of the pain process through central and peripheral mechanisms [53]. The opioid receptor antagonist naloxone did not alter the antinociceptive activity of the fenugreek seed fraction (Fb), indicating that opioid receptors do not play a role in the pain-relieving effects of Fb. This finding contrasts with that of morphine, whose analgesic effects are reduced by naloxone. To investigate whether glutamate receptors are involved in the analgesic activity of the F_b fraction, a glutamate-induced nociception model was used. Glutamate is the primary excitatory neurotransmitter in the nervous system and is essential for nociception and pain management [54]. Direct injection of glutamate into the lumbar region or footpad of a mouse leads to an instant nociceptive reaction, characterized by biting and licking [55]. Nociception caused by intraplantar glutamate injection is primarily due to the stimulation of both metabotropic and ionotropic glutamate receptors [56, 57]. A study by Mandegari et al. revealed that treatment with total extract (100 mg/kg), AKC (5 mg/kg), and morphine (2.5 and 5 mg/kg) greatly reduced the incidence of painful behaviors during the early part of the formalin test [35]. Additionally, our findings demonstrated that the administration of F_b (2.5 mg/kg; i.p.) reduced paw licking induced by glutamate, indicating a significant antinociceptive impact (Fig. 6). Studies have suggested that pain responses triggered by glutamate involve mechanisms at the peripheral, spinal, and supraspinal levels, which act through the stimulation of N-methyl-D-aspartate (NMDA) and non-NMDA receptors, as well as the release of NO or related compounds [58]. The release of NO, in turn, can promote the generation or release of proinflammatory agents, resulting in an intensified inflammatory response [50]. This study provides evidence that the analgesic properties of the F_b fraction in the glutamate test may be attributed to interference with the glutamatergic pathway or the inhibition of NO generation. Furthermore, the current study indicated that pretreatment with the F_b fraction did not impact the locomotor activity of the animals in the open field test.

Additionally, IL-1β plays a crucial role in the initiation and escalation of inflammatory responses and is involved in various cellular processes [59, 60]. TNF-α is also involved in the regulation of inflammatory reactions and stimulates a range of inflammatory mediators, such as chemokines and other cytokines [61]. Numerous studies have demonstrated that a number of proinflammatory molecules, e.g., IL-1β and TNF-α, are major contributors to pain [62, 63]. A study by Mandegari et al. demonstrated the significant anti-inflammatory effects of the aqueous fraction of fenugreek in a carrageenan-induced paw edema

model. Compared with the controls, the aqueous fraction (20 mg/kg) and ibuprofen (200 mg/kg) effectively reduced edema throughout the trial, with the total extract showing the strongest suppression at one hour [35]. Furthermore, our results indicated that pretreatment with fenugreek significantly lowered IL-1β and TNF-α levels in mice after LPS injection, highlighting its potent anti-inflammatory properties. Research has also revealed that various fractions of fenugreek seeds reduce iNOS expression in LPS-stimulated macrophages, suggesting that the suppression of iNOS may contribute to the plant's anti-inflammatory mechanisms [64].

Conclusion

This study explored the analgesic and anti-inflammatory properties of fenugreek extract, with a particular focus on its potential as a natural alternative for pain management. The analgesic effects of the main flavonoid fractions in a formalin-induced pain model in mice were shown to be independent of the opioid system due to the lack of reversal of its effects by naloxone. In addition, its anti-inflammatory effect was observed by reducing the levels of inflammatory cytokines (TNF-α and IL-1β) following lipopolysaccharide (LPS) injection. In conclusion, given the side effects associated with conventional pain medications such as NSAIDs and opioids, the use of fenugreek extract presents a promising avenue for developing safer pain management alternatives. In conclusion, the present work indicated that the methanolic extract of fenugreek seeds has significant antinociceptive and anti-inflammatory properties. These effects may be mediated, at least in part, through the NO pathway and glutamate receptors. However, further research is needed to elucidate the precise mechanism of action.

Declarations

Acknowledgment

This article has also been derived from the thesis of Pharm.D. students in Kerman University of Medical Sciences, Faculty of Pharmacy, Kerman, Iran.

Fariba Sharififar and Shiva Amiri conceived and designed the study and provided overall supervision. Faegheh Farhadi, Arefeh Mohammadi, Atefeh Hajializadeh, and Hosein Mardani performed the experimental procedures and collected the data. Data analysis and interpretation were carried out by Faegheh Farhadi, Shiva Amiri, Moazamehosadat Razavinasab, and Majid Jafari-Sabet. The original draft of the manuscript was written by Arefeh Mohammadi, Atefeh Hajializadeh, and Shiva Amiri. Critical revision and editing of the manuscript were performed by Fariba Sharififar, Faegheh Farhadi, Moazamehosadat Razavinasab, Majid Jafari-Sabet, Shiva Amiri, and Neda Mohamadi. All authors reviewed the final version and gave their approval for submission.

Ethical statement:

This study was approved by the ethics code IR.KMU.AEC.1402.009, IR.KMU.AEC.1402.063, IR.KMU.AEC.1402.095 in the ethics committee of Kerman University of Medical Sciences, Iran.

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Author Contribution

Fariba Sharififar and Shiva Amiri conceived and designed the study and provided overall supervision. Faegheh Farhadi, Arefeh Mohammadi, Atefeh Hajializadeh, and Hosein Mardani performed the experimental procedures and collected the data. Data analysis and interpretation were carried out by Faegheh Farhadi, Shiva Amiri, Moazamehosadat Razavinasab, and Majid Jafari-Sabet. The original draft of the manuscript was written by Arefeh Mohammadi, Atefeh Hajializadeh, and Shiva Amiri. Critical revision and editing of the manuscript were performed by Fariba Sharififar, Faegheh Farhadi, Moazamehosadat Razavinasab, Majid Jafari-Sabet, Shiva Amiri, and Neda Mohamadi. All authors reviewed the final version and gave their approval for submission.

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Figures

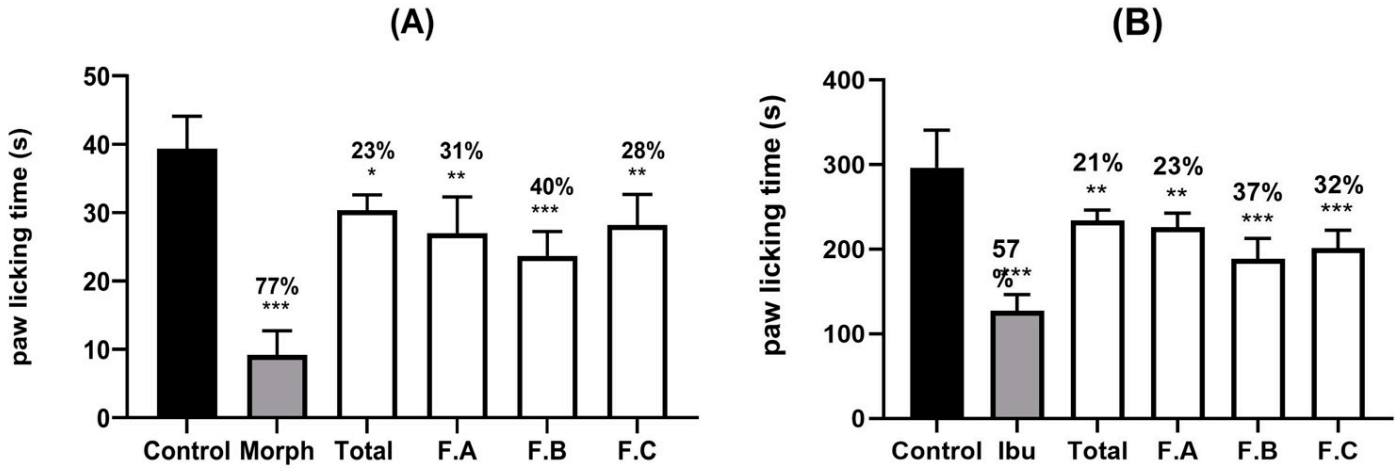


Figure 1

(A) Effect of the whole extract and three primary fractions (Total, F.A, F.B, and F.C) (5 mg/kg, i.p.)

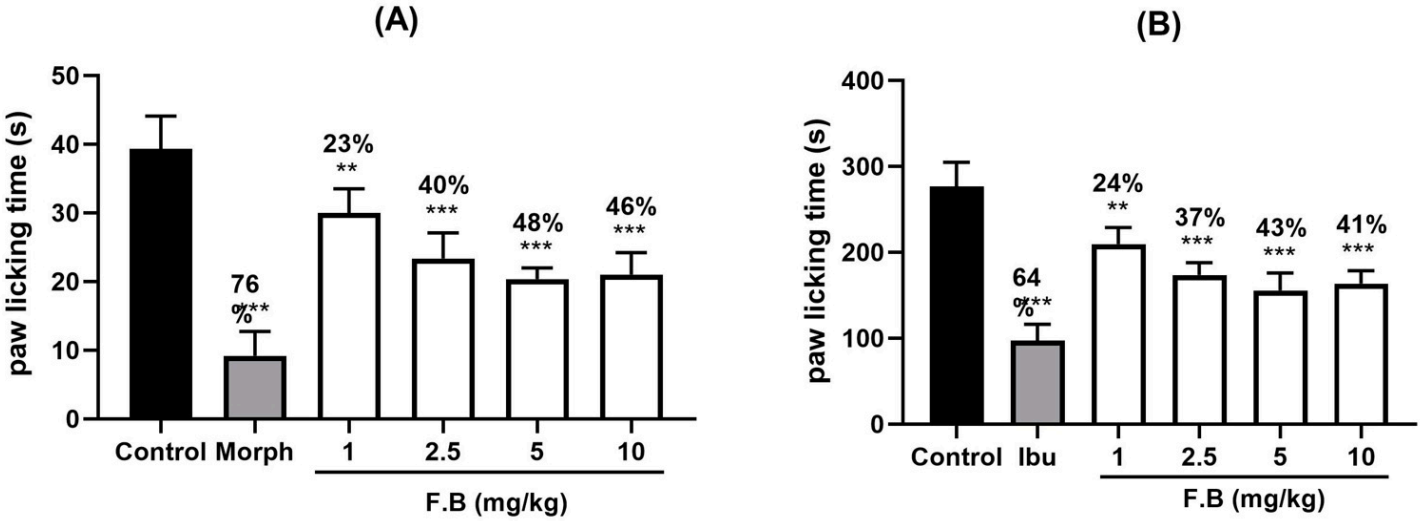


Figure 2

(A) Effect of F.B. (1, 2.5; 5 or 10 mg/kg, i.p.) on formalin-induced nociception (early phase) in mice. (B) Effect of F.B. (1, 2.5; 5 or 10 mg/kg, i.p.) on formalin-induced nociception (late phase) in mice. Control: Control group received only the vehicle used to dilute the compound. Morph (Morphine, 5 mg/kg, s.c.) and Ibu (Ibuprofen, 10 mg/kg, i.p.) were used as positive control. Each column represents the mean ± S.E.M (n = 6/group). Numbers above the bars indicate the percentage of analgesia. One-way ANOVA with post hoc Tukey's test (**p < 0.01 and ***p < 0.001 vs control).

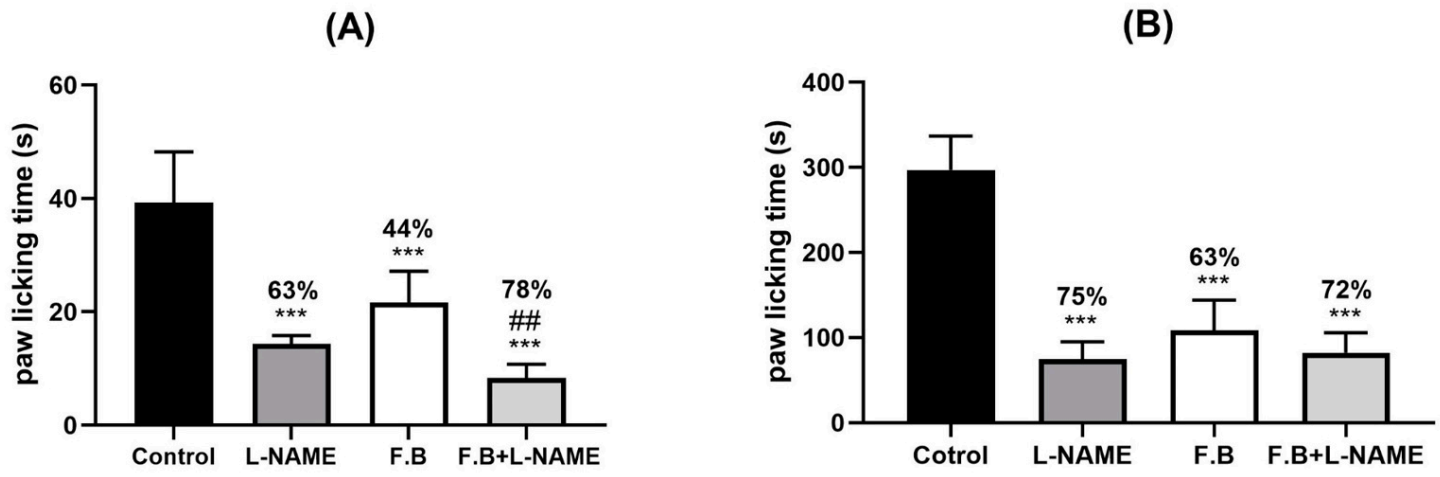


Figure 3

(A) Effect of L-NAME on the antinociceptive activity of F.B. (2.5 mg/kg, i.p.) in the formalin-induced nociception model (early phase). (B) Effect of L-NAME on the antinociceptive activity of F.B. (2.5 mg/kg, i.p.) in the formalin-induced nociception model (late phase). Control group received only the vehicle used to dilute the compound. L-NAME group recieved L-NAME (30 mg/kg, i.p.), F.B.+ L-NAME group recieved F.B. (2.5 mg/kg, i.p.) and L-NAME (30 mg/kg, i.p.). Each column represents the mean ± S.E.M (n = 6/group). Numbers above the bars indicate the percentage of analgesia. One-way ANOVA with post hoc Tukey's test ***$p < 0.001$ vs control, ##$p < 0.001$ vs F.B.+L-NAME).

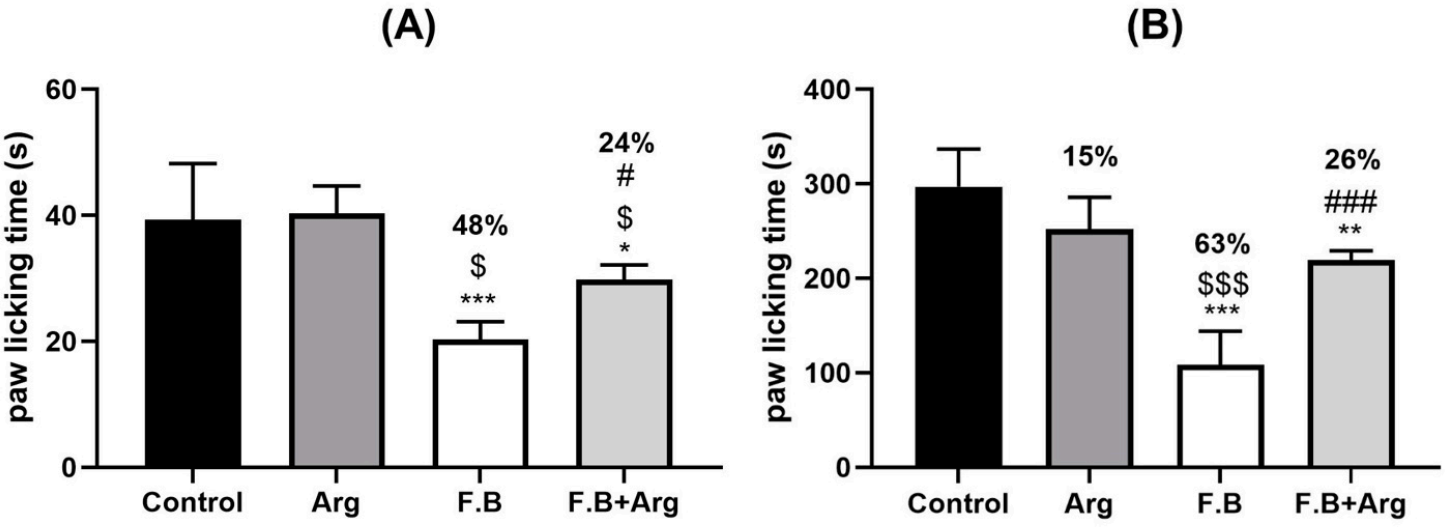


Figure 4

(A) Effect of L-Arginine on the antinociceptive activity of F.B. (2.5 mg/kg, i.p.) in the formalin-induced nociception model (early phase). (B) Effect of L-Arginine on the antinociceptive activity of F.B. (2.5 mg/kg, i.p.) in the formalin-induced nociception model (late phase). Control group received only the

vehicle used to dilute the compound. Arg group recieved L-Arginine (200 mg/kg, i.p.), F.B.+Arg group recieved F.B. (2.5 mg/kg, i.p.) and L-Arginine (200 mg/kg, i.p.). Each column represents the mean ± S.E.M (n = 6/group). Numbers above the bars indicate the percentage of analgesia. One-way ANOVA with post hoc Tukey's test (**p < 0.01 and ***p < 0.001 vs control, $p < 0.05 and $$$p < 0.001 vs Arg, #p < 0.05 and ###p<0.001 vs F.B.).

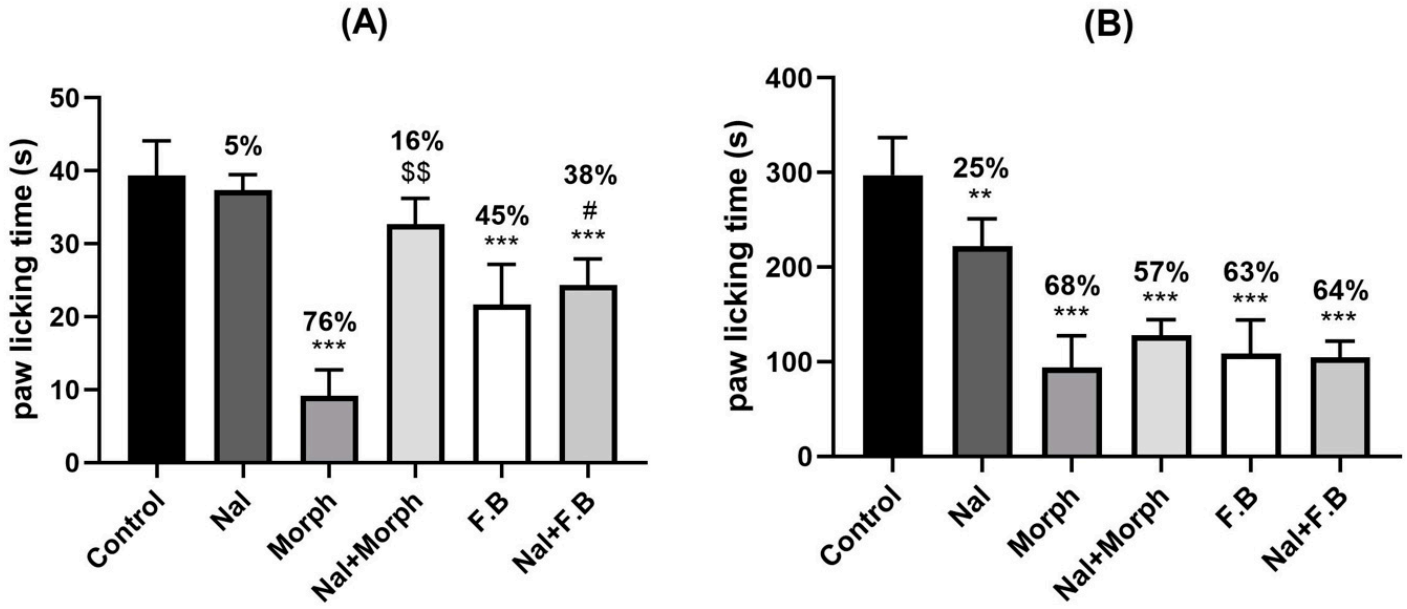


Figure 5

Effect of Naloxone on the antinociceptive activity of F.B. (2.5 mg/kg, i.p.) in the formalin-induced nociception model. Control group received only the vehicle used to dilute the compound. Morph group recieved morphine (5 mg/kg, s.c.) and Nal group recieved naloxone (5 mg/kg, i.p.). Each column represents the mean ± S.E.M (n = 6/group). Numbers above the bars indicate the percentage of analgesia. One-way ANOVA with post hoc Tukey's test (**p < 0.01 and ***p < 0.001 vs control, $$$p$ < 0.01 vs Morph, #p < 0.05 vs Nal+Morph).

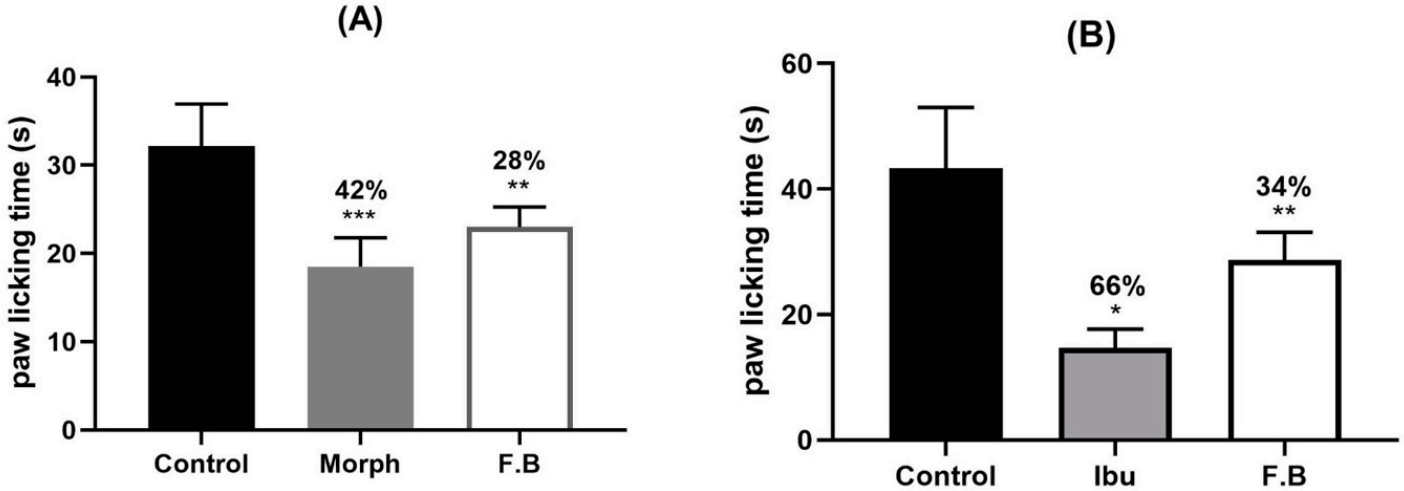


Figure 6

(A) Effect of F.B. (2.5 mg/kg, i.p.) on glutamate-induced nociception (early phase) in mice. (B) Effect of F.B. (2.5 mg/kg, i.p.) on glutamate-induced nociception (late phase) in mice. Control: Control group received only the vehicle used to dilute the compound. Morph (Morphine, 5 mg/kg, s.c.) and Ibu (Ibuprofen, 10 mg/kg, i.p.) were used as positive control. Each column represents the mean ± S.E.M (n = 6/group). Numbers above the bars indicate the percentage of analgesia. One-way ANOVA with post hoc Tukey's test (*p < 0.05, **p < 0.01 and ***p < 0.001 vs control)

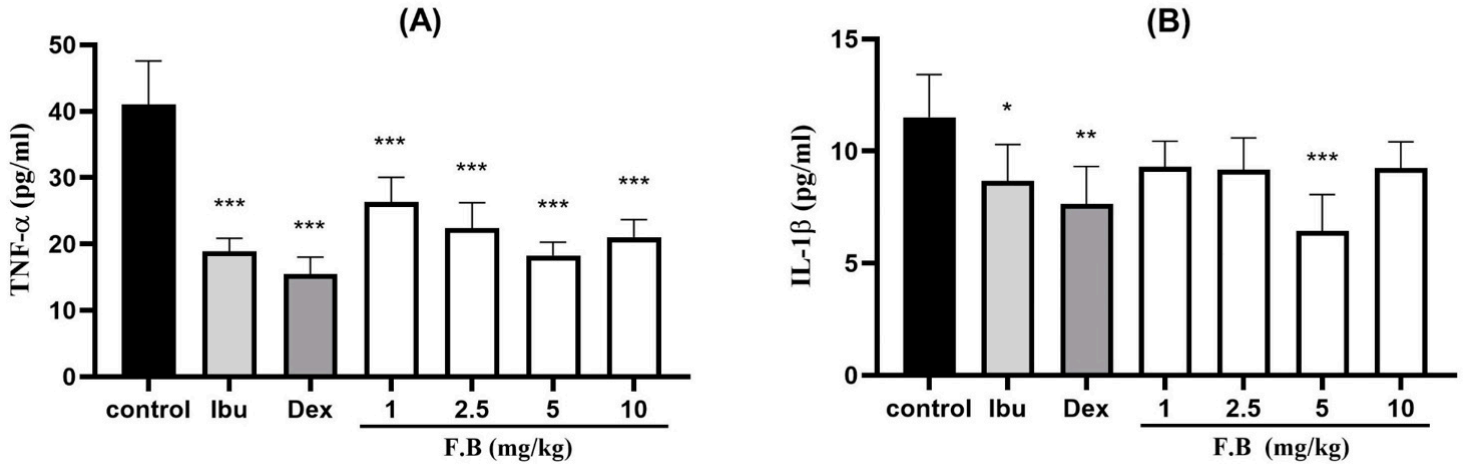


Figure 7

Effect of F.B (1, 2.5, 5, or 10 mg/kg, i.p.) on LPS-induced secretion of inflammatory cytokine TNF-α (A) and IL-1β (B) in mice. The control group received only LPS (10 ng/mL; i.pl.). Ibu (ibuprofen, 10 mg/kg body weight; i.p.) and Dex (dexamethasone, 10 mg/kg body weight; i.p.) were used as positive controls. Each column represents mean ± S.E.M (n = 6/group). (* $p < 0.05$, ** $p < 0.01$, *and* *** $p < 0.001$ vs the control group).

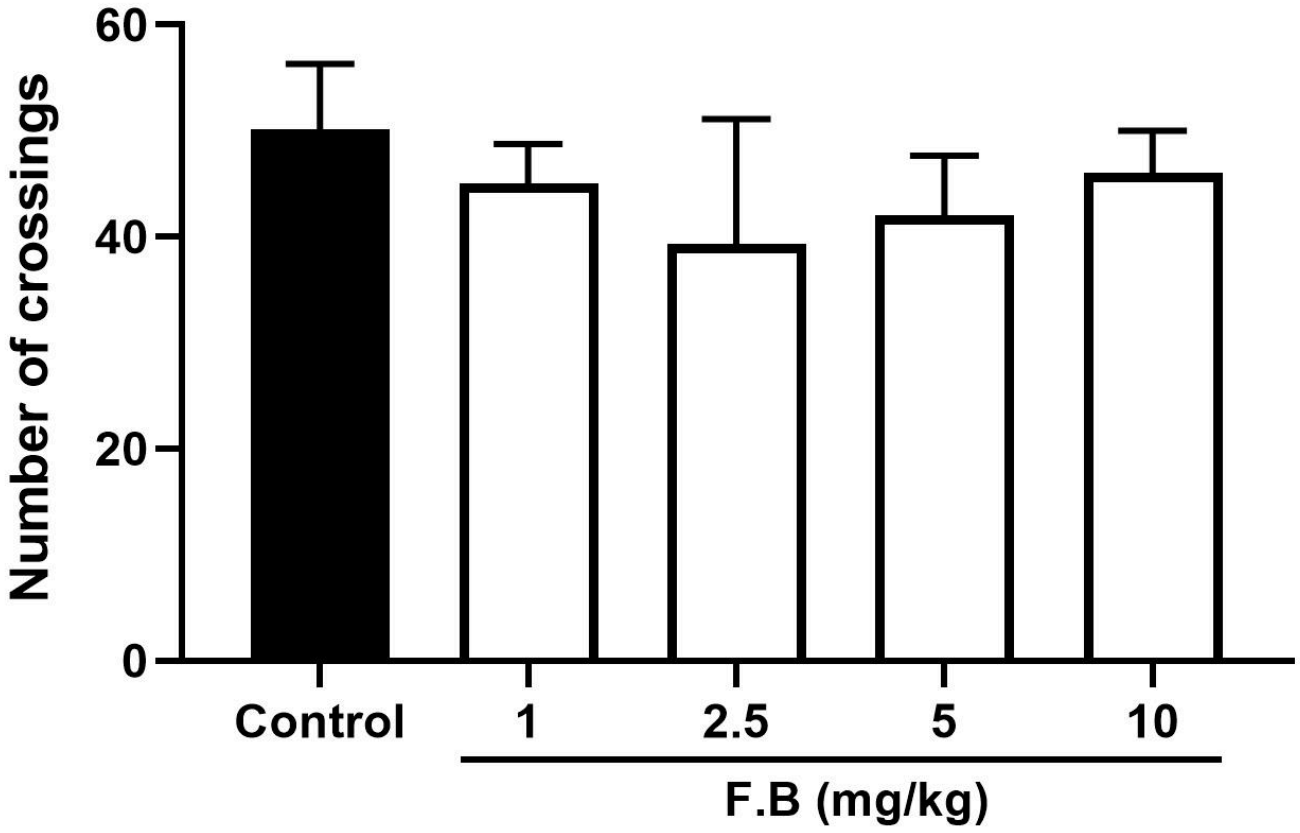


Figure 8

Effect of F.B. (1, 2.5; 5 or 10 mg/kg, i.p.) on locomotor activity of mice (n = 6/group) in the open field test. Control group received only the vehicle used to dilute the compound. Each column represents the mean ± S.E.M (n = 6/group). One-way ANOVA with post hoc Tukey's test.