Anti-inflammatory and antinociceptive properties of the leaves of *Eriobotrya japonica*

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**A B S T R A C T**

Aim of the study: The leaves of *Eriobotrya japonica* Lindl. have been widely used as a traditional medicine for the treatment of many diseases including coughs and asthma. The present study was designed to validate the anti-inflammatory and antinociceptive properties of the n-BuOH fraction of *E. japonica* (LEJ) leaves.

Materials and methods: The anti-inflammatory properties of LEJ were studied using IFN-γ/H9253/LPS activated murine peritoneal macrophage model. The antinociceptive effects of LEJ were assessed using experimental models of pain, including thermal nociception methods, such as the tail immersion test and the hotplate test, and chemical nociception induced by intraperitoneal acetic acid and subplantar formalin in mice. To examine the possible connection of the opioid receptor to the antinociceptive activity of LEJ, we performed a combination test with naloxone, a nonselective opioid receptor antagonist.

Results: In the IFN-γ and LPS-activated murine peritoneal macrophage model, LEJ suppressed NO production and iNOS expression via down-regulation of NF-κB activation. It also attenuated the expression of COX-2 and the secretion of pro-inflammatory cytokines like TNF-α and IL-6. Moreover, LEJ also demonstrated strong and dose-dependent antinociceptive activity compared to tramadol and indomethacin in various experimental pain models. In a combination test using naloxone, diminished analgesic activities of LEJ were observed, indicating that the antinociceptive activity of LEJ is connected with the opioid receptor.

Conclusions: The results indicate that LEJ had potent inhibitory effects on the inflammatory mediators including nitric oxide, iNOS, COX-2, TNF-α and IL-6 via the attenuation of NF-κB translocation to the nucleus. LEJ also showed excellent antinociceptive activity in both central and peripheral mechanisms as a weak opioid agonist. Based on these results, LEJ may possibly be used as an anti-inflammatory and an analgesic agent for the treatment of pains and inflammatory diseases.

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1. Introduction

*Eriobotrya japonica* Lindl., also known as ‘loquat’, belongs to the Rosaceae family. The plant is an evergreen shrub or small tree with narrow leaves that are dark green on the upper surface and have a lighter color under surface. The plant originated in south-eastern China and later became naturalized in Korea, Japan, India and many other countries. In Korea, the leaves of *E. japonica* have been widely used as a traditional medicine with beneficial effects for pain and chronic inflammatory diseases including headache, low back pain, dysmenorrhea, asthma, phlegm and chronic bronchitis (Ito et al., 2000).

Various triterpenes, sesquiterpenes, flavonoids, tannins and megastigmane glycosides were analyzed from LEJ, and some of them have been found to possess anti-tumor, antiviral, hypoglycemic and anti-inflammatory properties (Shimizu et al., 1986; De Tommasi et al., 1991; Ito et al., 2000; Taniguchi et al., 2002; Kim and Shin, 2009).

During the inflammatory process, macrophages produce nitric oxide, pro-inflammatory enzymes and cytokines. Because overproduction of these inflammatory mediators might cause inflammatory damage, many studies have attempted to find materials from traditional plant-derived medicines which selectively modulate these mediators (Lee et al., 2005). Previously, triterpene acids from LEJ have been reported to have an inhibitory effect on inflammatory cytokine secretion and the MAPK signal transduction pathway (Huang et al., 2009). An anti-inflammatory effect in rat model of chronic bronchitis has also been observed (Ge et al., 2009). In addition, Banno et al. (2005) demonstrated that LEJ has anti-inflammatory activity in a TPA-induced inflammation model. These reports strongly suggest that LEJ is an anti-inflammatory agent. However, there have been no investiga-

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tions of LEJ’s anti-inflammatory activity in the IFN-γ/LPS activated murine macrophage model.

Despite recent advances in developing new therapies, there is still a need for effective painkillers. In this regard, new drugs originated from natural products have received a lot of attention and many plant-derived compounds have effective antinociceptive activities (Calixto et al., 2000). The plant used in this study, LEJ, was traditionally used as a painkiller. Nevertheless, there is no scientific report available in the literature on the antinociceptive activity of LEJ. Therefore, in the present study, we investigated the anti-inflammatory and antinociceptive effects of LEJ.

2. Materials and methods

2.1. Plant material

The plant materials were purchased from Hainyakupsa (Chonbuk, South Korea) in October 2009. The plant was identified by Dr. Dae Keun Kim, College of Pharmacy, Woosuk University, Republic of Korea. A voucher specimen (WOPE057) has been deposited at the Department of Oriental Pharmacy, College of Pharmacy, Woosuk University.

2.2. Extraction and fractionation of plant material

We extracted the dried sample (2000 g) using 12,000 ml of MeOH with 2 h sonication. The extract was concentrated into 60.7 g (yield: 3.035%) using a rotary evaporator. Then, the sample was subjected to successive solvent partitioning to yield n-hexane (0.83 g), CH2Cl2 (16.4 g), EtOAc (21 g) and n-BuOH (15.4 g) soluble fractions. Because the preliminary experiments for anti-inflammatory and antinociceptive activity of the fractions showed that the n-BuOH fraction was the most potent, further studies were conducted using the n-BuOH fraction.

2.3. Animals

ICR mice (six-week-old males and females) weighing 20–25 g and C57BL/6 mice (five-week-old) weighing 18–22 g were supplied by Damul Science (Dajeon, Korea). All animals were housed at 22 ± 1 °C with a 12 h light/dark cycle and fed a standard pellet diet with tap water ad libitum. For the purpose of isolating peritoneal macrophages, the C57BL/6 mice were given intraperitoneal (i.p.) injections three days earlier with 2.5 ml of thioglycollate (TG) solution. The experimental protocols complied with the recommendations of the International Association for the Study of Pain (Zimmermann, 1983).

2.4. Anti-inflammatory study

2.4.1. Isolation and culture of mouse macrophages

TG-elicited macrophages were harvested three days after i.p. injection of TG and isolated. Using 8 ml of PBS containing 10 U/ml heparin, peritoneal lavage was performed. Then, the cells were distributed in FBS-free DMEM and maintained at 37 °C in a humidified atmosphere of 5% CO2. After 3 h, the cells were washed three times with PBS to remove non-adherent cells and equilibrated with DMEM that contained 10% heat-inactivated FBS before treatment.

2.4.2. Determination of cell viability

Cell respiration, an indicator of cell viability, was measured by the mitochondrial dependent reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan, as described by Mosmann (1983). The extent of the reduction of MTT to formazan within cells was quantified by measuring the optical density (OD) at 540 nm using an automated microplate reader (GENios, Tecan, Austria).

2.4.3. Measurement of nitrite concentration

Peritoneal macrophages (3 × 105 cells/well) were cultured with various concentrations of LEJ. The cells were then stimulated with rIFN-γ (20 U/ml). After 6 h, the cells were finally treated with LPS (10 µg/ml). NO synthesis in cell cultures was measured with a microplate assay method. To measure nitrite, 100 µl aliquots were removed from conditioned medium and incubated with an equal volume of Griess reagent at room temperature for 10 min. The absorbance at 540 nm was determined by a microplate reader. The quantity of NO2− was calculated by using sodium nitrite as a standard.

2.4.4. Preparation of nuclear extracts

Nuclear extracts were prepared as described previously (Baek et al., 2002). Briefly, the cells were allowed to swell by adding lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride). Pellets containing crude nuclei were resuspended in extraction buffer (20 mM HEPES pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) and incubated for 30 min on ice. The samples were centrifuged at 12,000 rpm for 10 min to obtain the supernatant containing nuclear extracts.

2.4.5. Western blot analysis

Whole cell lysates were made by boiling peritoneal macrophages in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% sodium dodecyl sulfate (SDS), 20% glycerol and 10% 2-mercaptoethanol). Proteins in the cell lysates were then separated by 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose paper. The membrane was then blocked with 5% skim milk for 2 h at room temperature and then incubated with anti-NO, anti-IFN-γ, anti-NF-κB (Santa Cruz Biotechnology, CA, USA) and anti-COX-2 (Pierce Biotechnology, IL, USA). After washing the membrane in phosphate buffered saline (PBS) containing 0.05% tween 20 three times, the blot was incubated with HRP-conjugated anti-rabbit and anti-mouse (Amersham Biosciences, Little Chalfont, UK) and the target proteins were visualized by an enhanced chemiluminescence detection system (Millipore Corporation, MI, USA).

2.4.6. Assay of cytokine release

Peritoneal macrophages (3 × 105 cells/well) were treated with various concentrations of LEJ. The cells were then stimulated with rIFN-γ (20 U/ml) plus LPS (10 µg/ml) and incubated for 24 h. The amount of TNF-α and IL-6 in the supernatants (from 3 × 105 cells/ml, culture medium DMEM with 10% FBS) were measured by a sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocol (BD biosciences, CA, USA). Absorption of the avidin–horseradish peroxidase color reaction was measured at 405 nm and compared to the serial dilutions of recombinant mouse TNF-α and IL-6, which were used as standards.

2.5. Antinociceptive study

2.5.1. Grouping and drug administration

Animals were randomly assigned into several groups, each consisting of eight or ten mice for analgesic tests. Negative controls were treated with the same volume of distilled water which was used for reconstituting the drug. Positive controls were treated with standard drugs: tramadol (i.p.) or indomethacin (p.o.). Treatment groups in each test were treated orally with different doses of LEJ.
2.5.2. Acute toxicity test

To evaluate possible toxicity, the acute toxicity test was carried out. Mice (n = 6) were tested by administering different doses of LEJ and the doses were increased or decreased according to the response of the animal (Bruce, 1985). The control group received only the equal volume of distilled water. All of the groups were observed for any gross effect or mortality for a 24 h period.

2.5.3. Tail immersion test

In the present study, the tail immersion test was performed according to the procedures used by Wang et al. (2000) with minor modification. Briefly, the lower two-thirds of mouse’s tail was immersed on a water bath set at temperature of 50 ± 0.2°C. The reaction time, i.e. the amount of time it takes the animal to withdraw its tail, was measured 0, 30, 60, 90 and 120 min after the administration of LEJ (250, 500 mg/kg; p.o.), tramadol (10 mg/kg; i.p.) and vehicle (D.W). To avoid tissue injury, the cut-off time was 20 s.

2.5.4. Hot plate test

The hot plate test (Franzotti et al., 2000) was carried out on groups of male and female mice using a hot plate apparatus, maintained at 55 ± 1°C. Only mice that showed initial nociceptive responses (licking of the forepaws or jumping) between 7 and 15 s were used for additional experiments. The chosen mice were pre-treated with LEJ (250, 500 mg/kg; p.o.) or vehicle (D.W), and 30 min later the measurements were taken. A tramadol (10 mg/kg; i.p.) treated animal group was included as a positive control. To examine the possible connection of endogenous opioids to antinociceptive activity, LEJ and tramadol were investigated in groups of mice pre-treated with naloxone (5 mg/kg; i.p.). The cut-off time was set at 30 s to minimize skin damages. The reaction time was calculated as described for the tail immersion test.

2.5.5. Acetic acid-induced writhing test

Antinociceptive activity of LEJ was detected as previously described (Olajide et al., 2000). The response to an intraperitoneal injection of acetic acid solution (1% in 0.9% saline), which consisted of abdominal constrictions and hind limb stretching, was measured for each mouse starting 5 min after the acetic acid injection and was measured for an additional 20 min. Each experimental group was treated orally with vehicle (D.W), LEJ (250, 500 mg/kg) or indomethacin (10 mg/kg) 1 h prior to the acetic acid injection. To examine the possible connection of endogenous opioids to the antinociceptive activity, the LEJ group was pretreated with naloxone (5 mg/kg; i.p.).

2.5.6. Formalin test

In the formalin test (Santos and Calixto, 1997), groups of mice were treated orally with vehicle (D.W) or LEJ (250, 500 mg/kg). After 30 min, each mouse was treated with 20 μl of 5% formalin (in 0.9% saline, subplantar) into the right hind-paw. The duration of paw licking (s) was used as an index to measure the pain response during the 0–5 min period (first phase, neurogenic) and the 20–35 min period (second phase, inflammatory) after formalin injection. Tramadol and indomethacin were used as positive control drugs and were administrated 30 min before the test at a dose of 10 mg/kg, i.p. and p.o., respectively.

2.6. Statistical analysis

The results are expressed as the mean ± S.D. or mean ± S.E.M. depending on the experiments. Data between groups were analyzed by a Student’s unpaired two-tailed t-test and p-values less than 0.01 were considered significant. The intensity of the bands obtained from Western blotting studies was estimated with ImageQuantTL (GE Healthcare, Sweden) and the values were expressed as mean ± standard error.

3. Results

3.1. Effects of LEJ on NO production

To determine the effect of LEJ on the production of NO in rIFN-γ/LPS-activated mouse peritoneal macrophages, nitrite accumulation was measured by the Griess reaction. We pre-treated the cells in the presence or absence of various concentrations of LEJ, then stimulated the cells with rIFN-γ (20 U/ml) and LPS (10 μg/ml). The amount of NO in unstimulated cells was 3.3 ± 0.5 μM. When mouse peritoneal macrophages were primed for 6 h with murine rIFN-γ and then treated with LPS for 48 h, NO production was increased about thirty folds (90 ± 0.5 μM). As shown in Fig. 1, LEJ suppressed NO production in a dose-dependent manner and about 87.7% (p < 0.001) inhibition of NO production occurred at a concentration of 500 μg/ml.

3.2. Effects of LEJ on cell viability

To determine the effects of LEJ on the viability of mouse peritoneal macrophages, we carried out a MITT assay. When the cells were treated with various concentrations of LEJ (125, 250, 500 μg/ml), there was no effect on cell viability (data not shown). Therefore, the inhibitory effect of LEJ on NO production was not due to the cytotoxicity of LEJ.

3.3. Effects of LEJ on iNOS and COX-2 expression

To investigate the mechanism of the LEJ on the inhibition of NO production, the following experiment was performed. We investigated the effect of LEJ at a translational level with Western blotting. As shown in Fig. 2, the expression of iNOS protein was markedly increased after the 24 h rIFN-γ (20 U/ml) and LPS (10 μg/ml) challenge. This enhanced expression of iNOS protein was significantly reduced by LEJ in a dose-dependent manner. β-Actin, a cytosolic protein, was used as a control to confirm that there were no differences in protein level between each group. Because an increase in COX-2 expression is associated with inflammatory responses, we
also investigated the inhibitory effect of LEJ on COX-2 expression. As seen in Fig. 2, the increased amount COX-2 protein expression from rIFN-γ (20 U/ml) plus LPS (10 μg/ml) activation was suppressed by LEJ dose-dependently.

### 3.4. Effects of LEJ on cytokine secretion

We examined the inhibitory effect of LEJ on rIFN-γ/LPS-induced TNF-α and IL-6 secretion. Mouse peritoneal macrophages secreted low levels of TNF-α and IL-6 after a 24 h incubation with medium alone. The basal levels of TNF-α and IL-6 did not change when the cells were incubated with LEJ alone. When the cells were treated with rIFN-γ (20 U/ml) plus LPS (10 μg/ml) for 24 h, TNF-α and IL-6 levels drastically increased in the cells and pre-treatment of cells with various concentration of LEJ (125, 250, 500 μg/ml) significantly inhibited TNF-α (Fig. 3A) and IL-6 (Fig. 3B) induction in activated mouse peritoneal macrophages.

### 3.5. Effects of LEJ on NF-κB translocation

Many previous studies demonstrated that NF-κB is an important transcriptional factor for the expression of various inflammatory mediators (Henkel et al., 1993; Ghosh et al., 1998). Thus, we evaluated the influence of LEJ on the distribution of the NF-κB subunit (p65). Nuclear and cytosolic extracts were prepared and subjected to immunoblot analysis. As shown in Fig. 4, co-incubation with LPS plus LEJ the NF-κB protein level decreased in the nucleus and increased in the cytosol, respectively. These findings strongly suggest that LEJ inhibited the LPS-stimulated transcriptional activity of NF-κB by suppressing phosphorylation-dependent proteolysis of IκB-α in the cells.

### 3.6. Acute toxicity

To test possible toxicity of LEJ in animals, 2000 mg/kg of LEJ was administered to the mice. The treated mice did not present any behavioral alterations, convulsions or death during the assessment.
Effects of LEJ on NF-κB translocation by LPS-stimulated peritoneal macrophages. Peritoneal macrophages (5 × 10⁶ cells/well) were pretreated with LEJ or 0.05% DMSO for 30 min and then stimulated with rIFN-γ (20 U/ml) for 2 h. After 1 h stimulation with LPS (10 μg/ml), the nuclear extracts were prepared and samples were analyzed by Western blotting as described in the Method section and quantified by densitometry.

3.7. Analgesic activity of LEJ in the tail immersion test

In the tail immersion test, LEJ was antinociceptive in a dose-dependent manner (Table 1). The most prominent analgesic activity for the lower concentration of LEJ (250 mg/kg) was observed 90 min after oral administration (23.06%). In contrast, pre-treatment with a high concentration of LEJ (500 mg/kg) significantly delayed reaction times to a nociceptive stimulus 60 min after oral administration (47.72%, p < 0.01). Tramadol (10 mg/kg), the reference drug, also exhibited powerful activity which was recorded 30 min after drug treatment (56.60%, p < 0.01). However, no differences in time lattencies were observed in the vehicle-treated control group.

3.8. Analgesic activity of LEJ in the hot plate test

In the hot plate test, pre-treatments of LEJ generated significantly increased analgesia in a dose-dependent manner. The analgesic effects of LEJ (250, 500 mg/kg) occurred between 30 and 90 min and maximum analgesia was reached at 60 min (71.62% and 77.92%, respectively) (Table 2). Tramadol also caused significant antinociception (98.20%). In combination studies using naloxone, an opioid receptor antagonist, the analgesic activity of tramadol was diminished by naloxone to 19.37%. Interestingly, naloxone antagonized LEJ (500 mg/kg) antinociception (−55.50%) only at the 30 min time point but not at 60, 90 or 120 min.

3.9. Analgesic activity of LEJ in the acetic acid test

An intraperitoneal injection of 1% acetic acid into mice causes 44.75 ± 2.85 writhing in an interval of 20 min and mice treated with LEJ showed a significant decrease (when compared to the vehicle-treated group) in the mean number of writhing (Table 3). The data showed that the protective effect of LEJ was dose-dependent. A 31.56% (p < 0.001) reduction was observed for the 250 mg/kg dose and a 71.50% (p < 0.001) reduction occurred for the 500 mg/kg dose. The reference drug, indomethacin (10 mg/kg), had 57.82% (p < 0.001) inhibition which is lower than the inhibition observed for the high concentration of LEJ. In combination studies, similar to the hot plate test, naloxone significantly antagonized LEJ antinociception.

3.10. Analgesic activity of LEJ in the formalin test

In the formalin test, the vehicle-treated mice had mean licking times of 111.22 ± 6.62 s in the first phase (0–5 min) and 113.44 ± 9.67 s in the second phase (15–30 min). As shown in Table 4, mice pre-treated with LEJ showed a reduced response to formalin in both the first phase (neurogenic-induced) (29.36%, 42.87%) and the second phase (inflammatory) (49.44%, 74.84%), for the 250 and 500 mg/kg doses, respectively. The reference drug, tramadol, also significantly blocked the pain formalin-response in both phases (first-phase, 53.66 ± 7.17 s and second-phase, 13.57 ± 4.93 s). However, indomethacine was significantly active (59.72%, p < 0.001) only in the second phase.

4. Discussion

The dried leaves of E. japonica Lindl. (Rosaceae) are a well-known oriental medicine, containing various beneficial compounds, with diverse pharmacological effects and has been used to treat many different diseases. Nevertheless, the previous reports on LEJ are still not enough to understand how LEJ treatment works on inflam-
Cyclooxygenase-2 (COX-2), another key enzyme in inflammation, is the rate-limiting enzyme that catalyzes the formation of prostaglandins (PGs) from arachidonic acid. Levels of PGs increase early in the course of the inflammation (Wallace, 1999). Since, COX-2 is induced by stimulation in inflammatory cells, inhibitors of COX-2 induction might candidate for a nonsteroidal anti-inflammatory drug (NSAID). We documented the increased production of COX-2 protein by macrophages exposed to IFN-γ and LPS. IFN-γ and LPS in combination with LEJ led to a reduction in COX-2 expression. Thus, it seems quite reasonable to speculate that LEJ may inhibit PGE₂ production. However, further studies are required to determine whether LEJ is a selective inhibitor of COX-2.

Cytokines are the physiological messengers of the inflammatory response. The macrophage-derived mediators, TNF-α and IL-6 are thought to play an important role in the inflammatory response based on the appearance of these cytokines at inflammatory sites and their ability to induce certain mechanisms in the inflammatory response (Park et al., 2002). TNF-α has been documented as a pathogenic factor in several inflammatory diseases, including arthritic diseases, inflammatory bowel diseases, Type 1 diabetes mellitus, multiple sclerosis and Guillain–Barre syndrome (O’Shea et al., 2002). The secretion of IL-6 has been found to play a central role in the regulation of defense mechanisms, haematopoiesis and most importantly, the production of acute phase proteins (Park et al., 1999). Mouse peritoneal macrophages secreted low levels of TNF-α and LPS for 24 h, TNF-α and IL-6 levels drastically increased in these cells. However, pre-treatment with LEJ significantly inhibited the increase in TNF-α and IL-6 levels.

The induction of pro-inflammatory mediators is largely regulated by transcriptional activation (Park et al., 2006). Nuclear factor kappa B (NF-κB) is essential for the transcription of genes that encode a number of pro-inflammatory molecules which participate in the acute inflammatory response, including iNOS, COX-2, TNF-α and IL-6 (Muller et al., 1993). In unstimulated cells, NF-κB is present in the cytoplasm and interacts with the inhibitory...
protein, kC3. However, in an active state, following the induction of NF-κB by appropriate extracellular stimulation, NF-κB translo-
cates to the nucleus through the phosphorylation, ubiquitination and
degradation of IκBα. NF-κB also acts on the pro-inflammatory
gene promoter to activate transcription (Park et al., 2006). The
translocation of NF-κB to nucleus was markedly increased after the
IFN-γ and LPS challenge. This increase was significantly reduced by
LEJ. On the contrary, LEJ generated a potent increase in NF-κB
protein levels in the cytosol. These results strongly suggest that
LEJ suppressed various pro-inflammatory mediators via decreased
NF-κB translocation and DNA binding activities. Similar to the
results described above, Kim and Shin (2009) revealed that the anti-
inflammatory activity of the loquat leaf correlated with down
regulation of p38 MAPK, ERK and NF-κB activation in mast cells.
Huang et al. (2009) also demonstrated that the triterpene acids of
the loquat leaf attenuate the MAPK signal transduction pathway in
alveolar macrophages from rats with chronic bronchitis. Moreover,
Lee et al. (2008) reported that the triterpenes, especially urso-
lic acid, inhibited lipopolysaccharide-induced cytokines and inducible
enzyme production via the nuclear factor-kappaB signaling path-
way in lung epithelial cells. In the present study, we also isolated
triterpenes from the EtOAc fraction (data not shown). Therefore, it
could be assumed that not only triterpenes in the EtOAc fraction
but also other compounds in LEJ may possess anti-inflammatory
potential.

Next, we investigated the antinociceptive properties of LEJ.
Thermal nociception models such as tail immersion and the hot-
plate tests were used to determine central antinociceptive activity.
LEJ showed analgesic effect in both the tail immersion and hot plate
tests, implicating both spinal and supraspinal analgesic pathways.
In both tests, tramadol exhibited a rapid effect with a maximum
peak in a short amount of time, which is similar to the action of
opioid agonists (e.g. morphine). In contrast, LEJ reached the max-
imum analgesic level 60 min after administration. This difference
in the maximum analgesic point could be explained by the meth-
ods of drug administration (i.p. or p.o.) or the metabolic rate of
each drug. Moreover, in the hotplate test, the antinociceptive action
of tramadol was reduced, but was still present, when the non-
selective opioid receptor antagonist naloxone was applied. These
results correlate with previous studies that showed tramadol is a
partial opioid agonist with both opioid and non-opioid properties
(Yalcin and Aksu, 2005). Interestingly, the analgesic effect of LEJ was
also blocked by naloxone at the 30 min time point, suggesting that at
least some of the antinociceptive effects of LEJ are mediated via
activation of opioid receptors. However, at the other time points,
naloxone was unable to antagonize the analgesic effect of LEJ, which
indicates that LEJ-induced antinociception is largely affected by a
non-opioid mechanism of action. Thus, these results showed that
LEJ acted as a weak opioid receptor agonist.

The effects of LEJ on peripheral nociception were determined
using the acetic acid-induced writhing model which is frequently
used to estimate both the central and peripheral analgesic effects of
drugs (Fukawa et al., 1980). The acetic acid-induced writhing test has been associated with an increased level of prostaglandins
(PGs), especially PGE2, in peritoneal fluids (Derardt et al., 1980).
PGs induce abdominal contractions by activating and sensitizing
peripheral chemosensitive nociceptors (Dirig et al., 1998) which
are largely associated with the development of inflammatory pain
(Bleye et al., 1998). Therefore, one of the possible analgesic mechan-
isms related to this test is the inhibition of the COX enzyme.
Non-steroidal anti-inflammatory drugs exert their peripheral anal-
gesic potential through inhibition of PG synthesis, and in the
present study, indomethacin produced a significant decrease in the
writhing response. LEJ also showed potent inhibition of acetic acid-
induced abdominal contractions in a dose-dependent manner. The antinociceptive potential of LEJ could be explained by its inhibitory
action on COX-2 expression, which was described above. Moreover,
in a combination study, naloxone exhibited moderate inhibition of
the analgesic effect of LEJ. Therefore, it is likely that the opioid sys-
tem is also involved in the peripheral antinociceptive actions of LEJ,
at least in part.

Because the acetic acid-induced writhing test was not a distinct
test to indicate if the analgesic effects of LEJ resulted from cen-
tral or peripheral mechanisms, the formalin test was conducted. A
subcutaneous injection of formalin, used as a peripheral noxious
stimulus, causes biphasic nociceptive responses which involve two
different mechanisms (Hunskaar and Hole, 1987). The first phase
(neurogenic pain) is caused by the direct chemical stimulation of
nociceptive afferent fibers, predominantly C fibers, which can be
suppressed by opiates like morphine (Amaral et al., 2007). On the
other hand, the second phase (inflammatory pain) results from the
action of inflammatory mediators such as prostaglandins, soro-
toin, histamine and bradykinin in the peripheral tissues (Hunskaar
and Hole, 1987) and from functional changes in the spinal dorsal
horn (Hunskaar et al., 1995). The results of the present study have shown
that tramadol, a central analgesic drug, is effective in preventing
both the early and late phases of formalin-induced nociception,
whereas indomethacin, a NSAID, suppressed nociceptive activ-
ity mainly in the later phase. These results are quite reasonable
because many reports suggest that drugs which primarily act on
the central nervous system are inhibited equally in both phases,
whereas drugs which act peripherally, such as steroids and NSAIDs,
can slightly inhibit the early phase of the formalin test (Hunskaar
et al., 1985; Trongsakul et al., 2003; Vontagut et al., 2004). In this test,
it was observed that LEJ could reduce the duration of the paw licking
time in both the first phase and the second phase, demonstrating that
LEJ can suppress neurogenic and inflammatory nociception.
These data provided further confirmation that LEJ has a central
mechanism of action, which was shown in the tail-immersion and
hot plate tests. Furthermore, in agreement with the results from
the acetic acid test, LEJ also displayed better peripheral analgesic
effect than indomethacin. Based on these findings, it may be con-
cluded that LEJ has excellent antinociceptive properties involving
central and peripheral mechanisms.

In summary, in the IFN-γ and LPS-induced mouse peritoneal
macrophage model, LEJ showed significant inhibitory effects on
proinflammatory mediators including NO, IL-6, TNF-α, iNOS and
COX-2 via the down regulation of NF-κB translocation to the
nucleus. In several thermal and chemical nociception tests, LEJ
also exhibited potent antinociceptive activities on both central and
peripheral mechanism. In addition, a combination test with nalox-
one revealed that LEJ acts as a weak opioid receptor agonist. Based
on these various properties, LEJ may hold great promise for treating
inflammatory diseases and pain as an effective immunomodulatory
and analgesic agent.

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