Glabridin alleviates inflammation and nociception in rodents by activating BKCa channels and reducing NO levels.

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ABSTRACT

Inflammation, and the pain that accompanies it, is a natural response of the body. The licorice plant (*Glycyrrhiza glabra*) have demonstrated anti-inflammatory, anti-edematous, and anti-nociceptive effects of its extracts. The effective ingredient remains unidentified; however, one possibility is the unique isoflavone glabridin. The anti-nociceptive, and anti-inflammatory effects of glabridin and its possible mechanism with focus on the large conductance Ca$^{++}$-activated K$^+$ ($\text{BK}_{\text{Ca}}$) channels and L-Arginine-nitric oxide (NO) pathway were examined by using different tests. In order to determine the anti-edematous, anti-nociceptive, and anti-oxidative effects of glabradin, some tests such as the tail flick, hotplate, carrageenan-induced paw edema, air pouch, acetic-acid-induced writhing, formalin, and capsaicin tests, as well as toxicity and open field tests were made. Glabridin was administered to rats (n = 8) or mice (n = 8) for 3 days at 3 doses (10, 20, and 40 mg/kg). Glabridin inhibited cytokine production and showed an anti-nociceptive response via the activating of BKCa channels and downregulating NO level and partially transient receptor potential vanilloid-1 pathways. It also demonstrated anti-inflammatory effects by inhibiting cyclooxygenase (COX) activity, while showing no cytotoxicity. Glabridin, however, showed no anti-nociceptive effect in the neurogenic phase. Glabridin is a promising substance in terms of its anti-nociceptive and anti-inflammatory effects by disrupting peripheral NO production, inhibiting cGMP activation and activating BKCa channels and its lack of acute and subacute toxic effects.

**Keywords:** Glabridin; nociception; inflammation; large conductance Ca$^{++}$-activated K$^+$; N(ω)-nitro-L-arginine methyl ester; transient receptor potential cation channel subfamily vanilloid 1.
INTRODUCTION

Inflammation is a natural response of the body's immune system to various external stimuli, including those triggered by microorganisms, pathogens, trauma, stress, and chemicals.\(^1\) As a response to stimuli such as toxin and infection, inflammation is generally characterized by microvascular leakage, edema, vasodilation, cell migration to the inflamed site, oxidative stress, and the release inflammatory mediators.\(^2\) Among the cells migrating to the injury site are macrophages, which produce several different mediators, including nitric oxide (NO), histamine, serotonin, tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), leukotrienes, interleukins (especially IL-1 and IL-6), and peptides.\(^3\)–\(^5\) Prostaglandins (PGs) and thromboxanes (TXs) generated by cyclooxygenase (COX) also play a major role in inflammatory reactions.\(^6\)

Pain, which is one symptom of inflammation, is a biological response that protects the body from harmful stimuli. Nociceptors, which convey pain stimuli from the sensory nerve endings to the central nervous system, are specialized receptors that enable the detection of a pain stimulus as the primary pain unit and the transformation of the mechanical and chemical response into electrical conduction.\(^7\) Previous studies have revealed a relationship between nociception and pain that involves the interaction of molecules such as PGs, opioids, acetylcholine, interleukins, and cytokines.\(^8\) One way to prevent inflammation is to hinder the nociceptive effect;\(^9\) therefore, several pain-killer drugs, such as corticosteroids,\(^10\) nonsteroidal anti-inflammatory drugs (NSAIDs),\(^11\) cannabinoid 2 receptor agonists,\(^12\) immunosuppressive drugs,\(^13\) and even plant extracts,\(^8\) have been tested over the centuries by medical community. However, excessive and long-term use of many of these drugs, and especially the NSAIDs and corticosteroids, can have undesired side effects, such as gastrointestinal toxicity, addiction, drug resistance, and induction of Cushing syndrome.\(^14\) For this reason, treatment with active ingredients derived from plant extracts is becoming
popular in consumers and drug companies because of both relatively less side effects and increased medical compliance by consumers.\textsuperscript{15}

In traditional medicine, \textit{Glycyrrhiza glabra} is used to treat many diseases, ranging from coughs and colds to liver and intestinal tract disturbances and even cancer\textsuperscript{16–19} because it contains many active phytochemicals.\textsuperscript{20,21} One of these, the isoflavone glabridin, has demonstrated antioxidant and anti-inflammatory effects,\textsuperscript{22,23} but its mechanism of action has been remained to be unexplored especially the role of the large conductance Ca\textsuperscript{2+}-activated K\textsuperscript{+} (BK\textsubscript{Ca}) channels and L-arginine-nitric oxide-cyclic guanosine monophosphate (cGMP) pathway in pain models. Recently, studies have been conducted on the role of BK\textsubscript{Ca} channels in inflammation and pain transmission.\textsuperscript{24–26} Furthermore, there is evidence that BK\textsubscript{Ca} channels play a role in the relaxation of smooth muscle of glabridin.\textsuperscript{27} However, to date there is insufficient information on the anti-nociceptive and anti-inflammatory mechanism of action of glabridin.

The aim of the current study was to confirm that glabridin alleviates edema and reduces nociceptive and oxidative stress occurring during the inflammatory process in rodents and to uncover its mechanisms. Here we also aim to find out whether BK\textsubscript{Ca} channels have roles in the anti-nociceptive mechanism of glabridin. Therefore, the novelty of this study is due to the fact that BK\textsubscript{Ca} channels have a role in the anti-nociceptive effect of glabridin in various pain and inflammation models in rodent.

MATERIALS AND METHODS

Chemicals

The source of \textgreek{l}-carrageenan, acetic acid, formaldehyde, NS1619 (1,3-dihydro-1-[2-
hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one), iberiotoxin (IbTX), naloxone, diclofenac, L-arginine, methylene blue, acetylsalicylic acid, and dimethyl sulfoxide (DMSO) were Sigma-Aldrich (USA) while capsaicin, capsazepine, N(ω)-nitro-L-arginine methyl ester (L-Name) were obtained from Cayman chemical (Michigan, USA). Glabridin, [(R)-4-(3,4-dihydro-8,8-dimethyl-2H,8H-benzo[1,2-b:3,4-b’]dipyran-3-yl)-1,3-benzenediol] was purchased from Xi’an ZB Biotech Co., Ltd. (Shaanxi, China) and morphine hydrochloride was obtained from Osel İlaç San ve Tic A.Ş. (Turkey) and Deva Holding (Turkey), respectively; these were all 98% purity.

**Experimental animals**

Male Wistar-albino rats weighing 250 ± 10 g were used for tail flick, edema, and air pouch tests. Male BALB/C mice weighing 25 ± 5 g were used for tests such as toxicity, writhing, formalin, open-field, capsaicin, hotplate, and toxicity. The animals were provided by the Experimental Research Center of Adiyaman University. Ethical permission for the study was obtained from the Animal Experiments Local Ethics Committee (Ethics Committee decision no. 2019/028) of Adiyaman University, where the study was conducted. The animals were randomly housed in appropriate cages at 22 ± 2°C under a 12/12 h dark/light cycle with free access to tap water and commercial rat or mouse chow. All procedures were conducted in accordance with the Guide for Care and Use of Laboratory Animals.

**Experimental Design**

After the rats and mice were randomly divided into 9 series of 8 animals per group, they were taken to the laboratory where the experiments were to be performed and kept for four days for acclimatization. The groups were designated as shown in Figure 1.
administration of the drugs and the plans of the tests are indicated in Table 1.

**Acute toxicity test**

The acute toxicity effects of glabridin were evaluated by determining the LD$_{50}$ of glabridin by administering a range of doses from 100 to 400 mg/kg in male mice (n=8). The highest doses of glabridin were calculated based on the Guidelines for the Organization for Economic Cooperation and Development for testing chemicals.\(^{28}\)

Sub-chronic toxicity was evaluated by administering glabridin doses of 100, 200, and 400 mg/kg for 14 days in male mice (n=8) via intraperitoneal (i.p). The same procedure was performed by giving saline to the control group (n=8).

**Anti-nociception experiments**

*Tail flick test in rats*

The tail flick test was performed to measure the anti-nociceptive activity of glabridin at the spinal level.\(^{29}\) Briefly, the tail of an immobilized rat was restrained on the tail flick apparatus and a beam of light was focused on the tail as a radiant heat source. The tail-flick latency time was recorded manually. The source of heat-producing light was adjusted to give fundamental delays of 2 to 4 s without change. Animals whose baseline latency was outside the predetermined limits were excluded from the experiments. A cut-off time of 20 s was used to minimize injury to the animals. The latency times were measured at intervals of 30 min for a total of 8 measurements. The first measurement, called baseline time, was performed before any substance was given to the animals. The rats were administered vehicle (vehicle; 1% DMSO in distilled water, i.p. 0.2 ml volume: to solve glabridin), glabridin (10,
20, or 40 mg/kg, i.p), and other substances as illustrated Table 1 and below, 15 min prior to the tail flick test. The average of these measures was designated as the baseline as latency time (s: second). The maximum possible effect was expressed as a percentage which was given in text. In brief, the percentage of the maximum response (MPE) was calculated by dividing the difference between the post-drug latency and pre-drug latency by the difference between the cut-off period and the pre-drug latency and then multiplying by 100 (%MPE = [(reaction time of test − basal reaction time)/ (cut-off time-basal reaction time)]*100).

*Hotplate test in mice*

The basic purpose of the hotplate test was to measure nociceptive reactions, such as withdrawal of paws, jumping, or forepaw licking, at specific time intervals (0, 15, 30, 60, 90, 120, 150 and 180 min) after placement of mice on the surface of a hotplate device (UGO Basile) heated at a constant temperature of 55.0 ± 0.5°C. A cut-off contact time of 20 s was used to indicate complete analgesia and to avoid any paw tissue injury. The mice were administered vehicle, glabridin (10, 20, or 40 mg/kg, i.p), and other substances as mentioned below, 15 min prior to the hotplate test. MPE was calculated as described above.

*Acetic-acid-induced writhing test in mice*

The peripheral anti-nociceptive effects of glabridin were examined using the widely used acetic-acid-induced writhing test. This test records the number of abdominal contractions or writhing movements made by the animal after intraperitoneal injection of acetic acid. Although this test is nonspecific in terms of determining anti-nociceptive activity, it is considered useful as a screening test. For this purpose, mice were given i.p. 1% acetic acid (10 ml/kg) after other substances were administered. The mice were then placed in transparent cages and their responses were observed for 30 min. The mice were administered
vehicle, glabridin (10, 20, or 40 mg/kg, i.p), and other substances as mentioned Table 1 and Figure 1, 15 min prior to acetic acid injection. MPE was calculated as described above (showed in text).

Formalin test in mice

This test was used to demonstrate inflammatory and neurogenic pain. Briefly, after daily administration of glabridin for 3 days or immediately after administration of other substances as illustrated Table 1, 50 µl of diluted formalin (2.5% in saline) was administered to the right hind paw. The mice were then placed into the test container and the number of instances of licking the injected paw was recorded. Two distinct periods of intensive licking activity were identified and scored separately. The first period measured the neurogenic activity between 0 and 5 min after formalin injection, while the second period measured the inflammatory activity between 15 and 30 min after formalin injection.

Capsaicin test in mice

This test determines the number of licks over a 5 min period following capsaicin injection. This approach was based on a previously described model. Briefly, mice were given glabridin (10, 20, and 40 mg/kg, i.p) for 3 days or vehicle or saline 15 min prior to capsaicin (the transient receptor potential vanilloid agonist [TRPV]; 20 µmol/kg,10µl, intraplantar, i.pl) injection. To determine any TRPV involvement, mice were co-administered capsazepine (TRPV receptor antagonist; 50 µg/ml; 10 µl i.pl.) with capsaicin.

Involvement of opioid pathway

The possible involvement of opioid receptors was tested by administering naloxone (5 mg/kg, subcutaneously: s.c: non-selective opioid receptor antagonist) 15 minutes before the administration of morphine (5 mg/kg, s.c) or glabridin (40 mg/kg) in the tail flick, hot plate,
2.2.1. Involvement of \( BK_{Ca} \) channel

The possible involvement of \( BK_{Ca} \) channels was tested by administering IbTX (0.7 nmol/kg, i.p \( BK_{Ca} \) channels blocker) 15 minutes before the administration of NS1619 (10 mg/kg, i.p, \( BK_{Ca} \) channels opener) or glabridin (40 mg/kg) in the tail flick, hot plate, acetic acid-induced writhing test, and formalin tests. The doses of drugs were based on previously studies. \(^{26,31,36}\)

Involvement of L-arginine-nitric oxide pathway

The possible involvement of nitric oxide pathway was tested by administering L-arginine (NO precursor; 100 mg/kg, i.p) 15 minutes before the administration of L-Name (non-selective NO-synthase inhibitor; 30 mg/kg, i.p) or glabridin (40 mg/kg) in the acetic acid-induced writhing test, and formalin tests. The doses of drugs were based on previously study. \(^{37}\)

Involvement of cGMP pathway

The possible involvement of cGMP pathway was tested by administering methylene blue (a guanylate cyclase inhibitor; 20 mg/kg, i.p) 15 minutes before the administration of acetic acid in the acetic acid-induced writhing test. \(^{37}\)

Anti-inflammatory activity

Carrageenan-induced paw edema in rats

This procedure was conducted on rats as described previously\(^1\) with minor modification. \(^{12}\) Briefly, inflammation was induced by giving an i.pl. injection of carrageenan (100 µl, 1% w/v) into the right paw (n = 8). Control animals received a saline injection. Three days before carrageenan administration, three mouse groups were treated daily with glabridin
at doses of 10, 20, or 40 mg/kg. Edema was expressed as the increase in paw thickness (mm) after carrageenan injection relative to the pre-injection value for each animal. The basal thickness was determined by measuring the paw with electronic digital calipers just before giving the carrageenan and then at 0, 1, 2, 3, 4, 5, and 6 h after administering carrageenan; this time range corresponds to the peak edema time.

Leukocyte migration, cytokine production, and PGE2 levels determined by the air pouch test in rats

The aim of this test was to evaluate the effects of glabridin on leukocyte migration, cytokine production, and PGE2 levels, as described previously. Briefly, a region of the dorsum of the rat was injected at a single point with 20 ml sterile air from a filled syringe on the first day. This process was repeated, but with a 10 ml volume, for the next 2 days. Glabridin (at 10, 20, or 40 mg/kg) was also administered on these 3 days, before the air application step. The animals were given the test substances on the fourth day and then all animals, except for the control group, were treated with carrageenan (1 mL, 1% w/v) subcutaneously where the air had been injected. Six hours later, after sacrificing the animals, the subcutaneous cavity was washed with 5 ml of phosphate buffer solution containing 20 IU/ml heparin, 130 mM NaCl, 5 mM Na₂PO₄, and 1 mM KH₂PO₄ in distilled water adjusted to pH 7.4 and the exudate was collected. The materials in the cavity were collected through an incision and used to measure the levels of leukocytes, PGE2, and cytokines (e.g., TNF-α). The numbers of leukocytes were counted with an optical microscope. TNF-α and PGE2 levels in the exudates were measured with enzyme-linked immunoassay (ELISA) kits (abcam ab46070 and ab133021, respectively) following the manufacturer’s guidelines. Cell-free supernatants were obtained by centrifugation of the cavity materials at 10,000 g for 10 min at 4 °C and used for the ELISA measurements. All samples were assayed in duplicate, and
equivocal results were repeated.

*Involvement of L-arginine-nitric oxide pathway*

The possible involvement of nitric oxide pathway was tested by administering L-arginine 15 minutes before the administration of L-Name or glabridin (40 mg/kg) in the carrageenan-induced paw edema, and air pouch tests.

*Involvement of COX pathway*

The possible involvement of COX pathway was tested by administering acetylsalicylic acid (60 mg/kg), or diclofenac (2 mg/kg, i.p) 15 minutes before the administration of carrageenan in the carrageenan-induced paw edema, and air pouch tests.

*Open field test*

The aim of this test was to evaluate the effect of glabridin on the behavior of mice, such as sensuality and mobility. Prior to the experiments, the animals underwent training for 5 days as described previously. The movements of the mice on a flat surface, divided into 4 compartments with transparent plastic, were recorded with a video observation computer program at 5 min intervals.

The mice received a 40 mg/kg dose of glabridin daily for 3 days or they received diazepam (a central nervous system depressant, 1 mg/kg, i.p.) or vehicle administered i.p. 15 min before being placed on the test surface to determine any possible effects of glabridin on animal mobility and emotionality.

*Statistical analysis*

All statistical analyses were carried out using GraphPad prism 7.01 statistical software. All data were presented as mean ± standard deviation. The difference between groups was
compared using one-way ANOVA for the air pouch, acetic acid, and capsaicin tests, and using two-way ANOVA for the tail flick, hotplate, paw edema, formalin, and open field tests, followed by Bonferroni's multiple comparisons test. P < 0.05 was considered statistically significant.

RESULTS

Anti-nociceptive effects of glabridin

Tail flick test in rats

Figure 2a shows the results of the tail-flick latency test, which measures predominantly spinal-mediated anti-nociceptive effects. When compared to the basal latency, no significant increase in latency was observed following administration of glabridin at 10 mg/kg. However, glabridin at 20 mg/kg caused an increase in latency time at 15, 30, 60, and 90 minutes (P = 0.01, 0.002, 0.001 and 0.01, respectively), while a 40 mg/kg dose caused an increase in latency time at 15, 30, 60, 90, 120, and 120 minutes (P<0.001, < 0.001, <0.001, <0.001, =0.001, and =0.01, respectively), which peaked at 40 mg/kg (43.03% maximum possible effect) at 30 min, when compared to their basal latency time.

Hotplate test in mice

The hotplate test is another commonly used test of anti-nociceptive effects at the spinal level. The data are expressed as MPE%, and an anti-nociceptive effect is indicated by an increase in the latency time. As illustrated Figure 3a, glabridin at 10 mg/kg did not significantly increase the latency time when compared with the baseline. However, glabridin at 20 mg/kg caused a moderate increase in latency time at 15, 30, 60, 90 and 120 min (P<0.001, <0.001, <0.001, <0.001 and =0.04, respectively), while a 40 mg/kg dose caused an
increase in latency time at 15, 30, 60, 90, and 120 minutes (P<0.001).

**Glabridin effects on the acetic acid-induced writhing test in mice**

As shown Figure 4a, 20 and 40 mg/kg doses of glabridin led to reduce of the writhing induced by acetic acid treatment by 24.49% and 61.59%, respectively, while the 10 mg/kg dose of glabridin was not sufficient to reduce the number of writhing.

**Glabridin effects on the timing of formalin-induced paw licking in mice**

Figure 5a shows that formalin injection into the paws of mice caused the mice to lick their paws and to significantly increase the amount of time they spent licking their paws over both neurogenic and inflammatory phases. Glabridin did not reduce the time spent by mice to lick their paws in the neurogenic phase compared to vehicle group, whereas intermediate and high doses of glabridin (20, 40 mg/kg) caused this time to decrease in the inflammatory phase by 37.44%, 68.02%, respectively).

**Glabridin effects on the capsaicin test in mice**

Figure 6 shows that capsaicin injection into the paws caused mice to lick their paws and clearly increase the time they spent licking their paws over a period 5 min when compared to mice injected with saline. Capsazepine administration (50 µg/ml; 10 µl i.pl.) and glabridin (40 mg/kg) significantly reduced the time spent by mice to lick their paws by 47.90% and 38.81%, respectively, while the intermediate (20 mg/kg) and lowest (10 mg/kg) doses of glabridin did not change the amount of time the mice spent licking their paws when compared with the vehicle group.

**Elucidation of anti-nociceptive mechanism of glabridin in mice and rats**
Involvement of opioidergic system

As illustrated Figure 2b, 3b, and 5b, glabridin (40 mg/kg) and morphine when administered alone and together, both significantly inhibited nociceptive effect in the hot plate, tail flick, and formalin (in inflammatory phase for glabridin) tests. The pretreatment with 5 mg/kg doses of naloxone did not inhibit the anti-nociceptive effect of glabridin in that tests but inhibited the anti-nociceptive effect of morphine. It showed that opioidergic system has no role in the anti-nociceptive effect of glabridin.

Two important tests for evaluation of the possible involvement of spinal mechanisms in anti-nociception are the tail flick and hot plate models. Both of these tests, which are considered to be selective for the detection of central analgesia, analyze the response to a centrally acting anti-nociceptive effect without affecting thermal stimuli by the use of peripherally acting agents. The results of hot plate and tail flick tests revealed that intraperitoneal administration of the 20 and 40 mg/kg doses of glabridin in rats and mice showed dose-dependent analgesic effect. In contrast, the 10 mg/kg dose of glabridin was unable to inhibit the nociceptive response due to low bioavailability.

Involvement of $\text{BK}_{\text{Ca}}$ channels

As illustrated Figure 2c, 3c, 4b, and 5c, glabridin (40 mg/kg) and NS1619 ($\text{BK}_{\text{Ca}}$ channel opener) when administered alone and together, both significantly inhibited nociceptive effect in the tail flick, writhing, hot plate, and formalin (in inflammatory phase only) test. The pretreatment with IbTX ($\text{BK}_{\text{Ca}}$ channels blocker, 0.7 nmol/kg, i.p) inhibited the anti-nociceptive effect of glabridin and of NS1619 in that tests. Inhibition of the analgesic effect of glabridin in pain tests with IbTX proves that glabridin has a role in $\text{BK}_{\text{Ca}}$ channels.

Involvement of l-arginine-nitric oxide, cGMP pathway
As illustrated Figure 4c and 5d, glabridin (40 mg/kg, i.p) and L-Name (nitric oxide synthase inhibitor) when administered alone and together, both significantly inhibited nociceptive effect in the writhing, and formalin (in inflammatory phase for glabridin) test. The pretreatment with L-arginine (nitric oxide precursor) inhibited the anti-nociceptive effect of glabridin and of L-Name in both tests.

The involvement of cGMP pathway (Figure 4d) was analyzed with the pre-treatment of a guanylate cyclase inhibitor, methylene blue. When methylene blue was administered alone or together with glabridin, both significantly reversed the acetic acid-induced abdominal writhing of mice.

Glabridin administration at writhing and formalin tested doses decreased the number of writhing movements and promoted a dose-dependent anti-nociceptive activity. Furthermore, the administration of BKCA channel opener, L-Name, and methylene blue inhibited writhing behavior in mice. However, the inhibitory effect of glabridin on the mouse writhing behavior was reversed by both IbTX and L-Arginine. The probable results of this test show that the anti-nociceptive effect of glabridin is not mediated by opioid receptors but is mediated by BKCA channels and the L-arginine-NO pathway.

Anti-inflammatory effects of glabridin

Glabridin effects on the carrageenan-induced paw edema in rats

Figure 7a shows the results for evaluation of anti-inflammatory effect of glabridin using the carrageenan-induced paw edema model in rats. At 15 min after intraperitoneal injection of saline, or, vehicle, diclofenac (2 mg/kg, i.p) or glabridin (10, 20, and 40 mg/kg), the i.pl. injection of carrageenan caused a time-dependent increase in paw thickness, which
was maximal at 4 h. The 10 mg/kg dose of glabridin did not cause any decrease in paw thickness, while the 20 and 40 mg/kg doses of glabridin caused a significant decrease in paw thickness. The maximum anti-inflammatory activity achieved with the higher doses of glabridin was observed at 4 h. Diclofenac at 2 mg/kg achieved notable anti-inflammatory activity at all times points.

Figure 7b showed that paw thickening caused by carrageenan in rat paw was reduced 43.02% by glabridin (40 mg/kg), and 53.89% by L-Name, at 4 h. Pre-treatment with L-arginine reversed the reduction of paw thickness by glabridin and by L-Name.

Glabridin effects on leukocyte migration, cytokine production, and PGE2 levels determined by the air pouch test in rats

Table 2 shows that the administration of carrageenan into the air pouch led to the accumulation of leukocytes in the exudate obtained from the air pouch (vs saline group). This increase in leukocyte migration in the exudate was reduced 54.50% by glabridin (40 mg/kg, i.p.), 80.14% by diclofenac (2 mg/kg, i.p.), 84.69% by acetylsalicylic acid, 71.82% by L-Name, and 81.31% by combined L-Name with glabridin. Pre-treatment with L-arginine reversed the reduction of leukocyte migration by glabridin and by L-Name.

Table 2 shows that carrageenan administration to the air pouch significantly increased TNF-α production (322.21%) when compared to the saline control group. The 10 mg/kg dose of glabridin did not cause any changes in TNF-α production, whereas the 20 and 40 doses of glabridin, 2 mg/kg dose of diclofenac, 60 mg/kg dose of acetylsalicylic acid, 30 mg/kg dose of L-Name, and combined L-Name with glabridin (40 mg/kg) significantly reduced TNF-α production (by 29.72%, 40.79%, 71.52%, 73.68%, 55.49%, and 67.41%, respectively). Pre-treatment with 100 mg/kg dose of L-arginine reversed the reduction of TNF-α level by
glabridin and by L-Name.

Carrageenan administration to the air pouch significantly increased PGE2 production (407.90%) when compared to the saline control group (P<0.001). The lowest dose of glabridin did not cause any changes in PGE2 production, whereas the middle and highest dose of glabridin, diclofenac, acetylsalicylic acid, L-Name, and combined L-Name with glabridin (40 mg/kg) significantly reduced PGE2 production (by 19.57%, 44.43%, 60.97%, 62.76%, 54.89%, and 57.70%, respectively). Pre-treatment with L-arginine reversed the reduction of PGE2 level by glabridin and by L-Name (Table 2).

**Glabridin effects on the open field test in mice**

Figure 8 shows the responses of mice given the 40 mg/kg dose of glabridin or a 1 mg/kg dose of diazepam 15 min before the 5 min open field test. Glabridin has been shown not to cause behavioral changes, such as emotionality or alterations in motor activity. In addition, in the present study, statistically significant differences were found between the control and diazepam groups in terms of mobility (p=0.03) as well as depressant effect (p<0.001). Statistically significant differences were also noted between diazepam and glabridin in terms of their effects on mobility (p=0.009) and depressant effect (p<0.001).

**Acute toxicity test**

As illustrated Table 3, no evidence of toxicity or behavioral abnormalities, including excitability and/or sedation, was observed in the animals at these doses. No mortality or physical changes, such as convulsion, hyperactivity, respiratory rate, loss of reflex, sedation, changed food intake, loss of body weight, or allergic reactions, were observed after i.p. administration of glabridin. These results indicated that glabridin had no acute toxicity at doses up to 400 mg/kg.
DISCUSSION

Glabridin, an isoflavonoid, is one of the active components of the licorice. Although the anti-inflammatory properties of glabridin have been mentioned in previous literature,23) no comprehensive study has yet been conducted to explain the mechanism of these effects of glabridin in rodents. In view of the increasing popularity of plant-derived drugs in recent years, the novelty of the present study is that provided more pharmacological information on the mechanisms by which glabridin mediates its anti-nociceptive and anti-inflammatory effects.

The results presented here confirm that glabridin demonstrates both anti-nociceptive and anti-inflammatory activities in well-organized pain and inflammatory models in both rats and mice. Glabridin produced an analgesic effect by decreasing the time course of the pain threshold or reaction time in the hotplate and tail flick tests, and it also displayed anti-nociceptive effects by decreasing the mean number of writhing movements in response to acetic acid and the duration of paw licking in the formalin and capsaicin tests. Glabridin administration reduced pro-inflammatory cytokine production, PGE2 production, and leukocyte migration in the exudate obtained from the air pouch, and it reduced paw edema in the carrageenan test. Glabridin did not affect mouse mobility, nor did it cause any behavioral changes in the open field test,

The glabridin effects reflected those achieved by administration of L-Name (a nitric oxide synthase inhibitor), and morphine, which is consistent with the data reported by previously studies.26,30,37) These results suggest that BKCa channels and L-arginine-NO pathway may be the primary site exhibiting the analgesic effect of glabridin, but opioid receptors may not be involved in the anti-nociceptive effect of glabridin.
In the first pain phase of the formalin test, called the neurogenic phase, the C-fiber nociceptors, as well as the type A fibers, are directly stimulated by the release of substance P and NO.\textsuperscript{42)} In the second phase, called the inflammatory phase, pro-inflammatory mediators, such as histamine, substance P, and PGs, which interact with the nociceptors, are released.\textsuperscript{43)} Glabridin showed anti-nociceptive effect in only inflammatory phase.

Capsaicin binds to TRPV, which causes its opening and calcium entry, thereby activating nociceptors.\textsuperscript{44)} TRPV agonists, such as capsaicin, induce nociception by releasing algesic chemical mediators, such as substance P, while TRPV antagonists, such as capsazepine, exhibit an anti-nociceptive effect by inhibiting the secretion of these algesic agents, resulting in the inhibition of chronic pain\textsuperscript{45).} The anti-nociceptive effect of glabridin via TRPV channels can be indirect. Namely, Huang Y et al\textsuperscript{46)} showed that Freund's adjuvant-induced nociceptive effect is transmitted through dorsal horn lamina I neurons in rats. Chen et al\textsuperscript{25)} showed that BK\textsubscript{Ca} channels are suppressed in dorsal horn neurons, especially lamina I. The results of this study led us to hypothesize that by activating the BK\textsubscript{Ca} channels by glabridin, hyperpolarizing neurons in lamina I, reducing the excitability of neurons.

The i.pl. injection of carrageenan resulted in an immediate and intense plasma extravasation and vasodilation as a result of release of serotonin, histamine, COX1 and COX2 in the carrageenan-induced paw edema model. The serotonin and histamine are released after carrageenan administration, while COX1 and COX2 play a key role in the regulation of inflammation after carrageenan injection.\textsuperscript{12,47)} Moreover, El-Ashmawy et al indicated that glabridin reduced nitric oxide content and inducible nitric oxide synthase gene expression in the rat colon.\textsuperscript{48)} The results of the present study show that glabridin inhibited paw edema to a similar extent as observed with diclofenac and L-Name.

Various cytokines and mediators also influence the accumulation of plasma proteins.
and white blood cells, especially lymphocytes, at inflammatory sites. The air pouch is an important model for testing the accumulation of lymphocytes, PGs, TNF-α, and IL-1β, at an inflamed site. TNF-α, which is activated lymphocytes, is the main actors in the acute phase of inflammatory pain and inflammation. It also influences apoptosis and the production of eicosanoids, glucocorticoids, platelet activating factor, and PGE2, stimulates the expression or release of adhesion molecules and is involved in the activation of coagulation. In the present study, lymphocyte migration to the inflamed area was inhibited by glabridin, diclofenac, and acetylsalicylic acid, whereas pre-administration of L-Arginine inhibited the anti-migration effect of glabridin. The production of various cytokines is influenced by PGE2 and other cyclooxygenase metabolites. The evidence for this is that exogenously added PGE2 has no effect on the release of IL-1β, but it reduces IL-6 and TNF-α release. By contrast, treatment of mouse peritoneal macrophages with PGE2 can increase IL-6 release, but not that of IL-1 or TNF-α. In the present study, we demonstrated that glabridin administration induces a dose-dependent inhibition of PGE2 synthesis and release of TNF-α in the air pouch exudate.

Previous work has led to the assumption that NO has common physiological effects, such as nociception and anti-nociception, and that the underlying cause of these effects is an increased level of intracellular cGMP that activates soluble guanylyl cyclase. Glabridin reduced the number of writhing movements. Glabridin may therefore interact with COX2, rather than COX1. The combination of L-Arginine and glabridin inhibited the anti-nociceptive effect of glabridin. Therefore, a relationship may exist between glabridin and L-Arginine-NO pathway.

By stimulating nociceptors by pain, the pain signals are conveyed to the laminated I-II of the spinal cord by Aδ and C fibers of the dorsal root ganglion (DRG). When nociceptive
stimuli are conducted to laminate I-II of the spinal cord, calcitonin gene-related peptide (CGRP) and substance P (SP) are secreted from these nerve endings. Serotonin, norepinephrine, dopamine, opioids in the central terminals of the DRG neurons and the spinal cord neurons, B-type natriuretic peptide (BNP) negatively regulate the excitatory synaptic transmission of nociceptive signaling.\textsuperscript{54} Recently studies suggest that the role of BK\textsubscript{Ca} channels in pain transmission has a crucial role. Zhang et al showed that BNP inhibited the excitability of small DRG neurons by activating the BK\textsubscript{Ca} channels in formalin test\textsuperscript{42} and they also showed that the blocking effect of BNP on pain signals was abolished by the cGMP-dependent protein kinase (PKG) inhibitor in the mouse inflammatory pain. The results of this study show that the anti-nociceptive and anti-inflammatory effects of glabridin are not only mediated by BK\textsubscript{Ca} channels, but also mediated in the NO/cGMP pathway. Consistent with the results of this study, Lu et al noted that chronic inflammatory pain reduced hypersensitivity with the activation of BK\textsubscript{Ca} channels with NS1619, BK\textsubscript{Ca} channels opener.\textsuperscript{26} Moreover, they also noted that in the BK\textsubscript{Ca} channels knockout mice, the nociceptive effects of animals increased in inflammatory pain tests, but did not cause any change in the pain threshold in acute nociceptive tests, which supports the results of the formalin test of our study. In contrast, the results of our study showed that glabridin has anti-nociceptive effect in tail flick, hot plate, acetic acid induced, and capsaicin tests including inflammatory phase of formalin test. Consistent with the results of this study Liu CY et al\textsuperscript{55} noted that in order to understand the effect of BK\textsubscript{Ca} channels on nociceptive, sham and a chronic constriction injury of infraorbital branch of trigeminal nerve (ION-CCI) groups in rats were compared and significant down-regulation of BK\textsubscript{Ca} channels was noted in the ION-CCI group. In addition, NS1619, a BK\textsubscript{Ca} channel opener, has been shown to increase the mechanical pain threshold as a result of target injection into the trigeminal ganglion, which was blocked by the BK\textsubscript{Ca} channel inhibitor iberiotoxin. Glabridin decreases the number of acetic acid-induced writhing
by L-Name (NO synthase inhibitor) and methylene blue (cGMP inhibitor), and in the formalin test, glabridin decreases the licking time by L-Name, the mechanism of action of glabridin can be associated not only with activation of BK_Ca channels but also inhibition of the NO/cGMP pathway.

In this study, although no data was investigated regarding the NO / cGMP pathway mediating the thermal nociceptive effects of glabridin, with the information gathered above, it supports our hypothesis that the anti-nociceptive effect of glabridin is not only mediated by BK_Ca channels but also mediated in the NO/cGMP pathway.

The open field test was performed to determine whether glabridin administration affected motor impairment and emotionality. This determination was important because any effect of glabridin on motor activity or emotionality could affect the performance in several of the tests, including the formalin, tail flick hotplate, acetic acid, and capsaicin tests. The results of the open field test clearly demonstrated that glabridin does not affect motor activity or have any depressant effect.

CONCLUSION

The results of the present study show that glabridin is a promising substance in terms of its anti-nociceptive and anti-inflammatory effects. The mechanisms mediating these effects of glabridin can be 1) interruption of peripheral NO production 2) inhibition of cGMP activation 3) activation of BK_Ca channels. In addition, the use of glabridin at ten times the highest dose used in the toxicity test described here does not cause any behavioral changes in animals, nor does it cause macroscopic changes in major organs, such as the liver, kidney, and brain (data not shown), indicating that it is safe to use.
Conflict of Interest

The authors declare no conflict of interest.
REFERENCES

1) WINTER CA, RISLEY EA, NUSS GW. Carrageenin-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. *Proc. Soc. Exp. Biol. Med.*, **111**, 544–7 (1962).

2) Adnan M, Uddin Chy MN, Kamal ATMM, Barlow JW, Faruque MO, Yang X, Uddin SB. Evaluation of anti-nociceptive and anti-inflammatory activities of the methanol extract of Holigarna caustica (Dennst.) Oken leaves. *J. Ethnopharmacol.*, (2019).

3) Hunter CA, Jones SA. IL-6 as a keystone cytokine in health and disease. *Nat. Immunol.*, **16**, 448–457 (2015).

4) Palomo J, Dietrich D, Martin P, Palmer G, Gabay C. The interleukin (IL)-1 cytokine family – Balance between agonists and antagonists in inflammatory diseases. *Cytokine*, **76**, 25–37 (2015).

5) van Ryn J, Trummlitz G, Pairet M. COX-2 selectivity and inflammatory processes. *Curr. Med. Chem.*, **7**, 1145–61 (2000).

6) Gaddi A, Cicero AFG, Pedro EJ. Clinical perspectives of anti-inflammatory therapy in the elderly: the lipoxigenase (LOX)/cyclooxygenase (COX) inhibition concept. *Arch. Gerontol. Geriatr.*, **38**, 201–12 (2004).

7) Peana AT, D’Aquila PS, Chessa ML, Moretti MD., Serra G, Pippia P. (−)-Linalool produces antinociception in two experimental models of pain. *Eur. J. Pharmacol.*, **460**, 37–41 (2003).

8) Tao X, Ma L, Mao Y, Lipsky PE. Suppression of carrageenan-induced inflammation in vivo by an extract of the Chinese herbal remedy Tripterygium wilfordii Hook F.
9) Sneddon LU. Comparative Physiology of Nociception and Pain. *Physiology, 33*, 63–73 (2017).

10) Gentry C, Melarange R, Durie M, Moore G, Spangler R. Effect of Nabumetone, Diclofenac, Ibuprofen and an Anti-Inflammatory Corticosteroid, Dexamethasone, on Cartilage Metabolism in a Biochemically-Induced Model of Osteoarthritis. *Clin. Drug Investig., 11*, 49–59 (1996).

11) Smith MJH, Ford-Hutchinson AW, Walker JR, Slack JA. Aspirin, salicylate and prostaglandins. *Agents Actions, 9*, 483–487 (1979).

12) Parlar A, Arslan SO, Doğan MF, Çam SA, Yalçın A, Elibol E, Özer MK, Üçkardeş F, Kara H. The exogenous administration of CB2 specific agonist, GW405833, inhibits inflammation by reducing cytokine production and oxidative stress. *Exp. Ther. Med., 16*, 4900–4908 (2018).

13) Guo J, Wang W, Yao L, Yan F. Local Inflammation Exacerbates Cyclosporine A-Induced Gingival Overgrowth in Rats. *Inflammation, 31*, 399–407 (2008).

14) Hodgson E. The effects of corticosteroids and nonsteroidal anti-inflammatory drugs, including aspirin, on coagulation. *South African Fam. Pract., 57*, 9–12 (2015).

15) Ekor M. The growing use of herbal medicines: Issues relating to adverse reactions and challenges in monitoring safety. *Front. Neurol., 4 JAN* (2014).

16) Hussain H, Green IR, Shamraiz U, Saleem M, Badshah A, Abbas G, Rehman NU, Irshad M. Therapeutic potential of glycyrrhretinic acids: a patent review (2010-2017). *Expert Opin. Ther. Pat., 28*, 383–398 (2018).
17) Singh P, Singh D, Goel RK. Protective effect on phenytoin-induced cognition deficit in pentylenetetrazol kindled mice: A repertoire of Glycyrrhiza glabra flavonoid antioxidants. *Pharm. Biol.*, **54**, 1209–1218 (2016).

18) Fiore C, Eisenhut M, Ragazzi E, Zanchin G, Armanini D. A history of the therapeutic use of liquorice in Europe. *J. Ethnopharmacol.*, **99**, 317–324 (2005).

19) Aiello F, Armentano B, Polerà N, Carullo G, Loizzo MR, Bonesi M, Cappello MS, Capobianco L, Tundis R. From Vegetable Waste to New Agents for Potential Health Applications: Antioxidant Properties and Effects of Extracts, Fractions and Pinocembrin from Glycyrrhiza glabra L. Aerial Parts on Viability of Five Human Cancer Cell Lines. *J. Agric. Food Chem.*, **65**, 7944–7954 (2017).

20) Alwan AM, Nesrullah Z, Faraj E. Study the Effect of Ethanolic Extract of Glycyrrhiza glabra on Pathogenic Bacteria. (2015).

21) Yang R, Yuan BC, Ma YS, Zhou S, Liu Y. The anti-inflammatory activity of licorice, a widely used chinese herb. *Pharm. Biol.*, **55**, 5–18 (2017).

22) Kang JS, Yoon YD, Cho IJ, Han MH, Lee CW, Park S-K, Kim HM. Glabridin, an Isoflavan from Licorice Root, Inhibits Inducible Nitric-Oxide Synthase Expression and Improves Survival of Mice in Experimental Model of Septic Shock. *J. Pharmacol. Exp. Ther.*, **312**, 1187–1194 (2004).

23) Yokota T, Nishio H, Kubota Y, Mizoguchi M. The inhibitory effect of glabridin from licorice extracts on melanogenesis and inflammation. *Pigment cell Res.*, **11**, 355–61 (1998).

24) Tsantoulas C. Emerging potassium channel targets for the treatment of pain. *Curr.*
25) Chen SR, Cai YQ, Pan HL. Plasticity and emerging role of BKCa channels in nociceptive control in neuropathic pain. *J. Neurochem.*, **110**, 352–362 (2009).

26) Lu R, Lukowski R, Sausbier M, Zhang DD, Sisignano M, Schuh CD, Kuner R, Ruth P, Geisslinger G, Schmidtko A. BKCa channels expressed in sensory neurons modulate inflammatory pain in mice. *Pain*, **155**, 556–565 (2014).

27) Chanda D, Prieto-Lloret J, Singh A, Iqbal H, Yadav P, Snetkov V, Aaronson PI. Glabridin-induced vasorelaxation: Evidence for a role of BKCa channels and cyclic GMP. *Life Sci.*, **165**, 26–34 (2016).

28) OECD. OECD/OCDE 420 OECD GUIDELINE FOR TESTING OF CHEMICALS Acute Oral Toxicity-Fixed Dose Procedure INTRODUCTION. (2001).

29) Clark SJ, Folienfant RL, Smith TW. Evaluation of opioid-induced antinociceptive effects in anaesthetized and conscious animals. *Br. J. Pharmacol.*, **95**, 275–283 (1988).

30) Adeyemi OO, Ishola IO, Adesanya ET, Alohan DO. Antinociceptive and anti-inflammatory properties of Tetracera alnifolia Willd. (Dilleniaceae) hydroethanolic leaf extract. *J. Basic Clin. Physiol. Pharmacol.*, (2018).

31) Oliveira P de A, de Almeida TB, de Oliveira RG, Gonçalves GM, de Oliveira JM, Neves dos Santos BB, Laureano-Melo R, Côrtes W da S, França T do N, Vasconcellos MLA de A, Marinho BG. Evaluation of the antinociceptive and anti-inflammatory activities of piperic acid: Involvement of the cholinergic and vanilloid systems. *Eur. J. Pharmacol.*, **834**, 54–64 (2018).

32) Dubuisson D, Dennis SG. The formalin test: A quantitative study of the analgesic
effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain, 4*, 161–174 (1977).

33) Perkins ’ M N, Campbell EA. Capsazepine reversal of the antinociceptive action of capsaicin in vivo. (1992).

34) Kosarmadar N, Ghasemzadeh Z, Rezayof A. Inhibition of microglia in the basolateral amygdala enhanced morphine-induced antinociception: Possible role of GABAA receptors. *Eur. J. Pharmacol.*, **765**, 157–163 (2015).

35) Fatemi I, Amirteimoury M, Shamsizadeh A, Kaeidi A. The effect of metformin on morphine analgesic tolerance and dependence in rats. *Res. Pharm. Sci.*, **13**, 316–323 (2018).

36) Sordi R, Fernandes D, Assreuy J. Differential involvement of potassium channel subtypes in early and late sepsis-induced hyporesponsiveness to vasoconstrictors. *J. Cardiovasc. Pharmacol.*, **56**, 184–9 (2010).

37) Taghi Mansouri M, Naghizadeh B, Ghorbanzadeh B, Farbood Y. Central and peripheral antinociceptive effects of ellagic acid in different animal models of pain. *Eur. J. Pharmacol.*, **707**, 46–53 (2013).

38) Ristic D, Spangenberg P, Ellrich J. Acetylsalicylic acid inhibits α,β-meATP-induced facilitation of neck muscle nociception in mice — Implications for acute treatment of tension-type headache. *Eur. J. Pharmacol.*, **673**, 13–19 (2011).

39) Patil CS, Jain NK, Singh A, Kulkarni SK. Modulatory effect of cyclooxygenase inhibitors on sildenafil-induced antinociception. *Pharmacology*, **69**, 183–189 (2003).

40) Imam MZ, Sultana S, Akter S. Antinociceptive, antidiarrheal, and
neuropharmacological activities of Barringtonia acutangula. *Pharm. Biol.*, **50**, 1078–1084 (2012).

41) Murtala AA, Akindele AJ. Anxiolytic- and antidepressant-like activities of hydroethanol leaf extract of Newbouldia laevis (P.Beauv.) Seem. (Bignoniaceae) in mice. *J. Ethnopharmacol.*, 112, 2420 (2019).

42) Zhang FX, Liu XJ, Gong LQ, Yao JR, Li KC, Li ZY, Lin LB, Lu YJ, Xiao HS, Bao L, Zhang XH, Zhang X. Inhibition of inflammatory pain by activating B-type natriuretic peptide signal pathway in nociceptive sensory neurons. *J. Neurosci.*, **30**, 10927–10938 (2010).

43) Malmberg AB, Yaksh TL. Antinociceptive actions of spinal nonsteroidal anti-inflammatory agents on the formalin test in the rat. *J. Pharmacol. Exp. Ther.*, **263**, 136–146 (1992).

44) Sung YJ, Sofoluke N, Nkamany M, Deng S, Xie Y, Greenwood J, Farid R, Landry DW, Ambron RT. A novel inhibitor of active protein kinase G attenuates chronic inflammatory and osteoarthritic pain. *Pain*, **158**, 822–832 (2017).

45) Hoffmeister C, Trevisan G, Rossato MF, De Oliveira SM, Gomez MV, Ferreira J. Role of TRPV1 in nociception and edema induced by monosodium urate crystals in rats. *Pain*, **152**, 1777–1788 (2011).

46) Huang Y, Chen SR, Chen H, Pan HL. Endogenous transient receptor potential ankyrin 1 and vanilloid 1 activity potentiates glutamatergic input to spinal lamina I neurons in inflammatory pain. *J. Neurochem.*, **149**, 381–398 (2019).

47) Scoto GM, Aricò G, Ronsisvalle S, Parenti C. Effects of intraplantar Nocistatin and
(±)-J 113397 injections on nociceptive behavior in a rat model of inflammation. 

*Pharmacol. Biochem. Behav.*, **100**, 639–644 (2012).

48) El-Ashmawy NE, Khedr NF, El-Bahrawy HA, El-Adawy SA. Downregulation of iNOS and elevation of cAMP mediate the anti-inflammatory effect of glabridin in rats with ulcerative colitis. *Inflammopharmacology*, **26**, 551–559 (2018).

49) Boetkjaer A, Boedker M, Cui J-G, Zhao Y, Lukiw WJ. Synergism in the repression of COX-2- and TNFα-induction in platelet activating factor-stressed human neural cells. *Neurosci. Lett.*, **426**, 59–63 (2007).

50) Smith WL, Garavito RM, DeWitt DL. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. *J. Biol. Chem.*, **271**, 33157–60 (1996).

51) P JJ, Manju SL, Ethiraj KR, Elias G. Safer anti-inflammatory therapy through dual COX-2/5-LOX inhibitors: A structure-based approach. *Eur. J. Pharm. Sci.*, **121**, 356–381 (2018).

52) Williams JA, Shacter E. Regulation of macrophage cytokine production by prostaglandin E2. Distinct roles of cyclooxygenase-1 and -2. *J. Biol. Chem.*, **272**, 25693–9 (1997).

53) Ozdemir E, Bagcivan I, Durmus N, Altun A, Gursoy S. The nitric oxide-cGMP signaling pathway plays a significant role in tolerance to the analgesic effect of morphine. *Can. J. Physiol. Pharmacol.*, **89**, 89–95 (2011).

54) Millan MJ. Descending control of pain. *Prog. Neurobiol.*, **66**, 355–474 (2002).

55) Liu CY, Lu ZY, Li N, Yu LH, Zhao YF, Ma B. The role of large-conductance, calcium-activated potassium channels in a rat model of trigeminal neuropathic pain.
Cephalalgia, 35, 16–35 (2015).

56) Jankowska E, Hammar I. Interactions between spinal interneurons and ventral spinocerebellar tract neurons. J. Physiol., 591, 5445–5451 (2013).
Table 1. Treatment groups and doses of chemicals used in the study. Gla: glabridin, NS1619; the large conductance Ca\(^{2+}\)-activated K\(^+\) opener, iberiotoxin (IbTX); the large conductance Ca\(^{2+}\)-activated K\(^+\) blocker, L-Name; nitric oxide precursor, L-Arginine; nitric oxide inhibitor, ASA; acetylsalicylic acid, Car: carrageenan, s.c.: subcutaneous; i.pl.: intraplantar, i.p.: intraperitoneal.

| Test               | Animal Type | Experimental Group Names                                                                 |
|--------------------|-------------|------------------------------------------------------------------------------------------|
| Tail flick test    | rat         | Glab (5 mg/kg, i.p.) + Glab (50 mg/kg, s.c.) + Glab (100 mg/kg, s.c.)                   |
|                   | mouse       | Glab (5 mg/kg, s.c.)                                                                       |
|                   | rat         | Glab (50 mg/kg, s.c.) + Neom (5 mg/kg, s.c.)                                              |
|                   | mouse       | Glab (10 mg/kg, s.c.) + Neom (5 mg/kg, s.c.)                                              |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (50 mg/kg, s.c.)                                              |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (50 mg/kg, s.c.)                                              |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (100 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (100 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (200 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (200 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (500 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (500 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (50 mg/kg, s.c.)                                              |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (50 mg/kg, s.c.)                                              |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (100 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (100 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (200 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (200 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (500 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (500 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (50 mg/kg, s.c.)                                              |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (50 mg/kg, s.c.)                                              |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (100 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (100 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (200 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (200 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (500 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (500 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (50 mg/kg, s.c.)                                              |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (50 mg/kg, s.c.)                                              |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (100 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (100 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (200 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (200 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (500 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (500 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (50 mg/kg, s.c.)                                              |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (50 mg/kg, s.c.)                                              |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (100 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (100 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (200 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (200 mg/kg, s.c.)                                             |
|                   | rat         | Glab (5 mg/kg, i.p.) + Glab (500 mg/kg, s.c.)                                             |
|                   | mouse       | Glab (5 mg/kg, s.c.) + Glab (500 mg/kg, s.c.)                                             |

Gla: glabridin, NS1619; the large conductance Ca\(^{2+}\)-activated K\(^+\) opener, iberiotoxin (IbTX); the large conductance Ca\(^{2+}\)-activated K\(^+\) blocker, L-Name; nitric oxide precursor, L-Arginine; nitric oxide inhibitor, ASA; acetylsalicylic acid, Car: carrageenan, s.c.: subcutaneous; i.pl.: intraplantar, i.p.: intraperitoneal.
Table 2. Glabridin effects on lymphocytes migration, cytokine production, and PGE2 levels and determination of the participation of L-arginine-nitric oxide pathway and COX enzyme by carrageenan-induced air pouch test in rat. The rats were pretreated with saline, or vehicle, or glabridin (10, 20, 40 mg/kg), or diclofenac (2 mg/kg), or acetylsalicylic acid, or L-arginine (100 mg/kg, nitric oxide precursor), or L-Name (30 mg/kg, nitric oxide synthase inhibitor). Data are expressed as mean±SD and analysed by one-way ANOVA with repeated measurements. *P<0.05, **P<0.01, and ***P<0.001 vs vehicle. "aaa"P<0.001 vs L-Name.

| Injection to air pouch | Treatment          | dosage (mg/kg) | Lymphocytes (X10^6/ml) | TNF-α (X10^5/ml) | PGE2 (ng/ml) |
|------------------------|--------------------|----------------|------------------------|------------------|--------------|
| Saline                 | -                  | -              | 21.17±10.40***        | 3.06±0.26***     | 29.17±8.11***|
| Carrageenan            | vehicle            | -              | 314.67±59.35          | 12.92±1.07       | 148.17±17.44 |
|                        | glabridin          | 10             | 279.83±30.20          | 12.02±0.71       | 130.33±14.60 |
|                        | glabridin          | 20             | 246.17±15.94**        | 9.08±0.62***     | 119.17±14.16*|
|                        | glabridin          | 40             | 143.17±22.36***       | 7.65±0.63***     | 82.33±11.72  |
|                        | diclofenac         | 2              | 62.50±14.75***        | 3.68±0.19***     | 57.83±10.74**|
|                        | acetylsalicylic acid| 60            | 48.17±11.43***        | 3.40±0.25***     | 55.17±7.47** |
|                        | L-Name             | 40             | 88.67±17.19           | 5.75±0.94        | 66.83±7.73   |
|                        | L-Name+glabridin   | 30 + 40        | 55.67±5.79            | 4.21±0.68        | 62.67±6.41   |
|                        | L-Arginine+glabridin| 100 + 40     | 254.31±23.74**        | 10.74±0.94**     | 125.83±18.18**|
|                        | L-Arginine+L-Name  | 100 + 40       | 238.17±29.19**        | 10.01±0.97**     | 120.31±14.60**|
Table 3. Acute and subacute toxicity of glabridin.

| Dose (mg/kg, i.p) (n=8) | Mortality | Sedation | Seizures | Respiratory change | Loss of reflex | Convulsion | Feed/bod weigt ratio (%) |
|-------------------------|-----------|----------|----------|-------------------|---------------|------------|--------------------------|
| Control                 | 0         | 0        | 0        | 0                 | 0             | 0          | 23.7%                    |
| 100                     | 0         | 0        | 0        | 0                 | 0             | 0          | 24.02%                   |
| 200                     | 0         | 0        | 0        | 0                 | 0             | 0          | 23.41%                   |
| 400                     | 0         | 0        | 0        | 0                 | 0             | 0          | 23.19%                   |
Figure 1. The drugs used and the plans of the tests. Gla: glabridin, veh: vehicle, NS1619; the large conductance Ca$^{++}$-activated K$^+$ opener, iberiotxin (IbTX); the large conductance Ca$^{++}$-activated K$^+$ blocker, L-Name; nitric oxide precursor, L-Arginine; nitric oxide inhibitor, ASA; acetylsalicylic acid, Car: carrageenan, s.c.: subcutaneous; i.pl.: intraplantar, i.p.: intraperitoneal.
Figure 2. The anti-nociceptive effect of glabridin (a) and determination of the participation of opioidergic system (b) and BKCa channels (c) by tail flick test in rat. The anti-nociceptive effect of 5 mg/kg morphine and glabridin (40 mg/kg, i.p) after non-selective opioid antagonist (naloxone 5 mg/kg, s.c) or co-administer glabridin (40 mg/kg) with morphine in rat (n=8) assessed by the tail flick test. Acute anti-nociceptive effect of 10 mg/kg NS1619 (the large conductance Ca++-activated K+ channels opener) and glabridin (40 mg/kg, i.p) after the large conductance Ca++-activated K+ channel blocker (iberiotoxin, IbTX, 0.7 nmol/kg, i.p) or co-administer glabridin (40 mg/kg) with IbTX in rat (n=8) assessed by the tail flick test. Data are expressed as mean±SD and analysed by two-way ANOVA with repeated measurements. *P<0.05, **P<0.01, and ***P<0.001 vs their baseline latency time. aP<0.05, aaaP<0.001 vs morphine. bbbP<0.001 vs NS1619 in their time, indicate statistically significant differences.
Figure 3. The anti-nociceptive effect of glabridin (a) and determination of the participation of opioidergic system (b) and BK_{Ca} channels (c) by hot plate test in mice.

The anti-nociceptive effect of 5 mg/kg morphine and glabridin (40 mg/kg, i.p) after non-selective opioid antagonist (naloxone 5 mg/kg, s.c) or co-administer glabridin (40 mg/kg) with morphine in mice (n=8) assessed by the hot plate test. Acute anti-nociceptive effect of 10 mg/kg NS1619 (the large conductance Ca^{++}-activated K^{+} channels opener) and glabridin (40 mg/kg, i.p) after the large conductance Ca^{++}-activated K^{+} channel blocker (iberiotoxin, IbTX, 0.7 nmol/kg, i.p) or co-administer glabridin (40 mg/kg) with IbTX in rat (n=8) assessed by the hot plate test. Data are expressed as mean±SD and analysed by two-way ANOVA with repeated measurements. *P<0.05, **P<0.01, and ***P<0.001 vs their baseline latency time. aP<0.05, aaaP<0.001 vs morphine. bbbP<0.001 vs NS1619 in their time, indicate statistically significant differences.
Figure 4. The anti-nociceptive effect of glabridin (a) and determination of the participation of BKCa channels (b), L-arginine-nitric oxide pathway (c), and cGMP (d) by acetic acid-induced writhing test in mice. The mice were pretreated with vehicle, or glabridin (10, 20, 40 mg/kg), or NS1619 (10 mg/kg, the large conductance Ca++-activated K+ channels opener), IbTX (0.7 nmol/kg, the large conductance Ca++-activated K+ channel blocker), or L-arginine (100 mg/kg, nitric oxide precursor), or L-Name (30 mg/kg, nitric oxide synthase inhibitor), or methylene blue (20 mg/kg, a guanylate cyclase inhibitor). Data are expressed as mean±SD and analysed by one-way ANOVA with repeated measurements. **P<0.01, and ***P<0.001 vs vehicle. ns: non-significant.
Figure 5. The anti-nociceptive effect of glabridin (a) and determination of the participation of opioidergic system (b), BK_{Ca} channels (c), and L-arginine-nitric oxide pathway (d) by formalin-induced hyperalgesia in mice. The mice were pretreated with vehicle, or glabridin (10, 20, 40 mg/kg), or morphine (5 mg/kg, s.c.), or naloxone (5 mg/kg, s.c.), or NS1619 (10 mg/kg, the large conductance Ca^{++}-activated K^{+} channels opener), IbTX (0.7 nmol/kg, the large conductance Ca^{++}-activated K^{+} channel blocker), or L-arginine (100 mg/kg, nitric oxide precursor), or L-Name (30 mg/kg, nitric oxide synthase inhibitor). Data are expressed as mean±SD and analysed by two-way ANOVA with repeated measurements. *: P<0.01, **: P<0.01, and ***: P<0.001. ns: non-significant, Nal: naloxone.
Figure 6. The anti-nociceptive effect of Glabridin on capsaicin-induced paw licking. Mice were treated with vehicle, glabridin (10, 20 and 40 mg/kg), capsazepine (TRPV receptor antagonist; 50 µg/ml; 10 µl i.pl.), saline. Data are expressed as the mean ± SD (n = 8). Statistically significant differences were found when comparing the treatment groups with the saline control group. ***P < 0.001 vs vehicle.
Figure 7. The anti-inflammatory effect of glabridin (a) and determination of the participation of L-arginine-nitric oxide pathway (b) by carrageenan-induced paw edema in rat. The rats were pretreated with saline, or vehicle, or glabridin (10, 20, 40 mg/kg), or diclofenac (2 mg/kg), or L-arginine (100 mg/kg, nitric oxide precursor), or L-Name (30 mg/kg, nitric oxide synthase inhibitor). Data are expressed as mean±SD and analysed by two-way ANOVA with repeated measurements. **P<0.01, and ***P<0.001 vs vehicle. &&P<0.01, and &&&P<0.001 vs L-arginine+gla40, ++P<0.01, and +++P<0.001 vs L-arginine+L-Name. ns: non-significant.
Figure 8. Glabridin effects on the open field test responses. Rats were treated with saline (control), or vehicle, or glabridin (40 mg/kg; Gla40), or diazepam (1 mg/kg, i.p). Data are expressed as the mean ± SD (n = 8). Statistically significant differences were found. *: P < 0.05, ***: P < 0.001, &&: P < 0.01, &&&: P < 0.001.