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Extract of *Glycyrrhiza glabra* root attenuates nociception in experimental pain models: The role of BKCa channels

[Extracto de raíz de *Glycyrrhiza glabra* atenúa la nocicepción en modelos experimentales de dolor: El rol de los canales BKCa]

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Abstract: The aim of this study was to evaluate the functional interaction of *Glycyrrhiza glabra* root extract (GGRE) on the large conductance Ca^{2+}-activated K^{+} (BKCa) channels expressed in the peripheral nervous system by using nociception and inflammation models in rodents *in vivo*. Besides toxicity studies and open field tests, nociception and inflammation tests were performed on rodents. Different doses of GGRE were given orally to rats and mice. Naloxone, indomethacin, morphine, NS1619 and iberiotoxin (IbTX) were administered. GGRE had both anti-nociceptive and anti-inflammatory activity in rats and mice. GGRE exhibited an analgesic effect by decreasing the time-course of the pain threshold or reaction time in some nociceptive tests. Furthermore, GGRE reduced level of pro-inflammatory cytokines, including TNF-α and IL-1β. As a conclusion, GGRE can alleviate the pain sensation of the afferent nerves and can reduce inflammation and associated pain by activating BKCa channels and reducing the levels of TNF-α, IL1β.

Keywords: Nociception; Neuropathic pain; *Glycyrrhiza glabra*; BKCa channels; Inflammation.

Resumen: El objetivo de este estudio fue evaluar la interacción funcional del extracto de raíz de *Glycyrrhiza glabra* (GGRE) en los canales de K^{+} (BKCa) activados por Ca^{2+} de gran conductancia expresados en el sistema nervioso periférico mediante el uso de modelos de nocicepción e inflamación en roedores *in vivo*. Además de los estudios de toxicidad y las pruebas de campo abierto, se realizaron pruebas de nocicepción e inflamación en roedores. Se administraron por vía oral diferentes dosis de GGRE a ratas y ratones. Se administraron naloxona, indometacina, morfina, NS1619 e iberiotoxina (IbTX). GGRE tenía actividad tanto antinociceptiva como antiinflamatoria en ratas y ratones. GGRE mostró un efecto analgésico al disminuir la evolución temporal del umbral del dolor o el tiempo de reacción en algunas pruebas nociceptivas. Además, GGRE redujo el nivel de citocinas proinflamatorias, incluidas TNF-α e IL-1β. Como conclusión, GGRE puede aliviar la sensación de dolor de los nervios aferentes y puede reducir la inflamación y el dolor asociado activando los canales BKCa y reduciendo los niveles de TNF-α, IL1β.

Palabras clave: Nocicepción; Dolor neuropático; *Glycyrrhiza glabra*; Canales BKCa; Inflamación.

INTRODUCTION

Inflammation is the body's reaction to tissue damage caused by infection, damaged cells, irritants, and mechanical and chemical stimulation (Winter *et al*., 1962). It can cause the loss of cells, tissue, and organs if the inflammatory response is excessive (Yang *et al.*, 2017). Many researchers have conducted studies to control excessive inflammatory responses for a long time. However, due to side effects of current anti-inflammatory drugs such as abnormal menstruation, facial flushing, and Cushing's disease, new agents have fewer side effects are needed. (Buchman, 2001).

The main reason of using anti-inflammatory drugs is reducing pain rather than edema, increased heat, or redness. Nociceptors, which play a role in conveying information regarding pain stimuli from nerve endings to the central nervous system, are specialized receptors that enable the detection of pain stimuli as the primary pain unit and the transformation of the mechanical and chemical response to electrical conduction. Previous studies have revealed that there is a relationship between nociception and pain, which involves various molecules, such as prostaglandin, opioids, acetylcholine, interleukins, and cytokines. One way to prevent inflammation is to hinder the nociceptive effect (Sneddon, 2017). Hence, to control inflammation, several drugs have been investigated, such as nonsteroidal anti-inflammatory drugs (NSAIDs) (Smith *et al*., 1979), corticosteroids (Gentry *et al.*, 1996), cannabinoid 2 receptor agonists (Parlar *et al.*, 2018). However, excessive and long-term use of NSAIDs and corticosteroids may cause adverse effects, such as gastrointestinal toxicity, addiction, drug resistance, and Cushing syndrome (Hodgson, 2015). Complementary and alternative treatments containing active ingredients derived from plant extracts such as *G. glabra* (Liquorice, Licorice) root extract (GGRE) are becoming more popular among consumers in both developing and developed countries because these extracts are relatively safer with fewer adverse effects than some drugs (Wang et al., 2014).

GGRE include wide variety of components such as saponins, flavonoids, phenolic compounds, coumarins and chalcones. The saponins in GGRE consist of glycyrrhizin (also known as glycyrrhizic or glycyrrhizinic acid), licorice saponin A3, licorice saponin G2, licorice saponin J2, licorice saponin C2 components (Hayashi & Sudo, 2009; Farag *et al*., 2012). Phenolic compounds in GGRE consist of liquiritin apioside, 5,8-dihydroxy-flavone-7-O-β-D-glucuroni-de (glychionide A), 5-hydroxy-8-methoxyl-flavone-7-O-β-D-glucuronide (glychionide B), galbrene, glabrone, glabraisoflavanone A, glabraisoflavanone B, isoviolanthin, 5,7-dihydroxyflavanone, and rhamnoliquiritin (Li *et al*., 2005; Farag *et al*., 2012), glabridin, hispaglabridin (A and B), 4′-O-methylglabridin, isoprenylchalcone, liquiritigenin, isoliquiritigenin and formononetin, glycosides (Chin *et al*., 2007; Martins *et al*., 2015).

As we can see from the literature review, several studies show that *G. glabra* and its root extract have various pharmacological effects. (Bhandage *et al.*, 2009; Maleki *et al.*, 2017; Maione *et al.*, 2019). Previously it has been shown that the large conductance Ca^{++}-activated K^+ (BK_{Ca}) channels are involved in both anti-oxidant and anti-inflammatory processes (Dai *et al.*, 2017; Parlar *et al.*, 2020), In addition, BK_{Ca} channels, which are proven to be present in dorsal root ganglion (DRG) neurons, play a role in anti-nociceptive efficacy (Lu *et al.*, 2014). Glabridin, one of the main components of GGRE, is known to activate BK_{Ca} channels (Chanda *et al.*, 2016). However, the role of BK_{Ca} channels in GGRE's anti-inflammatory and anti-nociceptive mechanism has not been investigated. in this context, we considered complementing the previous work on *G. glabra* extracts, and established the hypothesis that the anti-nociceptive and anti-inflammatory effects of GGRE is largely attributable to glabridin, one of its components. Although the previous study on GREE (Bhandage *et al.*, 2009) demonstrated the anti-inflammatory and anti-nociceptive effects of GREE, a complete mechanism of action has not been revealed. Therefore, we aimed in the present study to clarify the underlying mechanism of anti-inflammatory and anti-nociceptive activity of GGRE, and to especially determine the role of BK_{Ca} channels.

MATERIALS AND METHODS

Animals

Eight-week-old male Wistar rats (180-220 g) and BALB/c mice (20-25 g) were provided by the Experimental Animal Center of Adiyaman University. The rats were used for edema and tail-flick tests, while the mice were used for the hot plate, open-field, acetic acid, formalin, and toxicity tests. Their housing conditions included a constant temperature (22 ± 2ºC) and food and water available *ad libitum*. The ethical approval documentation was received from the Animal Research Committee of

Adiyaman University (2019/035).

Preparation of GGRE

Dried *G. glabra* roots were purchased from an herbal drug store (Arifoğlu Baharat, Ankara), deposited in the Herbarium Library of Ankara Yildirim Beyazit University, and identified by one of the authors, Professor Seyfullah Oktay Arslan. To begin the extraction, 100 g of dried *G. glabra* roots were ground, weighed and mixed with 300 ml of ethanol for 45 min in a magnetic stirrer and left at +4°C for 24 h. A 25% yield was obtained after evaporation in a vacuum evaporator (R-3, BUCHI. Switzerland).

Experimental Design

As illustrated Figure No. 1, rats and mice were randomly divided into 7 series and different groups with 8 animals in each group They were taken to the laboratory where the experiments would be performed and allowed to acclimate for 4 d. The groups were named as shown in Table No. 1.

The doses of carrageenan (1%, 100µl intraplantar; (Parlar *et al.*, 2018), morphine (5 mg/kg subcutaneous [s.c.]; (Adeyemi *et al.*, 2019), naloxone (3.1 mg/kg intraperitoneal [IP]; (Adeyemi *et al.*, 2019), acetic acid (0.6%, 10 ml/kg IP (Kawaura *et al.*, 2011), formalin (2.5%, 50 µ; (Landa-Juárez *et al.*, 2016), and indomethacin (10 mg/kg orally [PO]; (Scapinello *et al.*, 2019)), NS1619 (10 mg/kg IP; (Lu *et al.*, 2014)), iberiotoxin (IbTX) (0.7 nmol/kg intravenously [IV]; (Sordi *et al.*, 2010)) were decided based on relevant studies. The control group was given saline, and the experiment was blinded for all the treatments. GGRE administration (50, 100, and 200 mg/kg) were started PO 3 d before the experiment, GGRE and other drugs were given as specified in the tests below.

TABLE No.1
Group names and doses of chemical in the study

Model names	Experimental Group Names
Toxicity test	Control, GGRE500, GGRE1000, GGRE2000.
Hot Plate test	Control, morphine (5 mg/kg, s.c), GGRE50, GGRE100, GGRE200, naloxone (3.1 mg/kg, IP)+GGRE200, IbTX (0.7 nmol/kg, IV)+ GGR200, NS1619 (10 mg/kg, IP), NS1619+GGRE200.
Tail flick test	Control, morphine (5 mg/kg, s.c), GGRE50, GGRE100, GGRE200, naloxone (3.1 mg/kg, IP)+GGRE200, IbTX (0.7 nmol/kg, IV)+ GGR200, NS1619, (10 mg/kg, IP), NS1619+GGRE200.
Open field test	Control, morphine (5 mg/kg, s.c), GGRE200, naloxone (3.1 mg/kg, IP)+GGRE200, IbTX (0.7 nmol/kg, IV)+GGRE200, NS1619 (10 mg/kg, IP)+GGRE200.
Acetic acid test	Acetic acid, morphine (5 mg/kg, s.c), GGRE50, GGRE100, GGRE200, naloxone (3.1 mg/kg, IP)+GGRE200, IbTX (0.7 nmol/kg, IV)+ GGR200, NS1619 (10 mg/kg, IP), NS1619+GGRE200.
Formalin test	Formalin, morphine (5 mg/kg, s.c), GGRE50, GGRE100, GGRE200, naloxone (3.1 mg/kg, IP)+GGRE200, IbTX (0.7 nmol/kg, IV)+ GGR200, NS1619 (10 mg/kg, IP), NS1619+GGRE200.
Edema test	Control, carrageenan, indomethacin (10 mg/kg, PO), GGRE50, GGRE100, GGRE200, IbTX (0.7 nmol/kg, IV)+GGRE200, NS1619 (10 mg/kg, IP)+GGRE200, NS1619.

GGRE: *Glycyrrhiza glabra* root extract, s.c.: subcutan, PO: peros, IP: intraperitoneal, IV: intravenous

GGRE's acute oral toxicity

The toxicity test was performed on mice according to the guidelines of the Organization for Economic Co-operation and Development (OECD). For this purpose, each animal (n=8 in each group) were administered doses of GGRE, 500, 1000, and 2000 mg/kg by oral gavage. Saline gavage was administered to one group as a control. All animals were then observed for specific parameters, such as mortality, behavioral changes, locomotion, hypothermia, muscular tonus, abdominal contortions, shaking, paw paralysis, salivation, bronchial secretion, convulsions for 4 h. 14 d later animals were observed again for all of the parameters listed above (data not shown).

Hot plate test

The hot plate test was performed to measure the latency of paw licking, jumping, and rotation movements of animals. Animals placed on a 55 ± 0.5ºC hot plate (with a 20 s cut-off) 30, 60, 90, 120, and 150 m after the administration of saline, 3 GGRE doses (50, 100, and 200 mg/kg, PO), morphine (5 mg/kg, s.c.),naloxone (3.1 mg/kg, IP) with GGRE (200 mg/kg, PO), naloxone with morphine, NS1619 (10 mg/kg, IP), IbTX (0.7 nmol/kg, IV), IbTX with GGRE (200 mg/kg, PO), NS1619 with IbTX or NS1619 with GGRE (200 mg/kg, PO) to assess the possible role of opioid receptors and BK_{Ca} channels (n=8) in mice as described previously (Sordi *et al.*, 2010). The blockers were given 15 min before other substances. To prevent tissue damage to the animals, they were allowed a maximum of 20 s on hot plate. Pre-dosing latencies were determined on at least two occasions.

Tail flick test

The tail-flick test performed to measure the anti-nociceptive activity of GGRE at the spinal level in rats (n=8) as described by D'amour *et al.* (1941), with minor modifications (May TF 0703 Tail-flick Unit, Commat, Ankara, Turkey). Briefly, the tails of immobilized animals were restrained by a tail-flick apparatus. The intensity of the radiant heat source was adjusted to give fundamental delays of 2-4 s without temperature changing. 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 measurements were performed at intervals of 30 min for a total of 6 measures. The first measurement was taken prior to administering the substances. The average of these measures was used as the baseline. After GGRE doses were given to the animals orally 3 consecutive days, saline, morphine, or NS1619 were administered at the same time with GGRE (50, 100, or 200 mg/kg PO) on the test day. IbTX or naloxone were given 15 min before these administrations to clarify GGRE's mechanism of action (Figure No. 1).

Open field test

Mice were subjected to the open-field test to assess the effect of GGRE on animal motor activity. Before the experiments, training was performed for 5 d. The procedure was based on a study conducted by Schulz (Schulz, 2018). The movements of mice (n=8) on a flat surface divided into 4 compartments with white chipboard were recorded via a video observation computer program (ANY-maze Behavioral Tracking Software 6.0) at 5 min intervals. GGRE (200 mg/kg) were administered for 3 consecutive days before the test. Saline, morphine, naloxone, IbTX, NS1619, or GGRE were administered 30 min before performing the test to investigate whether GGRE affected the animals' mobility.

Writhing test with acetic acid

The aim of writhing test is to determine whether BK_{Ca} channels and/or opioid receptors play a role in the effect of GGRE on acetic acid-induced visceral pain that scans both peripheral and central mechanisms (n=8) as described by Kawaura *et al.* (2011). Briefly, in this test, contractions of the abdominal wall followed by trunk twisting and extensions of the hind limbs and contact of the abdomen with the floor of the counting vessel were documented. For this purpose, the animals were given acetic acid (10 ml/kg IP, 0.6%) after GGRE administrations (50, 100, or 200 mg/kg PO), morphine or NS1619 administration. To observe the parameters described above, the animals were placed into a transparent cage individually for a period of 30 min. To investigate GGRE's mechanism of action, acetic acid, or morphine, or naloxone, or NS1619, or IbTX were administered same time GGRE (50, 100, or 200 mg/kg PO) administration to investigate GGRE's mechanism of action. GGRE doses were given to the animals orally 3 consequence days and 15 min before the chemicals were given (Figure No. 1)

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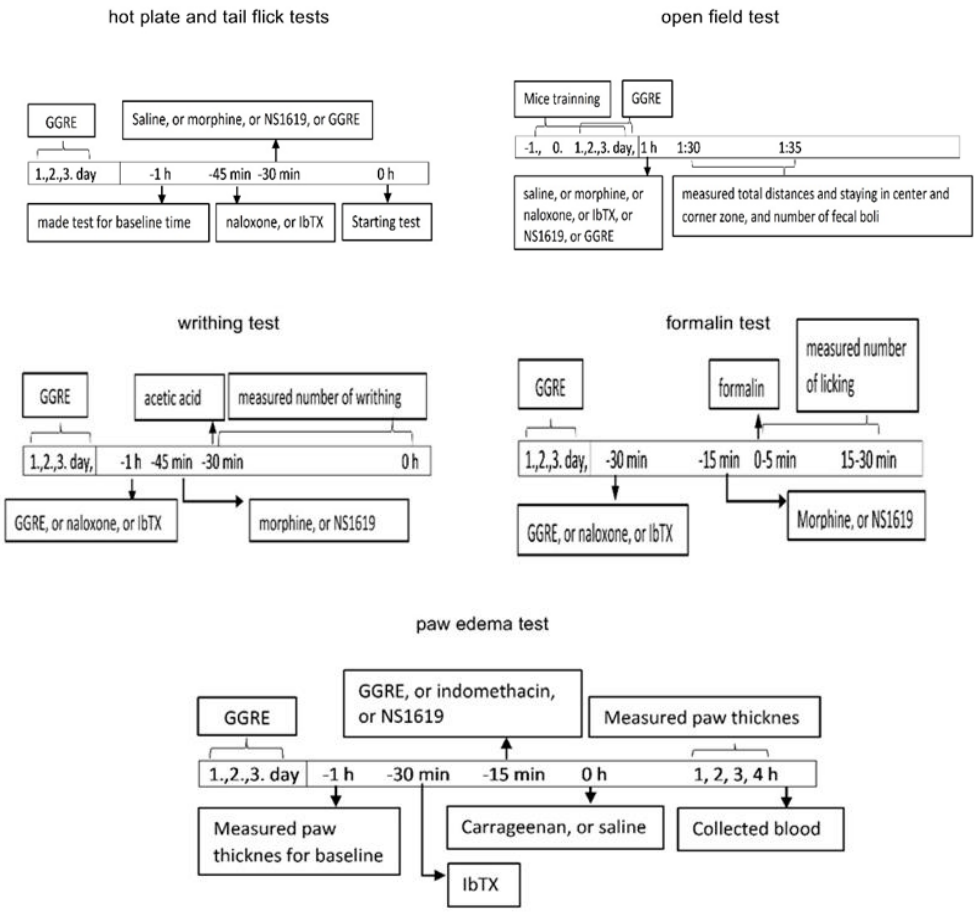

Figure No. 1
Drug application and plans of the tests
GGRE; *Glycyrrhiza glabra* root extract, NS1619; the large conductance Ca^{2+}-activated K^+ opener, iberiotoxin (IbTX); the large conductance Ca^{2+}-activated K^+ blocker

Formalin test

This test was used to demonstrate the effect of GGRE on inflammatory and non-inflammatory pain. As illustrated Figure No. 1, after administering GGRE, or other substances (IbTX, NS1619, morphine, naloxone) 50 µL of diluted formalin (2.5% in saline) was administered to the mice's (n=8) right hind paw. Then, the animals were placed into a container, and the duration of paw licking and biting was calculated as long as the animals remained in the container. The test was first performed to measure neurogenic activity for 0-5 min and 15-30 min after the formalin injection to measure inflammatory activity. To investigate GGRE's mechanism of action, formalin, or morphine, or naloxone, or NS1619, or IbTX were administered same time GGRE (50, 100, or 200 mg/kg PO) administration to determine the role of opioid receptors and of BK_{Ca} channels. GGRE doses were given to the animals orally 3 consequence days and 15 min before the chemicals were given.

Paw edema test with carrageenan

The edema test was conducted as previously described by Parlar *et al.* 2018). Briefly, inflammation was induced by giving an intraplantar injection of carrageenan (100 µl, 1% w/v [mg/mL]) into the paw of each rat (n=8) in all groups except the control group. GGRE groups (50, 100, or 200 mg/kg) were started to daily treatment with GGRE for 3 d prior to carrageenan administration. Edema was determined by comparing differences in paw thickness before and after carrageenan injection. To measure basal thickness, the paw was measured with electronic digital calipers, and then, after

administering carrageenan, its thickness was measured again after 0, 1, 2, 3, and 4 h, which corresponds to the peak edema time.

Indomethacin as a corticosteroid (10 mg/kg PO) (Scapinello *et al.*, 2019), NO1619 and IbTX were administered to determine the pathway of GGRE's anti-inflammatory effect. The difference between paw thickness and carrageenan thickness was recorded and expressed as %.

Measurement of Cytokine levels in serum

Immediately after the behavioural studies blood was collected from the animals to determine TNF-α and IL-1β serum levels. The blood samples were centrifuged at 3000 rpm for 10 min, and after the serum was obtained, it was stored at −80°C until analyzed. IL-1β and TNF-α levels were determined by ELISA kits (Thermo Fisher Scientific Inc.) and the results were expressed as pg/mL.

Phytochemical analyses

Determination of the glabridin level in the GGRE by High-Performance Liquid Chromatography (HPLC)

To identify the glabiridin in GGRE, Shimadzu HPLC 10AVP system and PDA detector were used. The separation was carried out using the 4,6 mm x 10 m column (Nucleosil 100-5 C 18, Macherey-Nagel) Chromatographic separation was carried out using two systems: phase A; methanol:water:formic acid (10:89:1 v/v/v) and phase B; methanol:water:formic acid (90:9.8:0.2 v/v/v). The analysis was performed using a linear gradient program. The flow rate was 1 ml/min and the injection volume was 5 µl. GGRE was dissolved in water and methanol at a 1:1 v/v ratio and injected into HPLC.

Screening of GGRE phytochemicals by GC-MS

Before GC-MS analysis of GGRE, it was derivatized as previously described by Hajji *et al*. (2010). Breifly, 100 µL of bis-(trimethylsilyl) acetamide was mixed with GGRE and then 20 µL of pyridine was added. The mixture was subsequently incubated at 80°C for 60 minutes and used for GC-MS analysis. For the analysis, GC 6890N/MS 5975 Inert XL mass selective detector and 7683B series auto-injector (Agilent-Technologies, Little Falls, CA, USA) was used.

Chemicals

Carrageenan, acetic acid, formalin, IbTX and NS1619 were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA), while the naloxone, morphine, and indomethacin were obtained from Hameln Pharma (Hameln, Germany), Osel Ilaç Sanayi (Istanbul, Turkey), and DEVA Holding (Istanbul, Turkey), respectively.

Statistical analysis

The anti-nociceptive and anti-inflammatory effects of GGRE were analysed by one-way ANOVA for the findings of the open-field, writhing, formalin and cytokine measurement tests, and two-way repeated ANOVA was used for the hot plate, tail-flick and paw edema tests' findings, followed by a Tukey HSD multiple comparisons test using GraphPad Prism 7.0 software (GraphPad Software Inc., La Jolla, California, USA). The Area Under Curve (AUC) values in hot plate, tail-flick, paw edema tests were subjected to one-way measures ANOVA, followed by the Dunnett's test. The results were presented as the mean ± standard deviation (SD). Differences with *p*-values less than 0.05 were considered to be statistically significant.

RESULTS

Hot plate test

GGRE dose–response analysis

As illustrated in Figure No. 2a, two-way ANOVA for repeated measures showed that GGRE dose-dependently and time-dependently increased latency time and induced analgesia [treatment: $F(3,126) = 34.85$, $p<0.001$; time: $F(5,42) = 36.41$, $p<0.001$; treatment×time interaction: $F(15,126) = 4.406$, $p<0.001$]. The results of Tukey's test demonstrated that there was a significant increase in latency time of GGRE (100 and 200 mg/kg) groups compared to control group in hot plate test at 30, 60, 90, and 120th min, but were not significant differences in baseline (0th min). The analgesic effects of the 100 and 200 mg/kg doses were greater more than the other doses at 90th min in hot-plate test. In addition, results of the one-way ANOVA followed by Dunnett's test for normalized AUC values were given in Figure No. 2b [$F(3,28) = 10.27$, $p<0.001$]. Post-hoc analysis showed that the doses of 100 and 200 mg/kg of GGRE significantly increased the latency time ($p<0.05$, and <0.001 respectively) whereas the dose of 50 mg/kg did not cause any significance ($p>0.05$) when compared to control group.

The effect of GGRE with or without naloxone in latencies in the hot plate test

As illustrated in Figure No. 2c, two-way ANOVA for

repeated measures showed that there was a statistically significant changes between the experimental groups tested [treatment: $F(3,126) = 191.4$, $p<0.001$; time: $F(5,42) = 97.58$, $p<0.001$; treatment×time interaction: $F(15,126) = 31.06$, $p<0.001$]. The results of Tukey's test demonstrated that experimental groups' latency time were increased compared to control group in hot plate test at 30, 60, 90, and 120th min, but in baseline (0th min). Analgesic effect of morphine has rapid onset (at 30 min) compared to GGRE (60 min). In addition, one-way ANOVA [$F(3,28) = 57.85$, $p<0.001$] followed by Dunnett's test for normalized AUC values were given in Figure No. 2d. Dunnett's test showed that naloxone+GGRE (200 mg/kg) significantly increased latency time ($p<0.001$) compared to morphine group. The naloxone abolished the analgesic effect of morphine but did not that of GGRE (200 mg/kg).

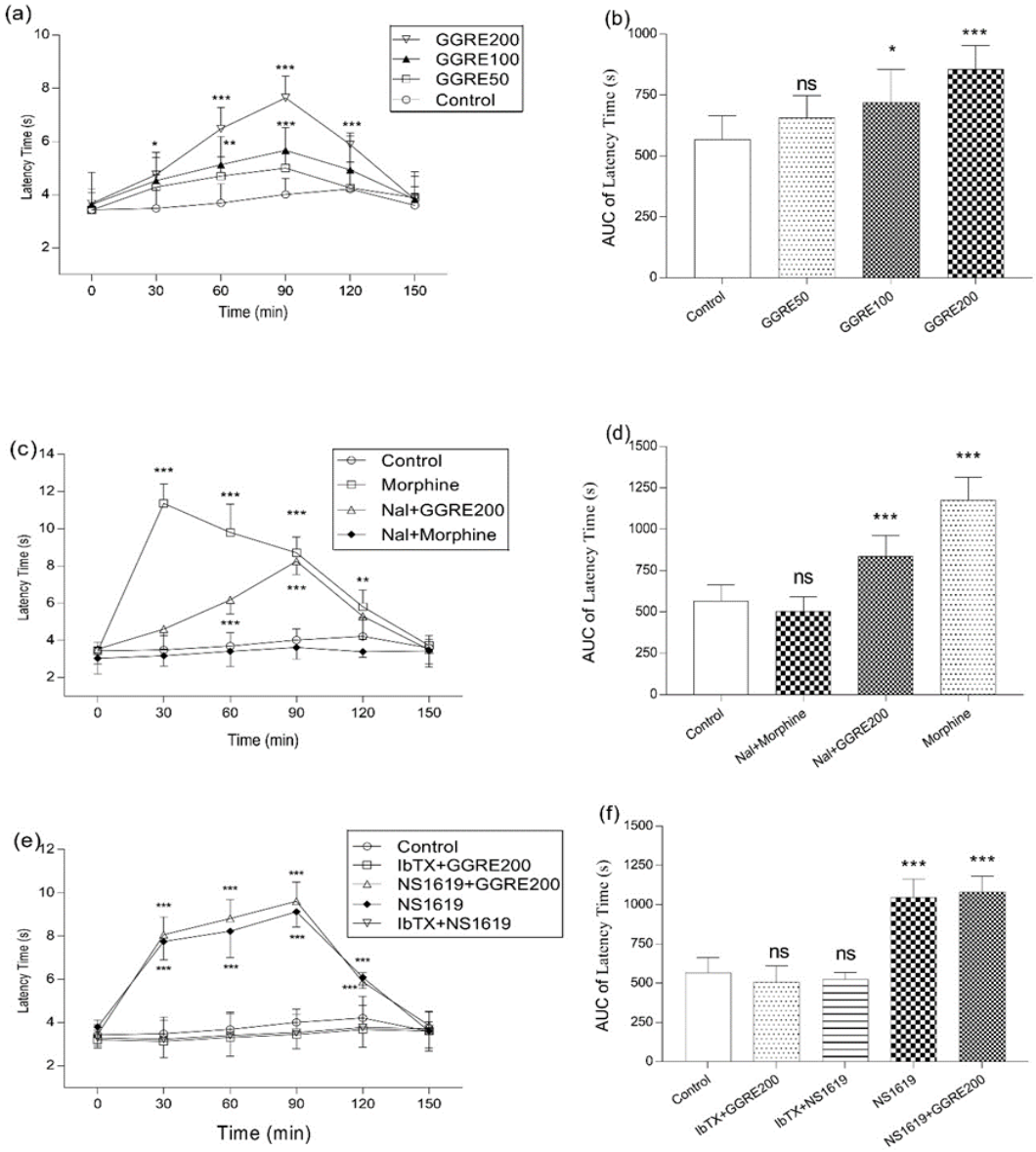

Figure No. 2
The effects of GGRE with or without morphine, NS1619, and IbTX on hot plate latencies
(a) The effect of different doses of GREE (50, 100 and 200 mg/kg) on latency time. (b) AUC calculated for the latency time. (c) The effect of morphine, GGRE (200 mg/kg) and naloxone on latency time. (d) AUC calculated for the latency time. (e) The effect of NS1619, GGRE (200 mg/kg) and IbTX on latency time. (f) AUC calculated for the latency time. Each symbol represents the mean (± SD) latency time for eight rats. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with the control group

The effect of GGRE with or without IbTX in latencies in the hot plate test

As illustrated in Figure No. 2e, two-way ANOVA for repeated measures showed that there was a statistically significant changes between the experimental groups tested [treatment: $F(4,168) = 254.9$, $p<0.001$; time: $F(5,42) = 36.98$, $p<0.001$; treatment×time interaction: $F(20,168) = 29.38$, $p<0.001$]. The results of Tukey's test demonstrated that both NS1619 and NS1619+GGRE significantly increased the latency time compared to control group. Pre-treatment of IbTX abolished the analgesic effect of NS1619 and GGRE. In addition, one-way ANOVA followed by Dunnett's post-hoc test for normalized AUC values were given in Figure No. 2f [$F(4,35) = 57.65$, $p<0.001$]. Post-hoc analysis revealed that GGRE+NS1619 and NS1619 significantly increased latency time when compared to control group. IbTX inhibited the analgesic effect of NS1619 and GGRE.

Tail flick test

GGRE dose–response analysis

As illustrated in Figure No. 3a, two-way ANOVA for repeated measures showed that GGRE dose-dependently and time-dependently increased latency time and induced analgesia [treatment: $F(3,126) = 33.81$, $p<0.001$; time: $F(5,42) = 44.73$, $p<0.001$; treatment×time interaction: $F(15,126) = 2.806$, $p<0.001$]. The results of Tukey's test demonstrated that there was a significant increase in latency time of GGRE (100 and 200 mg/kg) groups compared to control group in tail flick test at 30, 60, 90, and 120th min, but were not significant differences in baseline (0th min). The analgesic effects of the 100 and 200 mg/kg doses were greater more than the other doses at 90th min in hot-plate test. In addition, results of the one-way ANOVA followed by Dunnett's test for normalized AUC values were given in Figure No. 3b [$F(3,28) = 10.30$, $p<0.001$]. Post-hoc analysis showed that the doses of 100 and 200 mg/kg of GGRE significantly increased the latency time ($p<0.05$, and <0.001 respectively) whereas the dose of 50 mg/kg did not cause any significance ($p>0.05$) when compared to control group.

The effect of GGRE with or without naloxone in latencies in the tail-flick test

As illustrated in Figure No. 3c, two-way ANOVA for repeated measures showed that there was a statistically significant changes between the experimental groups tested [treatment: $F(3,126) = 168.8$, $p<0.001$; time: $F(5,42) = 36.22$, $p<0.001$; treatment×time interaction: $F(15,126) = 16.57$, $p<0.001$]. The results of Tukey's test demonstrated that experimental groups' latency time were increased compared to control group in tail flick test at 30, 60, 90, and 120th min, but in baseline (0th min). Analgesic effect of both morphine and GGRE were observed at 30 min. In addition, one-way ANOVA [$F(3,28) = 42.59$, $p<0.001$] followed by Dunnett's test for normalized AUC values were given in Figure No. 3d. Dunnett's test showed that naloxone+GGRE (200 mg/kg) significantly increased latency time ($p<0.001$) compared to morphine group. The naloxone abolished the analgesic effect of morphine but did not that of GGRE (200 mg/kg).

The effect of GGRE with or without IbTX in latencies in the tail-flick test

As illustrated in Figure No. 3e, two-way ANOVA for repeated measures showed that there was a statistically significant changes between the experimental groups [treatment: $F(4,168) = 100.2$, $p<0.001$; time: $F(5,42) = 9.733$, $p<0.001$; treatment×time interaction: $F(20,168) = 7.241$, $p<0.001$]. The results of Tukey's test demonstrated that both NS1619 and NS1619+GGRE significantly increased the latency time compared to control group. Pre-treatment of IbTX abolished the analgesic effect of NS1619 and GGRE. In addition, one-way ANOVA followed by Dunnett's post-hoc test for normalized AUC values were given in Figure No. 3f [$F(4,35) = 18.81$, $p<0.001$]. Post-hoc analysis revealed that GGRE+NS1619 and NS1619 significantly increased latency time when compared to control group. IbTX inhibited the analgesic effect of NS1619 and GGRE.

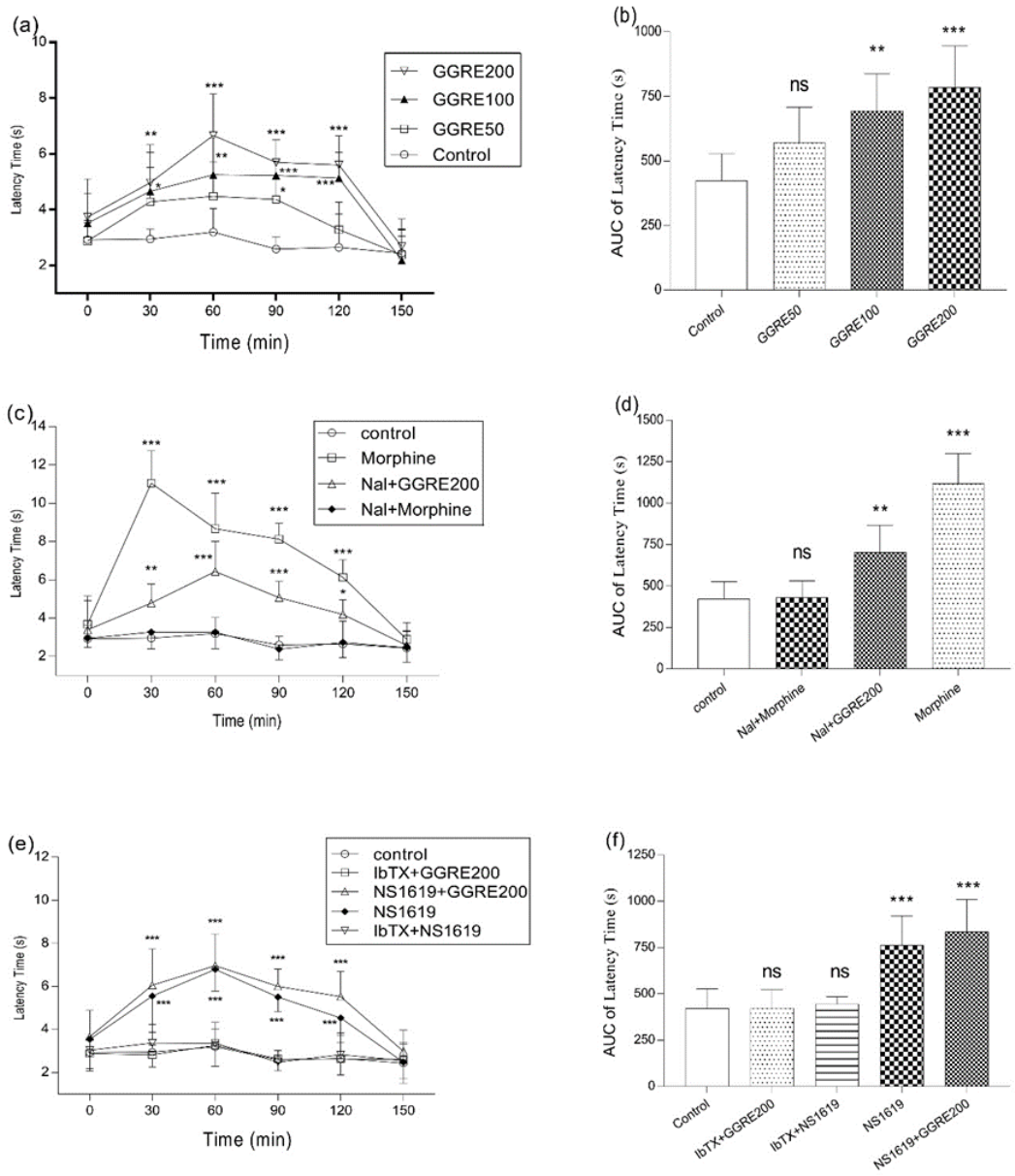

Figure No. 3
The effects of GGRE with or without morphine, NS1619, and IbTX on tail flick latencies
(a) The effect of different doses of GREE (50, 100 and 200 mg/kg) on latency time. (b) AUC calculated for the latency time. (c) The effect of morphine, GGRE (200 mg/kg) and naloxone on latency time. (d) AUC calculated for the latency time. (e) The effect of NS1619, GGRE (200 mg/kg) and IbTX on latency time. (f) AUC calculated for the latency time. Each symbol represents the mean (±SD) latency time for eight rats. *p<0.05, **p<0.01, ***p<0.001 compared with the control group

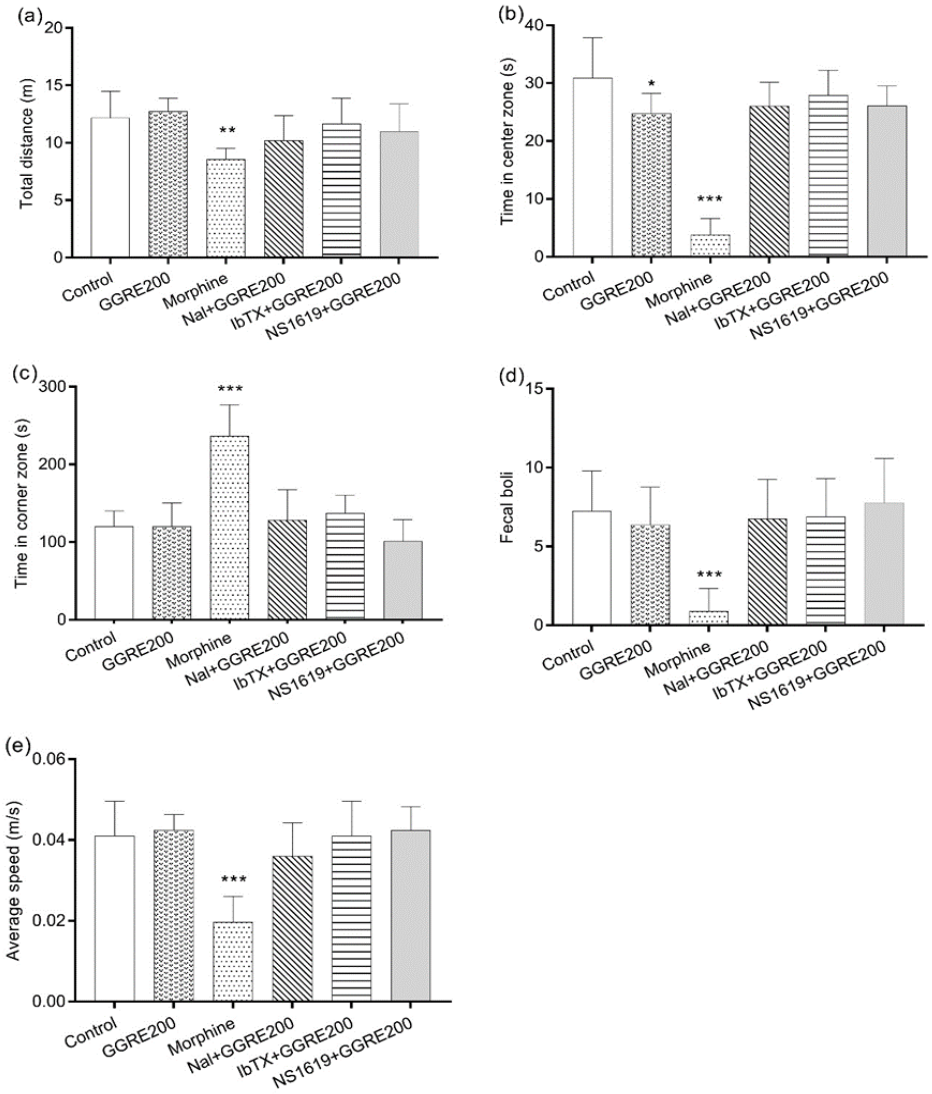

Figure No. 4
The effect of GGRE on the mice's mobility
Effect of administration of GGRE (200 mg/kg), morphine, naloxone+GGRE, IbTX + GGRE, and NS1619 + GGRE on behavioural parameters in mice exposed to the open-field test. (a) total distances, (b) time in central zone (c) time in corner zone (d) fecal boli and (f) average speed. Data are expressed as mean ± SD. Dunnett's test was used to determine significant differences between experimental groups and control group and *p<0.05 ** p<0.01, and *** p<0.001 indicate statistically significant differences

Open field test

As illustrated in Figure No. 4, GGRE and NS1619 had an insignificant impact on mice's motor activity, while morphine had a significant impact on mice's motor activity on open-field test. Using one-way ANOVA was results were found as total distance; $F(5,42) = 4.772$, $p<0.001$, Time in center zone; $F(5,42) = 39.92$, $p<0.001$, Time in corner; $F(5,42) = 19.49$, $p<0.001$, Fecal boli; $F(5,42)=9.055$, $p<0.001$ and Average speed; $F(5,42) = 12.46$, $p<0.001$.

Acetic acid test

As shown in Figure No. 5, the number of writhes over a period of 30 min were evaluated with one-way ANOVA. The results of Tukey's multiple comparison test showed that GGRE (200 mg/kg), NS1619, and morphine inhibited writhing [$F(10,77) = 35.00$, $p<0.001$]. The analgesic effect of morphine

was reversed by naloxone whereas that of GGRE did not. In addition, the effects of GGRE and NS1619 were inhibited by IbTX.

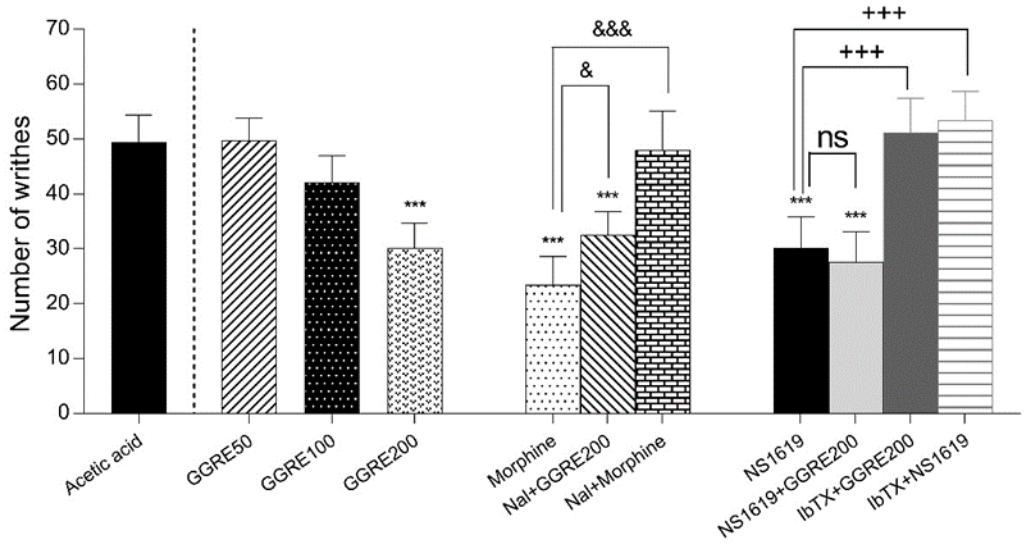

Figure No. 5
The effect of GGRE on Acetic acid induced writhing test
The mice were pre-treated with morphine, GGRE (50, 100 and 200 mg/kg), NS1619, naloxone+morphine, naloxone+GGRE200, IbTX+NS1619, IbTX+GGRE200 and NS1619+GGRE200 at 15 min before an intraperitoneal injection of acetic acid. The results are expressed as the mean ± SD (n=8). Tukey's test was used to determine significant differences between the experimental groups and acetic acid group and ***$p<0.001$ indicate statistically significant differences. $^{\&}p<0.05$ and $^{\&\&\&}p<0.001$ show statistically significance between morphine and the experimental groups. $^{+++}p<0.001$ show statistically significance between NS1619 and the experimental groups ns: non-significant

Formalin test

As presented in Figure No. 6, the licking time in the non-inflammatory phase (0-5 min) and the inflammatory phase (15-30 min) of formalin test were evaluated with one-way ANOVA [$F(10,77) = 18.35$, $p<0.001$ and $F(10,77) = 63.09$, $p<0.001$]. Tukey's multiple comparison test showed that, all of doses of GGRE did not change the paw licking time in the non-inflammatory phase($p>0.05$) whereas the GGRE (200 mg/kg), morphine, naloxone+GGRE (200 mg/kg), NS1619, and NS1619+GGRE (200 mg/kg) reduced the paw licking time in the inflammatory phase when compared to formalin group.

To test if the anti-nociceptive activity of GGRE involved the opioid system, we tested whether the opioid antagonist naloxone would reverse the analgesic effect of the GGRE. To this end, mice were pre-treated with naloxone 15 min before GGRE or morphine administration. With this protocol, naloxone completely abolished the inhibitory effect of morphine on the acetic acid-induced writhing and formalin-induced licking whereas it did not inhibit that of GGRE.

To test if the anti-nociceptive activity of GGRE involved the BK_{Ca} channel, we tested whether the BK_{Ca} channel blocker IbTX would reverse the analgesic effect of the GGRE. To this end, mice were pre-treated with IbTX 15 min before GGRE or NS1619 administration. With this protocol, IbTX completely abolished the inhibitory effect of NS1619 and GGRE on the acetic acid-induced writhing and formalin-induced licking.

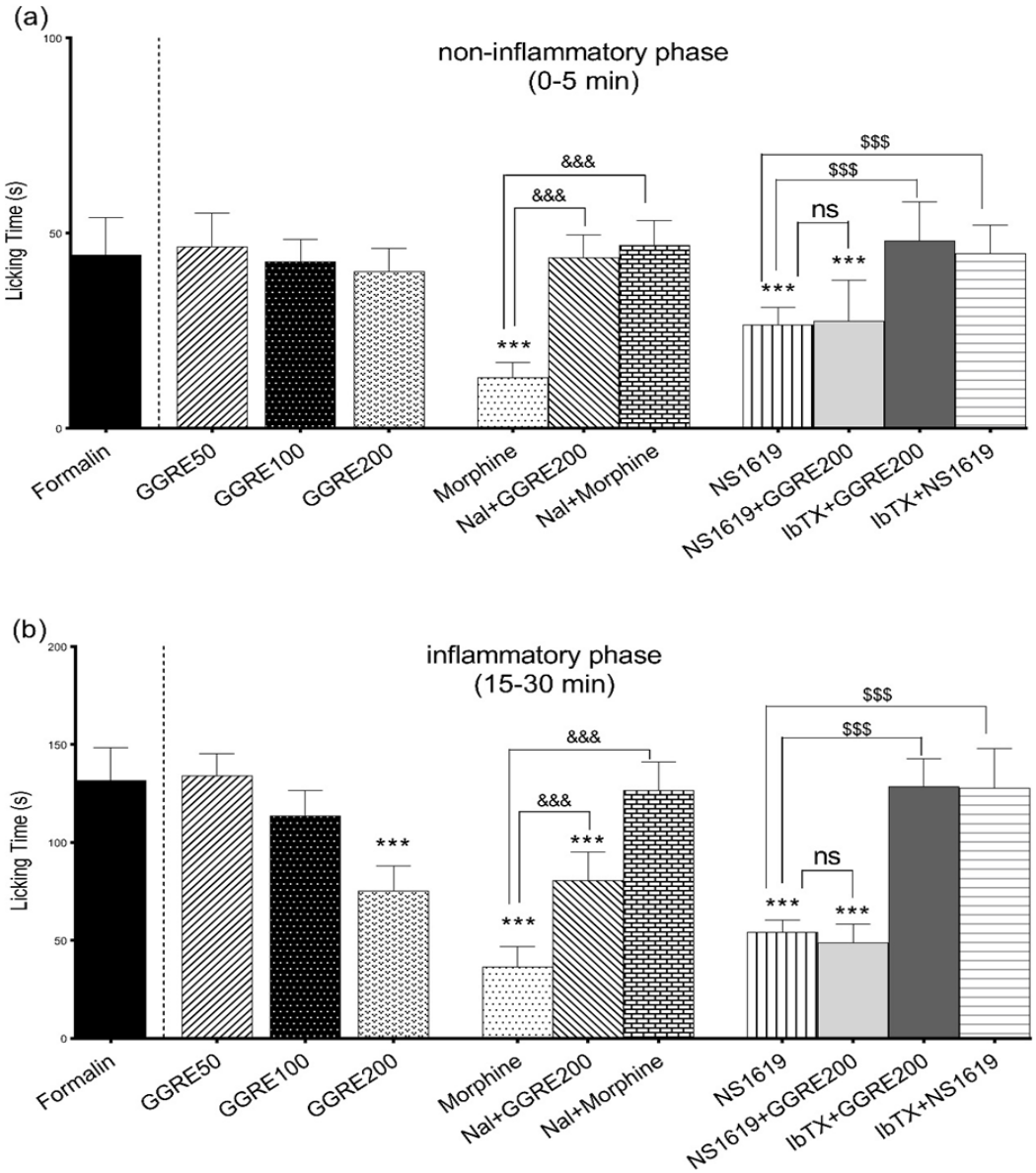

Figure No. 6

Comparison of the anti-nociceptive effects of GGRE, morphine, formalin, naloxone, NS1619, and IbTX in formalin test

The mice were pre-treated with morphine, GGRE (50, 100 and 200 mg/kg), NS1619, naloxone+morphine, naloxone+GGRE200, IbTX+NS1619, IbTX+GGRE200 and NS1619+GGRE200 at 15 min before an intraperitoneal injection of formalin. (a) non-inflammatory phase (0-5 min), (b) inflammatory phase (15-30 min). The results are expressed as the mean ±SD (n=8). Statistical significance was calculated by the analysis of variance followed by Tukey's test. ***p<0.001 relative to the formalin, $^{\&\&\&}p$<0.001 relative to the morphine, and ^{+++}p<0.001 relative to NS1619. ns: non-significant

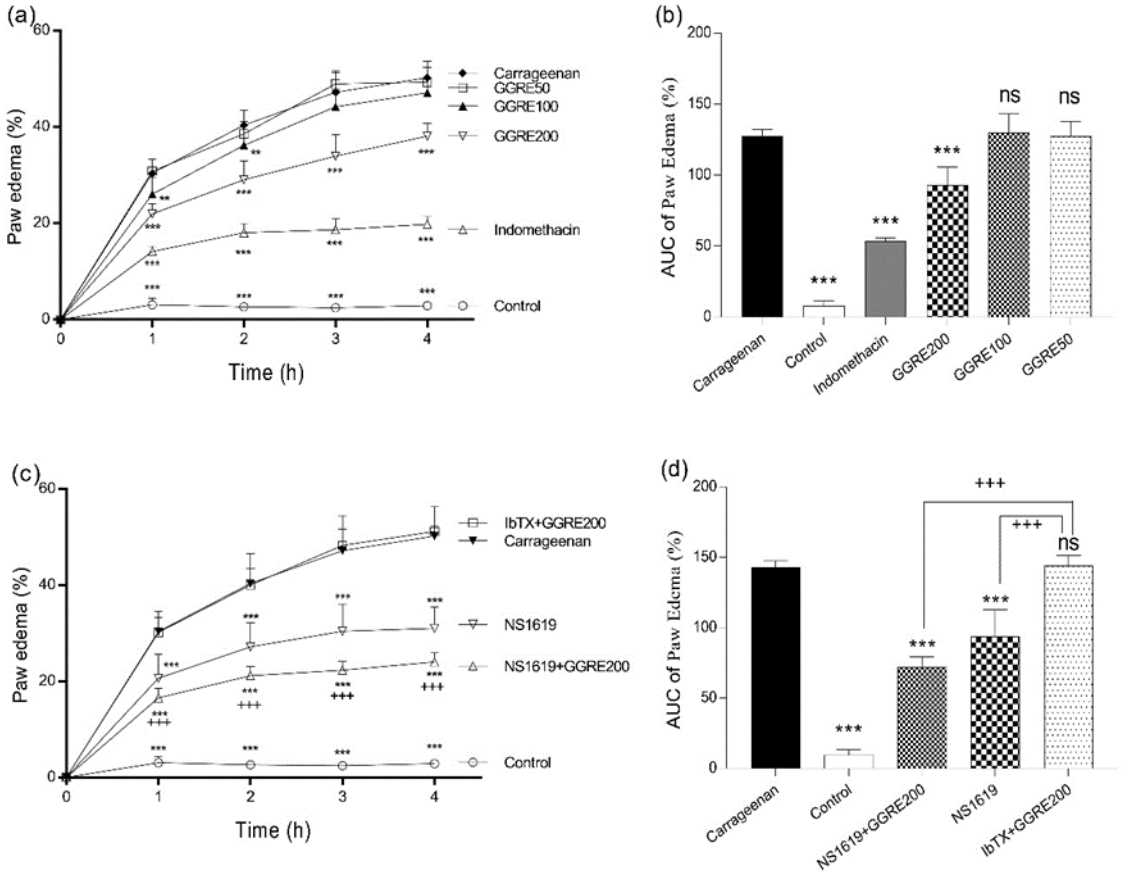

Figure No. 7
The effect of GGRE (50, 100, 200 mg/kg), control, indomethacin, carrageenan (car), IbTX and NS1619 on carrageenan-induced paw edema
(a) The effect of different doses of GREE (50, 100 and 200 mg/kg), carrageenan and indomethacin on rat paw edema. (b) AUC calculated for the paw edema. (c) The effect of GGRE (200 mg/kg), NS1619 and IbTX on rat paw edema. (d) AUC calculated for the paw edema. Each symbol represents the mean (±SD) paw edema (n=8). Statistical significance was calculated by the analysis of variance followed by Tukey's test. **p<0.01, ***p<0.001 compared with the carrageenan group. ^{+++}p<0.001 show statistically significance between IbTX+GGRE and the experimental groups ns: non-significant

Anti-edematous activity

GGRE dose–response analysis

As illustrated in Figure No. 7a, two-way ANOVA for repeated measures showed that GGRE dose dependently decreased paw edema [treatment: $F(5,175)=1005$, $p<0.001$; time: $F(4,35) = 1403$, $p<0.001$; treatment×time interaction: $F(20,175) = 78.38$, $p<0.001$]. The results of Tukey's test demonstrated that carrageenan increased paw thickness at 1, 2 3, and 4th h, which was reversed by indomethacin. GGRE caused a significant decrease in paw thickness dose-dependently but the dose of 50 mg/kg did not reduce the paw thickness. In addition, the one-way ANOVA followed by Dunnett's test for normalized AUC values in Figure No. 7b revealed that oral administration of GGRE dose-dependently decreased paw edema [$F(5,42) = 188.5$, $p<0.001$]. Post-hoc test revealed that only the 200 mg/kg dose of GGRE and indomethacin reduced the paw thickness significantly ($p<0.001$) whereas the other doses of GGRE did not change paw thickness compared to carrageenan group.

The effect of GGRE with or without IbTX on response on paw thickness

To test if the anti-inflammatory activity of GGRE

involved the BK_{Ca} channel, we tested whether the BK_{Ca} channel blocker IbTX would reverse the anti-inflammatory effect of the GGRE. To this end, rats were pre-treated with IbTX 15 min before GGRE or NS1619 administration. As illustrated in Figure No. 7c, two-way ANOVA for repeated measures showed that there was a statistically significant changes between the experimental groups [treatment: $F(5,140) = 626.6$, $p<0.001$; time: $F(4,35) = 484.4$, $p<0.001$; treatment×time interaction: $F(16,140) = 47.92$, $p<0.001$]. The results of Tukey's test demonstrated that IbTX completely abolished the anti-inflammatory effect of NS1619 and GGRE on the carrageenan-induced paw edema. In addition, one-way ANOVA followed by Dunnett's post-hoc test for normalized AUC values were given in Figure No. 7d [$F(5,35) = 192.1$, $p<0.001$]. Post-hoc analysis revealed that NS1619 and GGRE +NS1619 significantly decreased paw thickness ($p<0.001$). IbTX co-administration with NS1619 and with GGRE was non-significant ($p>0.05$) when compared to carrageenan group.

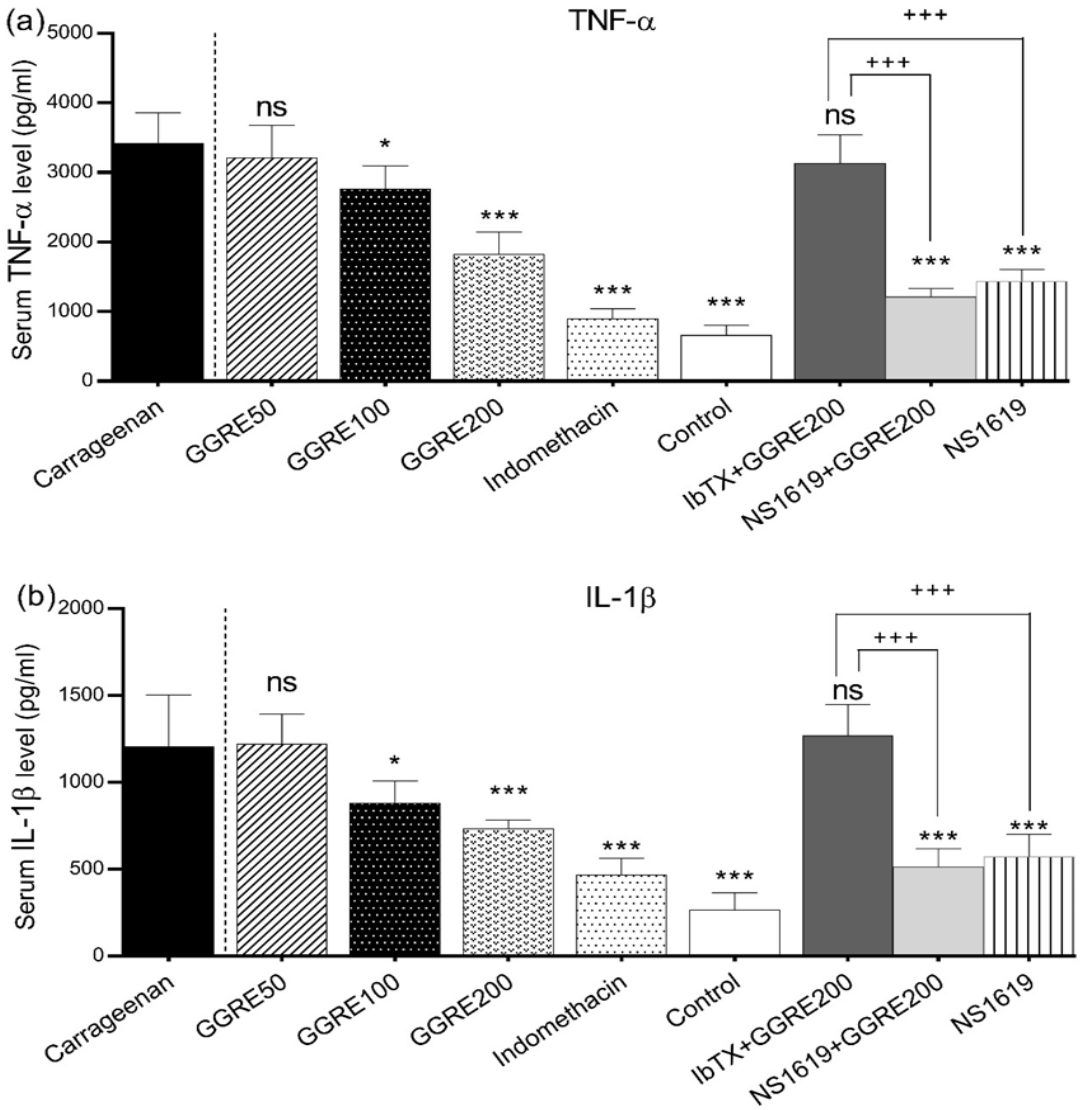

Figure No. 8
The effect of GGRE (50, 100, 200 mg/kg), control, indomethacin, carrageenan (car), IbTX and NS1619 on carrageenan-induced serum cytokines level
(a) serum TNF-α level. (b) serum IL-1β level. Each symbol represents the mean (± SD) cytokines level (n=8). Statistical significance was calculated by the analysis of variance followed by Tukey's test. *$p<0.01$, **$p<0.01$, ***$p<0.001$ compared with the carrageenan group. $^{+++}p<0.001$ show statistically significance between IbTX+GGRE and the experimental groups ns: non-significant

GGRE's effect on cytokine levels in carrageenan-induced paw edema in rats

Evaluating the effect of GGRE, indomethacin, NS1619, and IbTX on serum TNF-α and IL-1β levels in carrageenan-induced paw edema using the One-way ANOVA showed significant effects of the treatments (TNF-α [$F(8,45) = 72.50$, $p<0.001$] and IL-1β [$F(8.45) = 34.70$, $p<0.001$], Figure No. 8). The results of Tukey's test demonstrated that intraplantar carrageenan injection caused a significant increase in serum TNF-α and IL-1β levels in the carrageenan group when compared to the control group ($p<0.001$). Indomethacin, NS1619 and GGRE (200 mg/kg) decreased serum TNF-α and IL-1β levels compared with the carrageenan group ($p<0.001$). IbTX reversed the effect of GGRE and NS1619 on TNF- α and IL-1β levels.

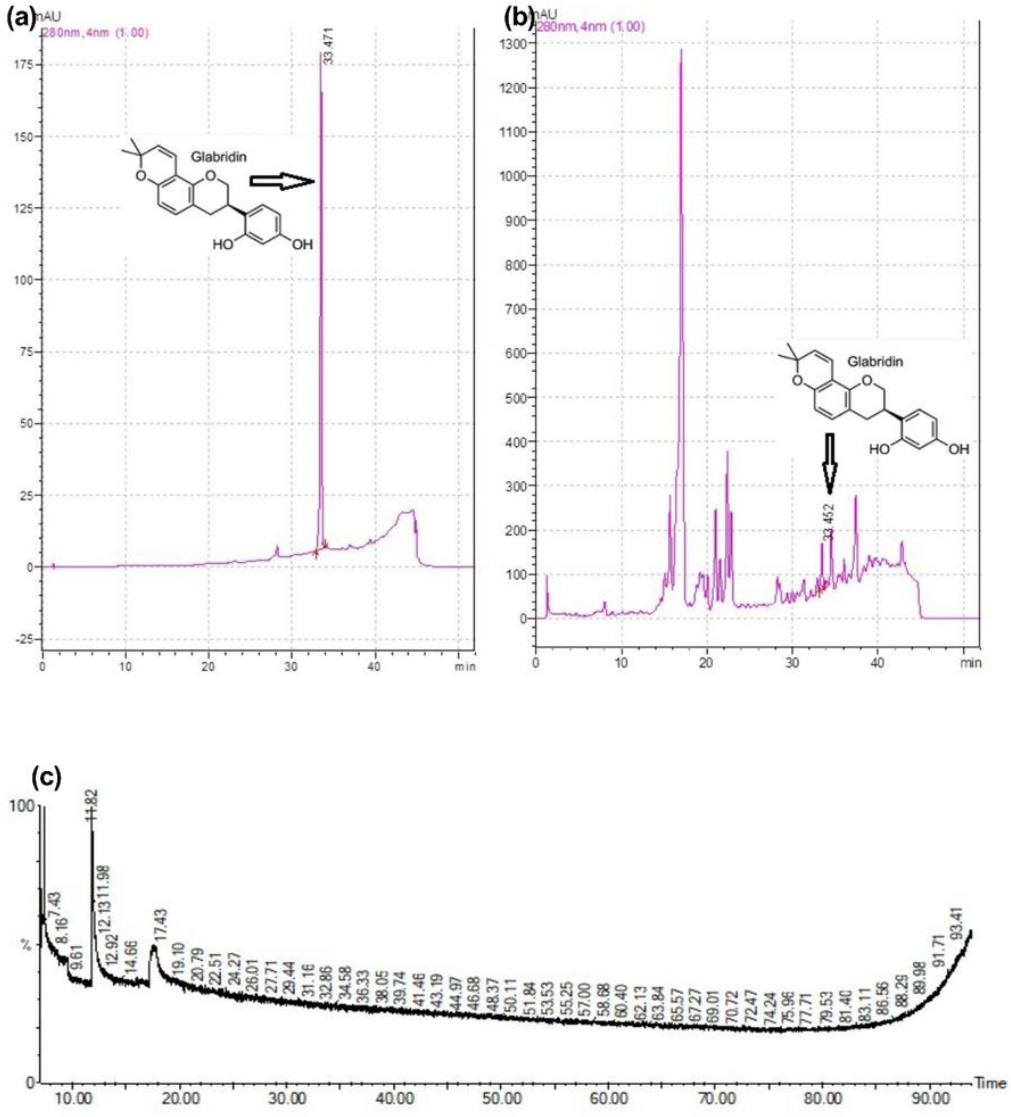

Figure No. 9
Phytochemical content of the GGRE by HPLC and GC-MS
In the figure it was shown the HPLC chromatogram of standard glabridin (a) and glabridin in GGRE (b) and was exhibited GC-MS chromatogram of phytochemicals determined in the derivatized GGRE (c)

Photochemical content of GGRE determined by HPLC and GC-MS

In the analysis made by using HPLC, Glabridin in GGRE was determined in value of 0.18 ± 0.01 mg/100mg in the GGRE (Figure No. 9a). Moreover, with scanning by GC-MS (Figure No. 9b), a total of 17 compounds were detected in the derivatised GGRE and they consisted of 92.817% of GGRE (Table No. 2). Oxirane, 2.4-hexadiyne and silanamine were determined as the main components in GGRE.

Table No. 2
Chemical composition of the GGRE. RT: retention time (as min), compound: listed in order of retention time

NO	RT	Compound	Area (%)
1	7,339	Silanamine, 1,1,1-trimethyl-N-	15,882
2	11,822	2,4-Hexadiyne	18,634
3	17,644	Oxirane, trimethyl-	50,588
4	43,913	2-Pentenedinitrile	3,659
5	45,041	2-Propenoyl chloride	0,928
6	48,488	1,4-Pentadien-3-one	0,273
7	49,643	Furazan, dimethyl	0,787
8	51,055	2-Chloroethyl 1-propynyl	0,743
9	51,734	Ethoxyacetylene	0,182
10	52,449	2-Propenoic acid	0,348
11	53,696	Ethene, chloro-	0,148
12	54,383	4-(4-Chlorophenyl)-3-	0,116
13	55,42	3,6-Dioxaoctanedioic acid	0,165
14	56,41	Ethenesulfonyl chloride	0,113
15	57,868	Hydrogen cyanide	0,132
16	58,491	Lupulon	0,066
17	59,656	3,8,12-Tri-O-acetylingol 7	0,053
		TOTAL	92,817

DISCUSSION

In this study it was demonsrated the role of BK_{Ca} channels on the anti-edema, analgesic and anti-nociceptive effects of an ethanolic extract of *G. glabra* root with evidences obtained from the hot plate, tail-flick, open-field, acetic acid, and formalin tests. *G. glabra* roots have been widely used in traditional medicine worldwide for 4,000 years. In some countries, such as Syria, Iraq, Iran, and Turkey, *G. glabra* decoction and infusionshave been used in herbal medicine. It is also used for detoxification and to treat injuries (Cherng *et al.*, 2006). Althouh it is known that *G. glabra* has anti-inflammatory effect, its mechanism is needed to be explained. Thus, the current study was conducted to reveal the mechanism of the anti-nociceptive and anti-inflammatory effects of GGRE.

The hot plate test was used to determine whether GGRE has an analgesic effect and to discuss its mechanism of action. The results indicate that the GGRE possesses an analgesic effect and exerts its maximal effect at about 90 min after oral administration. This effect was observed 30 min after the administration in another study (Bhandage *et al.*, 2009) but it was not clarified the mechanism of action. The difference might be due to using different routes of administration. In our study, the maximum analgesic effect of morphine given subcutaneously started within 30 min and its effect continued to 120 min after administration. In a previous study maximal effect of morphine was shown about60 min after the administration (Oliveira *et al.*, 2018). In the present study, maximum analgesic effect of 200 mg/kg of GGRE was observed 60 min after administration, indicating relatively rapid onset of analgesic effect. As in previous studies, co-administering morphine

with naloxone has inhibited analgesic effect of morphine by competing to bind opioid receptors responsible for morphine's analgesic effect (Sajid *et al.*, 2017). In this study, naloxone did not change the analgesic effect of GGRE, while inhibiting the effect of morphine indicates that GGRE' analgesic effect is not related to morphine receptors

By stimulating nociceptors by pain, fibers Aδ and C of the dorsal root ganglion (DRG) neurons convey the pain signals to the laminated I - II of the spinal cord. Substance P (SP) and calcitonin gene-related peptide (CGRP), expressed in the small DRG neurons and secreted from their afferent C and Aδ fibers in the laminate I-II of the spinal cord, plays an important role in response to nociceptive stimulation whereas in addition to the opioid peptides, serotonin, norepinephrine and dopamine 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 (Millan, 2002). Recent studies suggest that BK_{Ca} channels play a role in the transmission of pain. Zhang *et al.* (2010), showed that BNP inhibited the excitability of small DRG neurons by activating the BK_{Ca} channels in formalin test (Zhang *et al.*, 2010) 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 model of inflammatory pain induced by complete Freund's adjuvant. Glabridin, one of the main components of *G. glabra*, is known to alleviate inflammation by activating BK_{Ca} channels and increasing intracellular cGMP. To determine whether BK_{Ca} channels have a role in the anti-nociceptive and anti-inflammatory effects of GGRE or not, IbTX (a BK_{Ca} channel inhibitor) was given with either GGRE or NS1619 (BK_{Ca} channel opener). IbTX abolished the anti-nociceptive and anti-inflammatory effects of both NS1619 and GGRE.

The results of the tail-flick test support those of the hot plate test. In this study, using the tail-flick test, the morphine's maximum analgesic effect was seen after 30 min. This effect continued until 120 min and then disappeared within 150 min after administration. Previous studies have also found this time to be 30 min (Sajid *et al.*, 2017). The analgesic effect of 200 mg/kg of GGRE was seen at 30, 60, 90, and 120 min, while that of the 100 mg/kg GGRE was observed at 60, 90, and 120 min. Conversely, in a study used *G. glabra* extract (Bhandage *et al.*, 2009) found that its analgesic effect reached the peak level within 30 min, but this effectwas not seen at 60 min with orally administered GGRE. Wu *et al.* (2013), attributed this difference to the administration route in their pharmacokinetic study of GGRE (Wu *et al.*, 2013).

Lu *et al.* demonstrated that persistent inflammatory pain was inhibited by activating BK_{Ca} channels with NS1619 (Lu *et al.*, 2014). According to our results, the anti-nociceptive effect of GGRE, that was not abolished by naloxone, was abolished by IbTX. Therefore, BK_{Ca} channels might play an important role on anti-inflammatory mechanism of GGRE in pain.

The results of open-field test indicate that GGRE, NS1619, IbTX had no significant effect on the mice's locomotor system. Results of previous studies was similar to our study results (Seredenin *et al.*, 2013). In our study, morphine caused a decrease in mice's motor activity and anxiety, which is similar to the results of a previous study (Wang *et al.*, 2017). The present study showed that the effects of the morphine were reversed by naloxone, consistent with the results reported in the literature (Seredenin *et al.*, 2013). Since systemic administration of NS1619 does not readily cross the blood-brain-barrier, NS1619 might not cause anxiety or impair motor activity (Lu *et al.*, 2014).

Acetic acid is used in writhing tests since it causes, releasing certain pain-producing agents from the terminal nerve endings such as prostaglandin without tissue damage. This agent mediates pain signals to reach higher centers through spinal nerves. Based on these properties, the writhing test is applied to determine both central and peripheral analgesic effects (Yu *et al.*, 2019). In the present study, it was shown GGRE has antinociceptive effect at dose of 200 mg/kg, but was not as effective as morphine and NS1619. The anti-nociceptive effect of GGRE was not inhibited by naloxone whereas reversed by IbTX. As a result, the anti-nociceptive effect of GGRE might be mediated by BK_{Ca} channels, not opioid receptors. In a study using various inflammatory tests such as hot plate, capsaicin and formalin, it was founded that BK_{Ca} channel opener improves inflammatory pain by activation of channels in small DRG neurons (Lu *et al.*, 2014).

In a study it has been found that glycyrrhizin has an anti-inflammatory effect on lung injury induced by lipopolysaccharide (Lee *et al.*, 2019). *G. glabra* extract may not have an effect on release of substance P and bradykinin, both seen in the early non-inflammatory phase, but GGRE may exert its anti-nociceptive effect via inhibiting the release of

various chemicals, such as histamine, kinins, serotonin, and prostaglandins in the inflammatory phase, suggesting by Khaksa *et al*. (1996). In the present study, we used paw licking test with formalin to investigate anti-nociceptive effect of GGRE. Although morphine wasshown to has anti-nociceptive effect in bot phases, consistent with previous studies (Lu *et al.*, 2014) GGRE were evidenced has anti-nociceptive effect for 15-30 min in the inflammatory phase but in the non-inflammatory phase. Naloxone inhibited the anti-nociceptive effect of morphine in both phases. The anti-nociceptive effect of GGRE was reversed by IbTX, but not by naloxone. Based on these results, it revealed the role of BK_{Ca} channels on the monophasic anti-nociceptive effect of GGRE.

The paw edema model induced by carrageenan was used to evaluate the potential anti-inflammatory effect of GGRE on rats. The anti-inflammatory effect of GGRE was observed at 1 h after carrageenan administration and reached peak level at 3^{th} h and maintained for 4 h, in dose-dependent manner. In a similar study it was shown that this effect to be as much as that of indomethacin, a NSAID. (Lee *et al.*, 2019). They suggest that the anti-inflammatory effect of GGRE might be via inhibiting the synthesis and release of kinins and prostaglandins. Also, BK_{Ca} channels might play an important role of anti-inflammatory effect of GGRE. Lu *et al.*, showed that, BK_{Ca} channels play an important role in the control of inflammatory pain in sensory neurons, by activating BK_{Ca} channels with NS1619 in BK_{Ca} knockout mice (Lu *et al.*, 2014). In our study, as the anti-inflammatory effect of GGRE at 200 mg/kg was found as much as NS1619, BK_{Ca} channel opener, and inhibited by IbTX, we suggest that the anti-nociceptive effect of GGRE may be mediated by BK_{Ca} channels. In addition, IL-1β and TNF-α levels in serum were measured to support behavioral findings with biochemical findings.

TNF-α enhancing the release of leukotriene and kinins, causes lasting nociceptive response (McKinnon *et al.*, 2014). IL-1β is an essential cytokine in the regulation of the inflammatory response. It is directly related to hyperplasia, increasing the expression of adherence factors among endothelial cells to mediate leukocyte migration (Langer & Chavakis, 2009). Furthermore, Dai *et al.* (2017), demonstrated that activation of BK_{Ca} channels increases oxidant production and increases heme oxygenase-1 activity, preventing ischemia-reperfusion-induced inflammation and mucosal barrier disruption in the small intestine (Dai *et al.*, 2017)

The potential bioactivities including antioxidant, anti-inflammatory, antitussive, antimicrobial, antiviral, hepatoprotective, neuroprotective, sedative, antidepressive, oestrogenic, anticarcinogenic and antimutagenic of the bioactive components of *G. glabra* such as liquiritin, isoliquiritin, glycyrrhizin, liquiritigenin, isoliquiritigenin, 18β-glycyrrhetinic acid, liquiritin apioside, glycyrrhetic acid and glabridin are reported in a review by Pastorino *et al.* (2018). In the present study, glabridin content of *G. glabra* root extract was determined by HPLC. The glabridin, most investigated compound of *G. glabra*, has been found to have various bioactivities including anticancer (Hsieh *et al.*, 2016), antiobesity (Ahn *et al.*, 2013), antifungal (Fatima *et al.*, 2009), antinephritis and radical scavenging (Fukai *et al.*, 2003). In our study, however, none of the compounds determined in GGRE were tested in experimental pain models used. Therefore, in order to say whether those substances have a role in tested activities or not, they are -needed be tested individually in further studies.

CONCLUSION

GGRE may alleviate pain by activating BK_{Ca} channels in sensory afferent nerves without side effects on Central nervous system. The novelty of this study is that, GGRE has been shown to have a functional contribution to BK_{Ca} channels expressed in the peripheral nervous system in nociception and inflammation models in rodents *in vivo*. In addition, GGRE reduced inflammation and associated pain by activating BK_{Ca} channels, reducing cytokine levels such as TNF-α and IL1β.

The limitation of this study is that the roles of the cholinergic pathway, TRPV receptors, endothelial permeability, nitric oxide, oxidative stress markers have not been investigated.

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