

Analgesic and Anti-Inflammatory Activities of the Methanol Extract of *Kalanchoe gracilis* (L.) DC Stem in Mice

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Abstract: In this study, we evaluated the analgesic effect of the methanol extract of *Kalanchoe gracilis* (MKGS) stem in animal models by inducing writhing response with acetic acid and conducting formalin test. The anti-inflammatory effect of MKGS was also estimated on mice with λ -carrageenan induced paw edema model. In order to investigate the anti-inflammatory mechanism of MKGS, we analyzed the activities of glutathione peroxidase (GPx) and glutathione reductase (GRx) in the liver, and the levels of interleukin-1 β (IL-1 β), tumor necrosis factor (TNF- α), malondialdehyde (MDA) and nitric oxide (NO) in the edema paw tissue. In the analgesic tests, MKGS (0.5 and 1.0 g/kg) decreased both the acetic acid-induced writhing response and the licking time in the late phase of the formalin test. In the anti-inflammatory test, MKGS (0.1, 0.5 and 1.0 g/kg) decreased paw edema at the third, fourth, fifth and sixth hours after λ -carrageenan had been administrated. Furthermore, MKGS increased the activities of SOD and GRx in liver tissues and decreased MDA level in the edema paws three hours after λ -carrageenan was injected. MKGS also affected the levels of IL-1 β , TNF- α and NO induced by λ -carrageenan. All these results suggested that MKGS possessed analgesic and anti-inflammatory effects. The anti-inflammatory mechanism of

MKGS might be related to the lowering of MDA level in the edema paw via increasing the activities of superoxide dismutase (SOD) and GRx in the liver, as well as the decreases in the levels of TNF- α and NO, and the production of IL-1 β in inflamed tissues.

Keywords: *Kalanchoe gracilis*; Anti-Inflammation; Analgesia; Malondialdehyde; Nitric Oxide; Tumor Necrosis Factor- α ; Eupafolin.

Introduction

Kalanchoe gracilis (L.) DC (Crassulaceae) is a perennial, succulent and glabrous plant used as a medicinal herb in the treatment of injuries, pain, fever and inflammation in traditional Chinese medicine (Kao, 1981; Kan, 1958). In Taiwan, this herb, commonly known as Da-Huan-Hun, is also called “Da Hao Ji Zhao Huang” among the general public, meaning “chicken claws,” due to the morphology of the leaves (Huang, 1998). In previous phytochemical studies, major constituents of the aerial parts of *K. gracilitha* had been isolated, one coumarin and nine flavonoids, which were later identified as luteolin, quercetin, quercitrin, kaempferol, eupafolin and four glycosidic derivatives of eupafolin (Karin *et al.*, 1989). Eight bufadienolides were also analyzed, in which three were newly discovered compounds, kalanchosides A (1), B (2), and C (3) (Wu *et al.*, 2006).

Flavonoids are known to possess anti-inflammatory effects (Gabor, 1986; Lai *et al.*, 2009). Therefore flavonoid constituents of this herb may be important for its medicinal effects. Previous researches have shown that λ -carrageenan induced inflammatory effect is associated with free radicals. Free radicals, prostaglandin and NO will be released after λ -carrageenan is administered for 1–6 hours (Dudhgaonkar *et al.*, 2006). The edema effect usually reaches the maximum at the third hour (Kirkova *et al.*, 1992). In addition, the production of MDA is due to free radicals attacking the plasma membrane (Janero, 1990). Thus, inflammation would result in the accumulation of MDA (Lu *et al.*, 2007). Therefore, the aims of this study were to investigate the effects of KG stem methanol extract on pain induced by acetic acid and formalin, and inflammation induced by λ -carrageenan on mice. In order to evaluate the mechanisms of analgesic and anti-inflammatory effects of MKGS, we also analyzed the levels of β L-1 β , α NF- α , MDA and NO in edema paw tissues and the activities of SOD, GPx and GRx in the liver three hours after λ -carrageenan had been injected.

Materials and Methods

Plant Material and Crude Extract Preparation

KG was collected from farmlands and gardens in Chiayi County, Taiwan as described in Flora of Taiwan. The plants were identified by professor Hsin-Fu Yen, National Museum of Natural Science, Taichung, Taiwan. A plant specimen has been deposited in the Institute. After the stems were cut into small pieces, they were further crushed into slurry. The slurry of

KGS (12.8 kg) was extracted with methanol three times. The methanol extract was evaporated under reduced pressure with a rotary evaporator, yielding 3.01% (385 g) of crude methanol extract (MKGS), then stored in a light protected condition.

Chemicals

λ -carrageenan, indomethacin and Griess reagent were purchased from Sigma-Aldrich Chemical Co. Formalin was purchased from Nihon Shiyaku Industry Ltd. SOD, GPx, GRx and MDA assay kits were purchased from Randox Laboratory Ltd. The enzyme immuno-metric assay kit for mouse IL-1 β and TNF- α was obtained from Assay Designs Inc. LC grade methanol was from Labscan (Ireland). All other reagents used were of analytical grade.

Phytochemical Analysis of MKGS by LC/MS/MS

LC/MS/MS experiments were carried out on a Quattro Ultima tandem mass spectrometer coupled with a Waters 2695 Alliance LC & 996 PDA with an automatic liquid sampler and an injector. Data was acquired using MassLynx 4.0 software. Chromatographic separation was performed on a Cosmosil 5C18-MS (15 cm \times 4.6 mm I.D., 5 μ m) reverse phase column (Nacalai Tesque, Kyoto, Japan) with the injection volume of 10 μ l. The mobile phase consists of mixture of 0.25% formic acid (A) and methanol (B) using a gradient elution. The gradient program was set as follows: 0–10 min, 10% B to 30% B; 10–15 min, 30% B to 50% B; 15–20 min, 50% B; and 20–25 min, 50% B to 90% B. The flow rate was set at 0.5 ml/min. The interface between HPLC and mass spectrometer was atmospheric pressure ionization source with the electrospray inlet operated in the positive mode. The column effluent was split 1:1 into the photodiode detector and tandem mass spectrometer. The mass spectrometer parameters were set as follows: capillary voltage 3 kV, the ion source temperature 120°C, desolvation temperature 350°C, cone gas (nitrogen) flow 50 L/hours, desolvation gas (nitrogen) flow 500 L/hours, ion energy 1.0 V and multiplier 600 V. The parent ion ($[M+H]^+$) for each standard was obtained from MS scanning mode by tuning the cone voltage (V) while direct infusing the standard solution into the mass spectrometer. Subsequently, daughter ion scan mode was carried to get its daughter ions resulted from the fragmentation of the precursor ion. Different collision energy (eV) was applied to obtain the optimal daughter ion spectrum, which was used to establish the mass spectra library. Argon was used as collision gas at a pressure of $3\text{--}4 \times 10^{-3}$ mbar. In this study, the cone voltage/collision energy was set at 50 V/27 eV. The optimal parameters of daughter ion scan mode for them were saved as a mass file for the subsequent identification work. Multiple reactions monitoring (MRM) with specific parent/daughter ion transition was used for quantitation. Similarly, MRM method containing transition ions, dwell time, cone voltage and collision energy was set up for the following work. The transition (precursor to product ion) monitored was m/z 317.0 \rightarrow 302.0. The dwell time per transition was 0.25 sec.

Experimental Animals

Male ICR mice (20~25 g) were purchased from BioLasco Charles River Technology, Yilan, Taiwan. The mice were kept in the animal center of China Medical University at $22 \pm 1^\circ$, relative humidity $55 \pm 5\%$, with light dark cycles of 12 hours (08:00 to 20:00) for 1 week before the experiment. Animals were provided with rodent diet and clean water *ad libitum*. All studies were conducted in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. The experimental protocol was approved under the code 96-195-S by the Committee on Animal Research, China Medical University. Placebo groups were administered orally with 0.1 ml/10 g body weight (BW) saline. All tests were conducted under the guidelines of the International Association for the Study of Pain (Zimmermann, 1983).

Acetic Acid-Induced Writhing Response

The writhing test in mice was carried out using the method of Koster *et al.* (1959). The writhes were induced by intraperitoneal injection of 1.0 % acetic acid (v/v, 0.1 ml/10 g body weight). Different doses (0.1, 0.5 and 1.0 g/kg) of MKGS were administered orally to separate groups of mice, 60 min before the chemical stimulus. Indomethacin, the positive control, was administered 30 min prior to the acetic acid injection. The number of muscular contractions for each group was counted over a period of 10 min after the acetic acid injection, and was expressed as writhing number.

Formalin Test

The model used in this study was similar to that described previously (Shibata *et al.*, 1989). Twenty μ l of 5% formalin was injected subcutaneously into the right hind paw of each mouse. The time (in sec) spent on licking and biting on the injected paw was taken as an indicator for pain response. Responses were measured for 5 min after formalin injection (early phase) and once more 20–30 min after formalin injection (late phase). MKGS (0.1 g/kg, 0.5 g/kg and 1.0 g/kg, p.o.) were administered 60 min before formalin injection. Indomethacin (10 mg/kg, i.p.) was administered 30 min before formalin injection. The control group received the same volume of saline through oral administration.

λ -Carrageenan-Induced Mice Paw Edema

The anti-inflammatory activity of MKGS was evaluated by λ -carrageenan-induced edema test in the hind paws of mice. Male ICR mice (10 per group), were fasted for 24 hours before the experiment, provided only with free access to water. Fifty μ l of 1% λ -carrageenan suspension in saline was injected into the plantar side of the right hind paws of mice (Winter *et al.*, 1962). Paw volumes were measured at 1-, 2-, 3-, 4-, 5- and 6-hours after the administration using a plethysmometer. The degree of swelling was evaluated by the delta volume ($a-b$), in which a and b were the volumes of the right hind paw after and before the

λ -carrageenan treatment respectively. Indomethacin, used as a positive control, (Mascolo *et al.*, 1989), was administered 90 min after the λ -carrageenan injection. MKGS was administered 120 min after λ -carrageenan injection. In the secondary experiment, the whole right hind paw tissues and liver tissues were taken at the third hour. The right hind paws were rinsed in ice-cold normal saline, and immediately placed in cold normal saline four times their volumes to be homogenized at 4°. The homogenates were then centrifuged at 12,000 g for 5 min. The supernatants, which would later be used for the processing of IL-1 β , TNF- α , NO and MDA assays, were stored at -20°C in a refrigerator. Next, whole liver tissues were rinsed in ice-cold normal saline, and immediately placed in cold equal volume normal saline and homogenized at 4°C. The liver homogenates were then centrifuged at 3,000 rpm for 5 min. The supernatants were stored in the -20°C refrigerator, and would later be used in antioxidant enzyme (SOD, GPx, and GRx) activity assays.

NO Assay

NO was measured according to the method of Moshage *et al.* (1995). For nitrite determination, NO³⁻ was converted into nitrite after enzymatic conversion by nitrate reductase; NO²⁻ was measured by the Griess reaction (Green *et al.*, 1982). Values obtained by this procedure represented the sums of nitrite and nitrate.

MDA Assay

MDA was evaluated by the thiobarbituric acid reacting substance (TRARS) method (Tatum *et al.*, 1990). In brief, MDA reacted with thiobarbituric acid in an acidic environment in high temperature, forming a red-complex TBARS. The absorbance of TBARS was determined at 532 nm.

Measurements of Antioxidant Enzyme Activities

The following anti-oxidative enzymes were analyzed to detect antioxidant activities of MKGS by the methods described. SOD activity was determined according to the method of Misra and Fridovich (1972). At room temperature, 100 μ l tissue extract was added to 880 μ l (pH 10.2, 0.1 mM EDTA) carbonate buffer. Twenty μ l of 30 mM epinephrine (in 0.05 % acetic acid) was added to the mixture and measured at 480 nm for 4 min with a Hitachi U 2000 Spectrophotometer. The enzyme activity was expressed as the amount of enzyme that inhibited the oxidation of epinephrine by 50% which was equal to 1 unit. GPx activity was determined according to the method of Flohe and Gunzler (1984). A reaction mixture was composed of 500 μ l phosphate buffer, 100 μ l 0.01 M GSH (reduced form), 100 μ l 1.5 mM NADPH and 100 μ l GSH-Rx (0.24 units). The tissue extract (100 μ l) was added to the reaction mixture and incubated at 37° for 10 mins. Then 50 μ l of 12 mM *t*-butyl hydroperoxide was added to 450 μ l tissue reaction mixture and was measured at 340 nm for 180 sec. The molar extinction coefficient of 6.22×10^{-3} was used to determine the enzyme activity. One unit of the activity was equal to the millimole of NADPH

oxidized/min per mg protein. GRx activity was determined by following the method of Carlberg and Mannervik (1985) at 37°C. Fifty μl of NADPH (2 mM) in 10 mM Tris buffer (pH 7.0) was added in a cuvette containing 50 μl of GSSG (20 mM) in phosphate buffer. 100 μl of tissue extract was added to NADPH-GSSG buffered solution and was measured at 340 nm for 3 min. The molar extinction coefficient of 6.22×10^{-3} was used to determine GRx activity. One unit of the activity was equal to the millimole of NADPH oxidized/min per mg protein.

Measurements of IL-1 β and TNF- α

IL-1 β and TNF- α were measured by enzyme-linked immunosorbent assay. The assay was carried out in accordance with the manufacturer's instructions (Ataoglu *et al.*, 2002). The amount of IL-1 β and TNF- α were determined with respect standard curves (0–1000 pg/ml) constructed for each assay. The concentration of IL-1 β and TNF- α in each sample were expressed as picogram per milligram of protein (pg/mg) for cytokine concentration.

Statistical Analysis

All the data were represented as mean \pm SEM. Data were analyzed by the software of SPSS via one-way ANOVA followed by Scheffe's multiple range test. The criterion for statistical significance was $p < 0.05$.

Results

Phytochemical Analysis of MKGS

Eupafolin was used as the marker component for the standardization of flavonoid ingredients of MKGS by using LC/MS/MS. The retention time of eupafolin was found at 18.06 min in Figs. 1(A) and 1(C). The identity of eupafolin in MKGS was further confirmed by the same daughter ion pattern at 302.0 and 317.1 in Figs. 1(B) and 1(D).

Effect of MKGS on Acetic Acid Induced Writhing Response

The analgesic activity of MKGS in mice writhing responses induced by acetic acid is presented in Fig. 2. It was found that MKGS (0.1–1.0 g/kg) and indomethacin (10 mg/kg) caused significant ($P < 0.05$ –0.001) inhibitions on the writhing responses induced by acetic acid in comparison to the control.

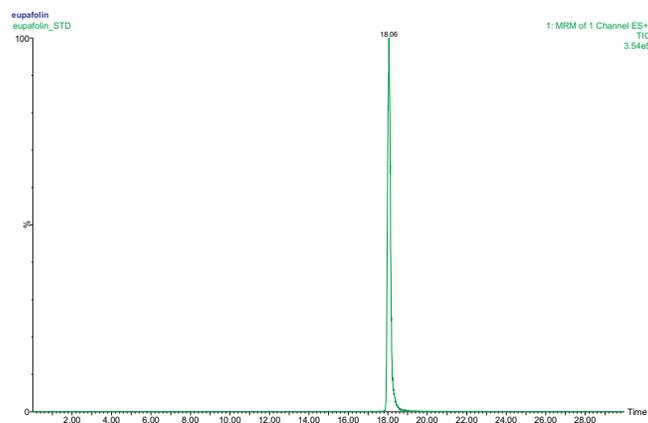
Formalin Test

In the early phase, there were no significant inhibitions produced at the doses of 0.1, 0.5, 1.0 g/kg MKGS compared to the control group (Fig. 3(A)). In the later phase, however, doses of 0.1, 0.5 and 1.0 g/kg MKGS significantly reduced nociceptive responses, similar

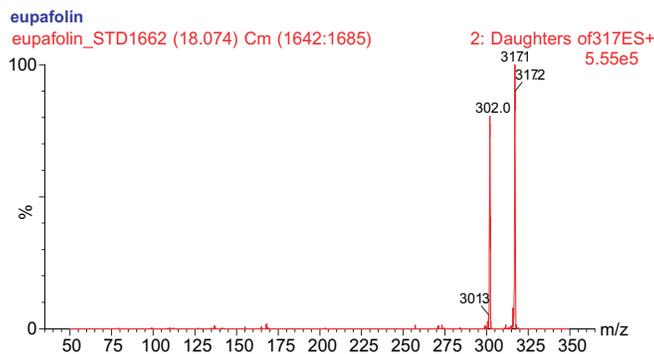
to indomethacin (10 mg/kg) (Fig. 3(b)). MKGS demonstrated a dose-dependent relationship in the late phase of formalin-induced pain.

Effects of MKGS on λ -Carrageenan-Induced Mouse Paw Edema

After the third, fourth, fifth, and sixth hours, λ -carrageenan-induced paw edema was significantly reduced in MKGS pretreated mice in a dose dependent manner ($p < 0.01$).



(A)



(B)

Figure 1. MRM chromatogram of eupafolin and MKGS and mass spectrum of eupafolin and MKGS from LC/MS/MS analysis. (A) The MRM chromatogram of eupafolin of LC/MS/MS analysis. (B) The full mass spectrum of eupafolin from m/z 50 to m/z 350. (C) The MRM chromatogram of MKGS of LC/MS/MS analysis. (D) The library search result for eupafolin. The peak at retention time of 18.1 min was processed by Masslynx 4.0 software and further compared to the mass spectra database. The R score means reverse peak search. The higher R score indicates higher similarity of mass spectra between unknown compound and pure compound in the mass database.

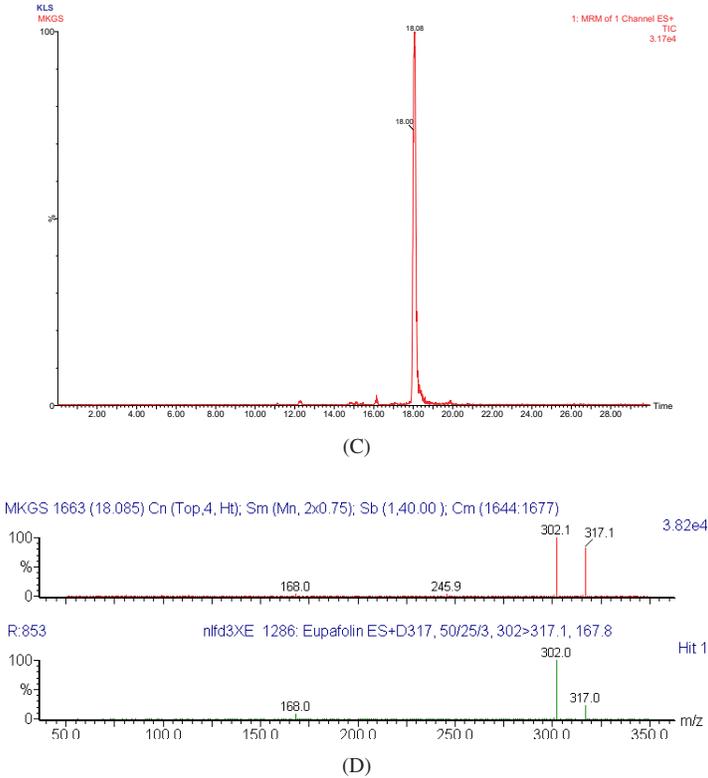


Fig. 1. (Continued)

Three hours after λ -carrageenan injection, anti-inflammatory effect of MKGS reached the maximum ($p < 0.01$). Anti-inflammatory effects of MKGS at the dose of 0.1, 0.5 and 1.0 g/kg were significant, similar to indomethacin (Fig. 4).

Effects of MKGS on NO Level

NO level in the λ -carrageenan induced edema paw was dramatically increased. However, MKGS at doses of 0.5 and 1.0 g/kg significantly decreased the NO levels. Similarly, indomethacin treatment at 10 mg/kg also dramatically decreased the NO level (Fig. 5).

Effects of MKGS on MDA Level

MDA level in λ -carrageenan induced edema paw was increased significantly. However, the MDA level was markedly decreased by treating with 0.1, 0.5 and 1.0 g/kg MKGS as well as 10 mg/kg indomethacin (Fig. 6).

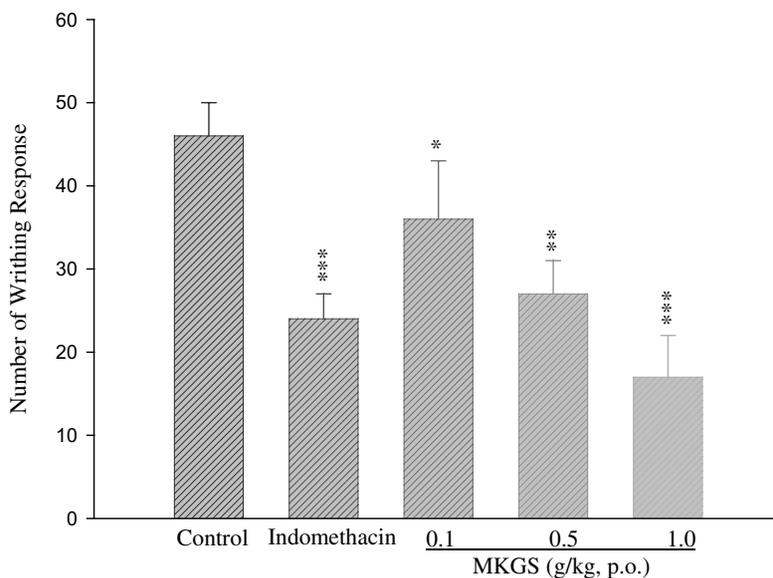


Figure 2. Analgesic effects of MKGS and indomethacin on acetic acid-induced writhing response in mice. Each value represented mean \pm SEM ($n = 6$). The number of muscular contractions was counted over a period of 10 min after acetic acid injection. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when comparing to the control group (one-way ANOVA followed by Scheffe's multiple range test).

Effects of MKGS on IL-1 β Level

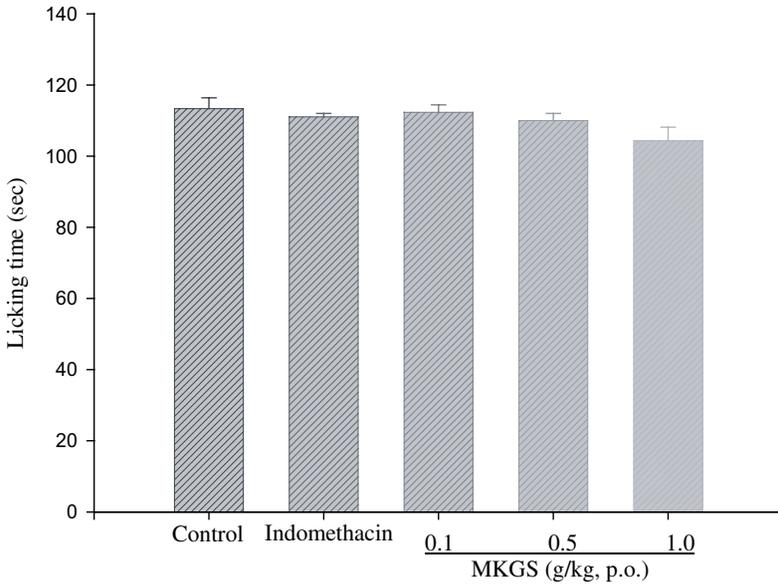
IL-1 β level in the edema paws was increased significantly due to λ -carrageenan injection. There were significant decreases in IL-1 β level when treating with MKGS (0.1, 0.5 and 1.0 g/kg) as shown in Fig. 7.

Effects of MKGS on TNF- α Level

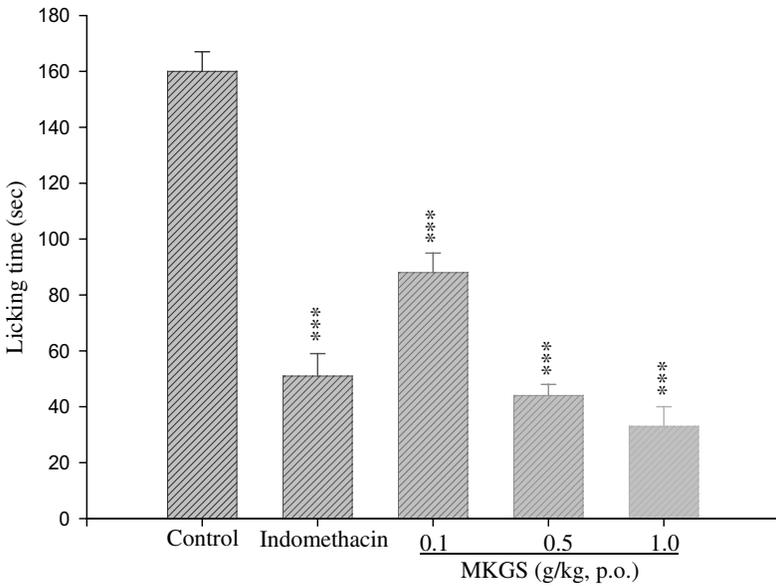
TNF- α level in the λ -carrageenan induced edema paws was raised pronouncedly. However, pretreatments with MKGS (0.1, 0.5 and 1.0 g/kg) significantly decreased the TNF- α level (Fig. 8).

Effects of MKGS on the Activities of Antioxidant Enzymes

Three hours after the intra paw injection of λ -carrageenan, liver tissues were analyzed for their biochemical parameters, such as SOD, GPx and GRx activities (Table 1). SOD, GPx and GRx activities in liver tissue were decreased significantly by λ -carrageenan administration. SOD and GRx activities were increased significantly after treatments with 0.1, 0.5 and 1.0 g/kg MKGS and 10 mg/kg indomethacin ($p < 0.001$).



(A)



(B)

Figure 3. Effects of MKGS and indomethacin on the (A) early phase and (B) late phase of formalin test in mice. Each value represented mean \pm SEM ($n = 6$). The time spent for licking and biting the injected paw was recorded separately at the 0–5 min (first phase or neurogenic pain) and the 20–30 min (second phase or inflammatory pain) as the indicators of pain. *** $p < 0.001$ as compared to the control group (one-way ANOVA followed by Scheffe's multiple range test).

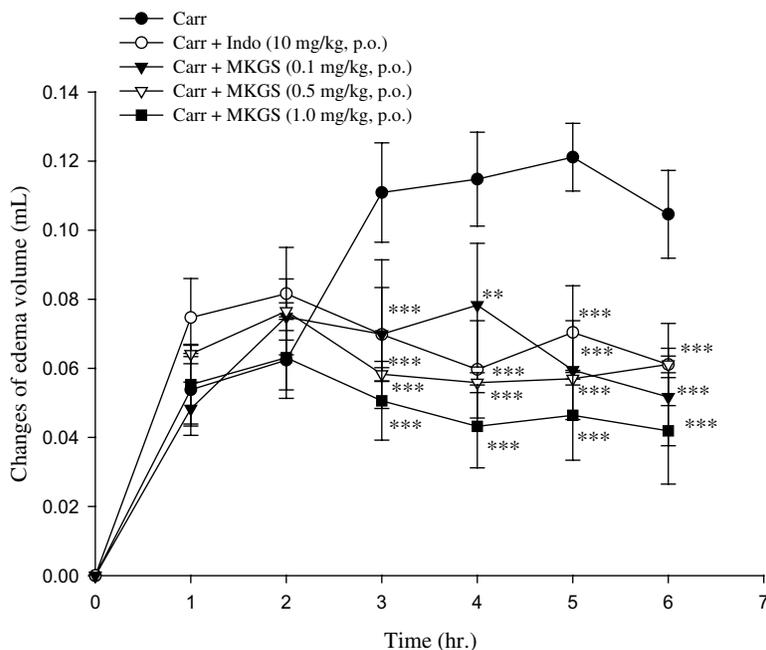


Figure 4. Effects of MKGS and indomethacin (Indo) on λ -carrageenan induced mouse hind paw edema. Each value represented mean \pm SEM (n = 6). Paw volume was measured 1-, 2-, 3- and 4-hours after the administration of the λ -carrageenan using a plethysmometer. ** $p < 0.01$, *** $p < 0.001$ as compared to the λ -carrageenan (Carr) group (one-way ANOVA followed by Scheffe's multiple range test).

Discussion

Anti-nociceptive activity of MKGS was examined by acetic acid-induced writhing responses. Although abdominal writhes induced by acetic acid might not be a very specific nociception model, they were general indications of anti-nociceptive activities of the extracts under study. Accordingly, the writhing response induced by intraperitoneal injection of acetic acid has been used to screen anti-nociceptive activity. Acetic acid is known to cause pain by liberating endogenous substances that excite nerve endings. As shown in Fig. 2, MKGS at 0.1, 0.5, and 1 g/kg, p.o. had inhibitory effects on the writhing response induced by acetic acid. Acetic acid-induced abdominal writhing is a sensitive method to evaluate peripherally acting analgesics. As shown in Fig. 2, we had more scientific evidences to suggest that MKGS possessed analgesic effects in acetic acid-induced writhing response model. Acetic acid-induced abdominal writhing response represented visceral pain; arachidonic acid produced during the biosyntheses of cyclooxygenase and prostaglandin played a mediator role in the nociceptive mechanism (Franzotti *et al.*, 2000). This model of response is thought to be mediated by peritoneal mast cells (Moshage *et al.*, 1995), acid sensing ion channels (Voilley, 2004) and the prostaglandin pathway. Results of the present study indicated that MKGS produced significant analgesic

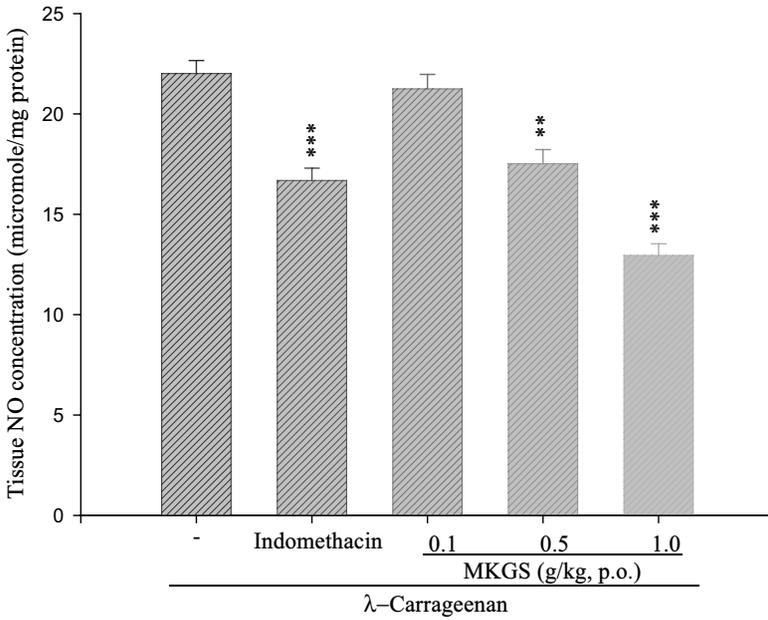


Figure 5. Effects of MKGS and indomethacin on nitrate/nitrite concentration of edema paw in mice. Each value represented as mean \pm SEM (n = 6). ** p < 0.01, *** p < 0.001 as compared to the λ -carrageenan (Carr) group (one-way ANOVA followed by Scheffe's multiple range test).

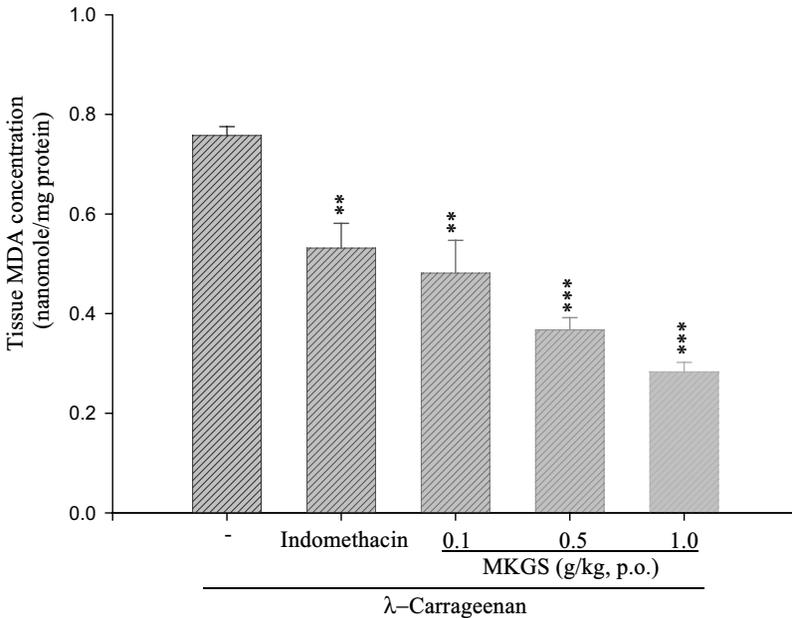


Figure 6. Effects of MKGS and indomethacin on the MDA concentration of mouse edema paws. Each value represented mean \pm SEM (n = 6). ** p < 0.01, *** p < 0.001 as compared to the λ -carrageenan (Carr) group (one-way ANOVA followed by Scheffe's multiple range test).

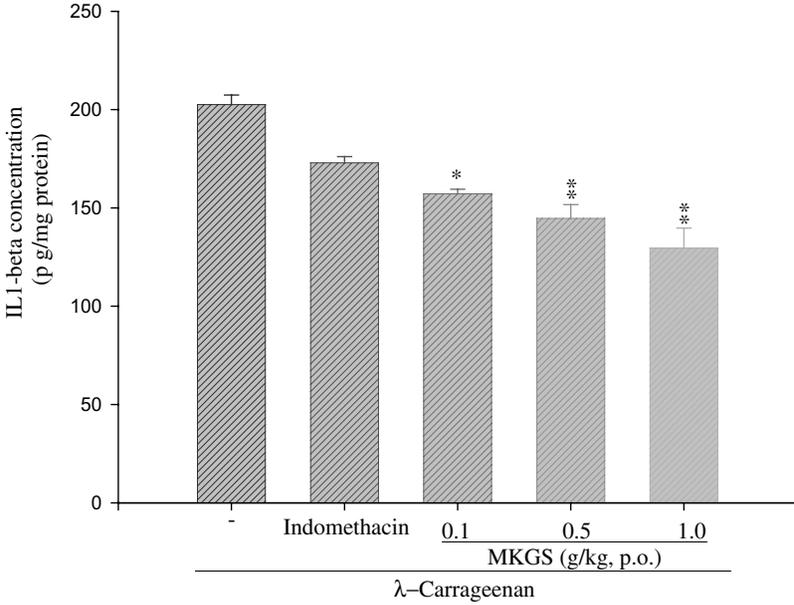


Figure 7. Effect of MKGS and indomethacin on IL-1 β concentration of mouse edema paws. Each value represented mean \pm SEM (n = 6). * p < 0.05, ** p < 0.01 as compared to the λ -carrageenan (Carr) group (one-way ANOVA followed by Scheffe's multiple range test).

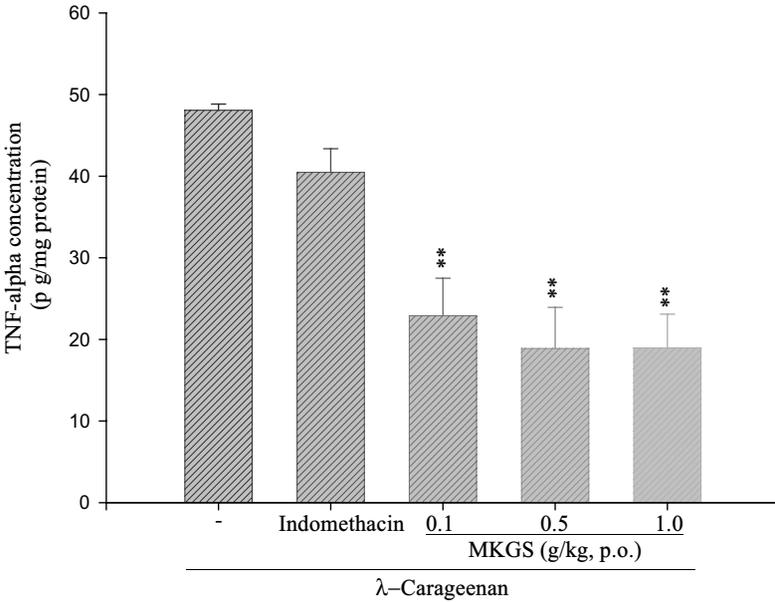


Figure 8. Effect of MKGS and indomethacin on TNF- α concentration in mouse edema paws. Each value represented mean \pm SEM (n = 6). ** p < 0.01 as compared to the λ -carrageenan (Carr) group (one-way ANOVA followed by Scheffe's multiple range test).

Table 1. Effect of MKGS and Indomethacin (indo) on SOD, GPx, and GRx Activities in Mouse Livers

Groups	SOD (U/mg Protein)	GPx (U/mg Protein)	GRx (U/mg Protein)
Carr	26.99 ± 2.52	1.03 ± 0.04	0.067 ± 0.007
Carr + Indo	41.41 ± 2.33***	1.17 ± 0.05	0.089 ± 0.009*
Carr + MKGS 0.1	35.77 ± 1.94***	0.98 ± 0.36	0.131 ± 0.034***
Carr + MKGS 0.5	38.04 ± 1.37***	0.99 ± 0.68	0.153 ± 0.030***
Carr + MKGS 1.0	38.25 ± 3.74***	1.08 ± 0.89	0.149 ± 0.024***

Each value represented mean ± SEM. * $p < 0.05$, *** $p < 0.001$ as compared to the λ -carrageenan (Carr) group (one-way ANOVA followed by Scheffe's multiple range test).

effects which may be due to interference with the synthesis of the arachidonic acid metabolite.

Current studies have shown that the early phase of formalin induced pain reflected the direct effect of formalin on nociceptors; on the other hand, inflammatory pain in the late phase appeared to be attributed to prostaglandin synthesis (Shibata *et al.*, 1989; Tjølsen *et al.*, 1992). Our result showed that MKGS exerted significant inhibitory effects on nociceptive responses in the late phase of the formalin induced inflammatory pain model. The formalin test provided a more useful model of clinical pain in which the late phase was dependent on peripheral inflammation and changes in central processing (Tjølsen *et al.*, 1992). Mediators, such as histamine, serotonin, prostaglandins, nitric oxide and bradykinin were involved in the late phase of the formalin test (Tjølsen *et al.*, 1992). There was powerful evidence that peripheral inflammation was involved in the late phase; the inhibitory effect of MKGS on nociceptive response in the late phase of formalin test suggested that the anti-nociceptive effect of MKGS could be due to its peripheral action.

λ -carrageenan induced hind paw edema as an *in vivo* model of inflammation is frequently applied to evaluate anti-edematous effects of natural products. In different animal models of acute inflammation, λ -carrageenan-induced inflammation has been used to study free radical generation in liver tissues after inflammatory states (Lu *et al.*, 2007). In our study, MKGS and indomethacin showed anti-inflammatory effects on λ -carrageenan induced mouse hind paw edema. Previous studies have reported that λ -carrageenan induced paw edema normally reaches the maximum at the third hour (Kirkova *et al.*, 1992), characterized by the presence of prostaglandins and other slow reacting compounds (Spector and Willoughb, 1963). Ueno *et al.* (2000) found that injecting λ -carrageenan into the rat paw induced liberation of bradykinin, which further induced the biosynthesis of prostaglandin and other autacoids. However, in the λ -carrageenan-induced rat paw edema model, prostanoids were produced through the serum expression of COX-2 by a positive feedback mechanism (Nantel *et al.*, 1999; Bonnie *et al.*, 2005).

Experimental results suggested that the mechanism of MKGS may be related to the inhibition of prostaglandin synthesis, as described for the anti-inflammatory mechanism of indomethacin in the inhibition of λ -carrageenan induced inflammation (Di *et al.*, 1971).

Furthermore, the current classification of anti-nociceptive drugs is usually based on their mechanism of action either on the central nervous system or on the peripheral nervous system (Planas *et al.*, 2000).

In research on mechanisms of inflammation, L-arginine-NO pathway has been proposed to play an important role in λ -carrageenan-induced inflammatory response (Salvemini *et al.*, 1996). Our present results also confirmed that NO production was significantly increased in λ -carrageenan induced hind paw edema model. The expression of the inducible isoform of NO synthase has been proposed as an important mediator of inflammation (Cuzzocrea *et al.*, 1997). Our data indicated the level of NO was dramatically decreased by treatments with 0.5 and 1.0 g/kg MKGS (Fig. 4). We suggested that the anti-inflammatory mechanism of MKGS may be through the L-arginine-NO pathway since MKGS displayed a significant inhibition of the NO production.

Current research has indicated that λ -carrageenan induced inflammatory effect is associated with free radicals (Lai *et al.*, 2009; Lin *et al.*, 2009). λ -carrageenan induced inflammatory response has been linked to neutrophil infiltration and the production of neutrophil-derived free radicals, such as superoxide, hydroxyl radicals and hydrogen peroxide, as well as due to the release of other neutrophil-derived mediators (Dawson *et al.*, 1991). Free radicals, prostaglandin and NO are released after λ -carrageenan is administered for 1–6 hours (Dudhgaonkar *et al.*, 2006).

(Janero, 1990) indicated that MDA production was due to free radicals attacking the plasma membrane. Therefore, inflammation would result in the accumulation of MDA. Glutathione is a well known oxyradical scavenger. Enhancing the level of glutathione reduces MDA production. Cuzzocrea suggested that endogenous glutathione played an important role against carrageenan induced local inflammation (Cuzzocrea *et al.*, 1999). In our study, MKGS treatment significantly increased SOD and GRx activities; on the other hand, MDA level was dramatically decreased. Carrageenan-induced inflammation is related to the production of free radicals. MDA production was due to free radicals attacking the plasma membrane (Janero, 1990). Therefore, we assumed that MKGS probably interrupted MDA production by increasing the activities of SOD and GRx.

The levels of prostaglandins PGE₂ and PGF₂ α had been increasing during the first 30 min after acetic acid injection in the writhing test (Deraedt *et al.*, 1980). Intraperitoneal administration of acetic acid induced the liberation not only the prostaglandins, but also the sympathetic nervous system mediators (Duarte *et al.*, 1988). Carrageenan induced edema is related to the production of histamine, leukotrienes, platelet-activating factor, and possibly cyclooxygenase products. Thus, results obtained from the writhing test using acetic acid were similar to those derived from the edematogenic test using carrageenan. On the other hand, MKGS decreased the production of inflammatory cytokines, IL-1 β and TNF- α . Therefore, we suggest that the anti-inflammatory mechanism of MKGS may be related to the reduction of IL-1 β and TNF- α (Kim *et al.*, 2008; Ge *et al.*, 2009).

In conclusion, MKGS possessed anti-nociceptive activities and anti-inflammatory effects. The anti-inflammatory mechanism of MKGS implies that MDA and NO levels in the edema paw could be decreased via increasing the activities of SOD and GR in the liver and decreasing the levels of IL-1 β and TNF- α in the edema paw. These findings offer

novel pharmacological information about MKGS, implying its plausible therapeutic roles for preventing and treating inflammatory diseases.

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