Anti-Inflammatory and Analgesic Activities of SKLJI, a Highly Purified and Injectable Herbal Extract of Lonicera japonica

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The parenteral route has many merits over the oral route, including greater predictability, reproducibility of absorption, and rapid drug action, but injectable phytomedicines are uncommon due to protein precipitating tannin and hemolytic saponin components. In this study, in an effort to develop a safe injectable analgesic phytomedicine, we prepared a tannin and saponin-free Lonicera japonica extract, SKLJI, through fractionation and column purification, and evaluated its anti-inflammatory and analgesic activities in in vivo experimental models of inflammation and pain. The removal of tannin and saponin resulted in loganin and sweroside-enriched SKLJI and it showed reduced hemolysis and protein precipitation. In efficacy tests, SKLJI inhibited croton oil- and arachidonic acid-induced ear edema, acetic acid-induced writhing, and carrageenan-induced rat hind paw hyperalgesia. Inhibition of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and 5-lipoxygenase (5-LO) activities by SKLJI appeared to be the mechanism underlying anti-inflammatory and analgesic efficacy. Loganin and sweroside also showed anti-inflammatory and analgesic activities, suggesting that they might be active principles in the efficacy of SKLJI. These results suggest that SKLJI is a viable candidate for a new anti-inflammatory and analgesic phytomedicine that can be administered by the parenteral route.

Key words: honeysuckle (Lonicera japonica Thunb.); anti-inflammatory; analgesic; sweroside; loganin

Parenteral administration of drugs permits greater predictability and reproducibility of absorption, and consequently, allows better-controlled management of therapeutic efficacy and potential side effects. In addition, parenteral drug forms are preferred to oral for a number of reasons, including rapid drug action, ease of treatment for disabled patients, and precise drug-level control.1,1 Hence, modern Chinese hospitals use as many as 147 Chinese herbal injection drugs,2 and in many countries parenteral phytomedicines are used, including glycyrrhizin, an extract of Glycyrrhiza glabra for the treatment of chronic hepatitis.3,4 In addition, many orally active herbal and phytomedicines are being developed as parenteral formulations.

Injectable nonsteroidal anti-inflammatory drugs (NSAIDs), including diclofenac and ketorolac, provide relief from pain.5 Injectable NSAIDs are preferable to oral NSAIDs in faster onset time and ease of treatment of inpatients as for post-operative pain management. However, injectable NSAIDs frequently bring about adverse side-effects in the gastrointestinal tract, liver, and kidneys, and can increase pre- and post-operative bleeding and allergic reactions even during short-term treatment,6,7 indicating an unmet need for new anti-inflammatory and analgesic drugs with improved safety profiles that can replace injectable NSAIDs.

The honeysuckle, Lonicera japonica Thunberg, (Caprifoliaceae) is a semi-evergreen vine shrub that grows naturally at the foot of mountains or levees in an altitude range of 50 to 600 meters in Japan, China, and Korea. It has long been used as an antidiote, diuretic, tonic, antipyretic, and anti-inflammatory agent.8 Its anti-inflammatory and analgesic activity has been demonstrated in a variety of experimental disease models8 and its active constituents have been isolated and reported,9,10 suggesting that it is a good candidate for an injectable analgesic and anti-inflammatory phytomedicine. However, some components of it, such as saponin and tannin, are known to evoke hemolysis and protein precipitation,11,12 preventing direct application of an injectable formulation. Hence, we prepared a tannin and saponin-free Lonicera japonica extract, SKLJI by removing saponin and tannin from a n-butanol extract of Lonicera japonica Thunberg through purification steps of one fractionation and two column works, to develop a safe injectable analgesic phytomedicine. Currently, SKLJI is being evaluated in nonclinical and clinical trials for efficacy and safety.

Here we are reporting the pharmacological properties of SKLJI relating to anti-inflammatory and analgesic effects using in vivo animal models. The possible mode of action was also explored with regard to effects on major inflammatory enzymes, cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and 5-lipoxygenase (5-LO).
Materials and Methods

Plant materials. Stems of Lonicera japonica were collected in November 2000 in China and were identified by Dr. Dae-Suk Han of the College of Pharmacy, Seoul National University, Seoul, Korea. Voucher specimen was deposited in the Life Science R&D Center of SK Chemicals Co., Ltd. (Suwon, South Korea).

Preparation of SKLJI. After the leaves were removed, the stems of Lonicera japonica (10 kg) were extracted twice with boiling water for 2.5 h. The water extract obtained was evaporated to 10 liters and partitioned twice with water-saturated n-butanol (30 liters). After evaporation and lyophilization, the n-butanol extract (1.9 kg obtained) was further purified by two-stage column chromatography. Briefly, n-butanol extract (1.4 kg, dispersed in water) was passed through a polyamide 6 resin (100 mm diameter column) and serially eluted with water (5.6 liters), 50% methanol (5.6 liters), and absolute methanol (5.6 liters). After evaporation of the solvents, 960 g was obtained from the water eluate, 223 g from the 50% methanol, and 111 g from the absolute methanol. The water-eluted fraction (480 g) was further purified by reversed-phase preparative HPLC and fractionated into eight parts. HPLC was performed at a flow rate of 400 ml/min at a detection wavelength of 254 nm at room temperature. The mobile phase was eluted as a linear gradient from 20% to 100% methanol over 22 min. The preparative HPLC fraction (11 ml) was further purified by reversed-phase preparative HPLC and fractionated into four parts. Finally, 15 mg of SKLJI was purified by preparative semi-preparative HPLC and fractionated into four parts. SKLJI was obtained at 0.4% yield. HPLC and LC/MS analysis showed that the major constituents were sweroside and loganin. The sweroside content was 23.1% and the loganin content was 16.7% in SKLJI.

Experimental animals. The mice used in testing were male ICR mice weighing 20–30 g. The rats were used as male Sprague-Dawley rats weighing 100–120 g (Charles River, Sekyo, Japan). They were maintained at 22 ± 2 °C, and 50 ± 5% relative humidity under a 12 h cycle of light and dark. They had access to water and food ad libitum.

Intravenous injection was done via the tail vein for 15 min before croton oil application. Ear edema was expressed as percentage thickness increase versus the contra-lateral untreated ears. A similar procedure was used in the arachidonic acid-induced ear edema models, except for the use of 2% arachidonic acid and a 1 h of contact time.

Assessment of anti-inflammatory activity. Croton oil and arachidonic acid-induced ear edema models were employed to evaluate the anti-inflammatory activities of the SKLJI extracts. Croton oil-induced ear edema was measured by the method described by Kim et al.16) with slight modifications. Briefly, 25 μl of 2.5% croton oil solution in acetone was topically applied to the right ears of ICR mice (the left ears were not treated). Ear thicknesses were measured 4 h after challenge using a dial thickness gauge. Extracts were administered by intravenous injection 15 min before croton-oil application. Ear edema was expressed as percentage thickness increase versus the contra-lateral untreated ears.

Assessment of analgesic activity. The writhing syndrome was elicited by intraperitoneal injection of 0.7% acetic acid (0.1 ml/10 g of body weight). Analgesic activity was evaluated by scoring of the writhing number for 10 min starting 5 min after acetic acid injection. Hyperalgesia was induced by subcutaneous injection of 100 μl of carrageenan (1% in saline) into the plantar surface of the rat hind paw after anesthesia with 5% halothane. Hind-paw hyperalgesia was measured as described by Randall and Selitto.17) Hyperalgesia was induced with 100 μl of carrageenan (1% in saline) subcutaneous injection into the plantar surface of the left hind paw of the rat. Paw pressure was measured using an Ugo Basile Analgesy meter (Stoelting, Wood Dale, IL) 4 h after carrageenan. The force at which a rat withdrew its hind paw, vocalized, or struggled was multiplied by 20 and recorded as the withdrawal force (g). SKLJI was injected intravenously at 20 min and acetylaminoeph (100 mg/kg) was orally administered 30 min before paw-pressure measurement.

Cell culture. Macrophage cell line RAW 264.7 was obtained from the Korean Cell Line Bank. The cells were grown in DMEM containing heat-inactivated 10% FBS and 100 μU/ml of penicillin-streptomycin (Invitrogen). Cells were cultured at 37 °C in a humidified 5% CO2/95% air incubator.

Assessment of COX-2 and iNOS expression. For COX-2 assay, RAW 264.7 cells were pre-incubated with and without SKLJI for 15 min. Aspirin (500 μM) was added before the test materials to eliminate basal cyclooxygenase-1. For induction of COX-2, the cells were further incubated with and without 1 μg/ml LPS for 16 h. After incubation, total cell extracts were analyzed for expressed COX-2 protein by western blot analysis.

For iNOS assay, RAW 264.7 cells were pre-incubated with and without SKLJI for 1 h. They were washed 3 times with phosphate buffered saline. They were further incubated with and without 1 μg/ml LPS and 10 units of IFN-γ for 12 h. After incubation, total cell extracts were analyzed for expressed iNOS protein by western blot.

Western blot analysis. For total cellular extraction, cells were washed twice and solubilized by addition of Laemmli sample buffer, the composition of which was Tris–HCl 187.5 mM, sodium dodecyl sulfate 6%, glycerol 30%, 2-mercaptoethanol 15%, pH 6.8. The sample was boiled at 95 °C for 5 min. Electrophoresis was performed on 10% acrylamide gels. Proteins were transferred electrophoretically from the gel onto Immobilon polyvinylidene difluoride membranes (GVHP Dapore filter: Millipore, Bedford, MA) by the semi-dry blotting method. The immunoblots were blocked for 3 h with 2% bovine serum albumin in Tris-buffered saline containing 0.05% Tween 20 (TBST) at 25 °C and incubated with anti-COX-2 antibody (murine), anti-iNOS antibody, and anti-beta-actin antibody at 1 μg/ml for 1 h at 25 °C. The immunoblots were washed several times and incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-murine IgG in TBST containing 2% BSA for 1 h at 25 °C. They were developed using ECL detection reagent and visualized by exposure to X-ray film (Sigma, St. Louis, MO).
Luciferase reporter gene assay for COX-2. The day before transfection, RAW 264.7 cells (1 x 10^6 cells/ml) were seeded and incubated at 37°C in a 5% CO2/air mixture. They were then transfected with 2.5 μg of pcDNA2.1/neo and 7.5 μg of luciferase reporter gene vector (pGL2) containing mouse COX-2 (3.2 kb) promoter using Superfect transfection reagent (Qiagen, Valencia, CA). At 48 h post-transfection, the cells were replated in selective media (DMEM containing 500 μg/ml of Geneticin), and the selective media were replaced at 2–3 d intervals. After 14 d of selection in Geneticin-containing media, the surviving colonies were monitored and analyzed for luciferase activity by stimulation with 100 ng/ml of LPS. The transfected cells were seeded for 24 h in 6-well plates (5 x 10^5 cells/well) and incubated with SKLJI and LPS for 8 h. The samples were then washed with PBS and lysed with 200 μl of cell culture lysis reagent (Promega, Madison, WI). The attached cells were scraped from the dish and centrifuged at 12,000 g at 4°C for 2 min. The resulting supernatant was used to measure luciferase activity by the Luciferase assay system (Promega) following the manufacturer’s instructions.

Measurement of nitrite. The nitrite concentration in the culture medium was measured as an indicator of nitric oxide (NO) production. For nitrite measurement, RAW 264.7 cells were cultured overnight at 1 x 10^6 cells/ml and then treated with LPS (0.1 μg/ml) alone or with LPS with various concentrations of SKLJI for 24 h. The cell supernatants were collected at the end of incubation for nitrite assay following a previous report. Briefly, an equal volume of Griess reagent was mixed with cell supernatant (100 μl aliquots) and the UV absorbance was measured at 540 nm. The concentration of nitrite was calculated from a standard curve obtained with known concentrations of sodium nitrite dissolved in DMEM.

Measurement of LTB4 production. Fresh venous blood was collected into a heparinized tube from healthy volunteers who had not taken any NSAIDs for at least 2 weeks. The blood (500 μl aliquots) was pre-incubated with either 2 μl of vehicle or SKLJI at 37°C for 1 h. followed by incubation with 5 μM calcium ionophore A23187 for 4 h at 37°C. After the determined incubation time, the samples were centrifuged at 1,500 g at 4°C for 10 min. The plasma LTB4 concentration was quantified by ELISA.

Statistical analysis. All values are expressed as mean ± SEM. Statistical significance of the difference was assessed by one-way ANOVA, followed by the Dunnet test. p values lower than 0.05 were considered significant. SigmaStat (Sigma Stat, Jandel, San Rafael, CA) was used for statistical analysis.

Results

Preparation of SKLJI and evaluation of its hemolytic effects
After the purification procedure from n-butanol extract of Lonicera japonica, SKLJI was obtained at 0.28% yield. No significant effect on hemolysis, protein precipitation, or blood clotting times was induced by SKLJI up to 1 mg/ml (Table 1), while the n-butanol extract induced hemolysis and protein precipitation (data not shown), indicating that tannin and saponin were removed by the purification steps. We also confirmed that intravenous injection of SKLJI at up to 150 mg/kg into the mice did not induce any significant adverse effects (data not shown).

Table 1. Safety Assessment of SKLJI for Parenteral Administration
<table>
<thead>
<tr>
<th>Treatment</th>
<th>Concentration (mg/ml)</th>
<th>Hemolysis (%)</th>
<th>Protein Precipitation</th>
<th>Clotting time (s)</th>
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</thead>
<tbody>
<tr>
<td>Saline</td>
<td>—</td>
<td>5.5 ± 0.4</td>
<td>ND</td>
<td>348 ± 13</td>
</tr>
<tr>
<td>SKLJI</td>
<td>0.1</td>
<td>5.9 ± 0.6</td>
<td>ND</td>
<td>343 ± 14</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5.6 ± 0.3</td>
<td>ND</td>
<td>337 ± 14</td>
</tr>
</tbody>
</table>

Rabbit red blood cells reconstituted in normal saline (1/10 dilution) were mixed with an equal volume of SKLJI (0.1, 1 mg/ml) and then incubated at 37°C for 1 h. Hemolysis was then determined by measuring the optical density of the supernatant at 540 nm after removal of RBCs by centrifugation. Experiments for protein precipitation and clotting time are described in “Materials and Methods.” The results are shown as means ± SEM of six animals.

Anti-inflammatory and analgesic activities of intravenous SKLJI injection
To evaluate the anti-inflammatory activities of SKLJI injection, croton oil- and arachidonic acid-induced mouse ear edema tests were conducted after SKLJI i.v. treatment. As shown in Fig. 1A and B, SKLJI and the positive control, diclofenac, significantly inhibited at all the test doses, the arachidonic acid and the croton oil-induced mouse ear edema. SKLJI showed an inhibitory effect at a much lower dose than diclofenac, suggesting that SKLJI i.v. potently inhibits inflammatory responses.

To evaluate the analgesic activities of SKLJI injection, an acetic acid-induced mouse writhing test and a carrageenan-induced rat paw hyperalgesia test were conducted. As shown in Fig. 2A, SKLJI i.v. injection significantly reduced the writhing number at doses of 0.1 and 1 mg/kg. Diclofenac also significantly reduced the number of writhing movements at intravenous doses of 4.5 and 45 mg/kg. As for carrageenan-induced paw hyperalgesia, SKLJI significantly increased the threshold pressure at 10 mg/kg (Fig. 2B). Acetaminophen, the positive control, administrated orally, showed a statistically significant effect only at 100 mg/kg.

Inhibition effects of SKLJI on COX-2, iNOS and 5-LO
Cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) are representative enzymes involved in the progress of inflammation. To determine whether the anti-inflammatory effects of SKLJI can be mediated by modulation of COX-2 or iNOS, lipopolysaccharide (LPS)-induced expression, COX-2 and iNOS was determined by western blot analysis in a murine macrophage cell line, RAW264.7, after treatment with SKLJI. This indicated that unstimulated RAW 264.7 cells do not express COX-2 or iNOS proteins, whereas the addition of LPS induced substantial levels of COX-2 and iNOS synthesis in these cells (Fig. 3A). However, RAW264.7 cells pretreated with SKLJI showed concentration-dependent inhibition of COX-2 and iNOS protein expression, indicating that SKLJI can suppress LPS-induced COX-2 and iNOS expression. This was further confirmed by the COX-2 luciferase reporter gene assay on RAW 264.7 cells, where COX-2 mRNA expression was significantly inhibited by SKLJI (Fig. 3B). In addition, LPS-induced NO production in RAW 264.7 macrophages was suppressed by treatment with SKLJI (Fig. 3C), confirming the inhibitory effects of SKLJI on COX-2 and iNOS. Another important inflammatory enzyme, 5-lipoxygenase (5-LO), was also inhibited.
by SKLJI at concentration above 10 µg/ml (Fig. 3D), indicating that SKLJI can manifest general suppression of the activities of inflammatory enzymes.

**Anti-inflammatory and analgesic activities of loganin and sweroside**

HPLC analysis with pure standards, sweroside and loganin, revealed that the major constituents of SKLJI were sweroside and loganin (Fig. 4). Sweroside and loganin were identified by comparison with commercially available standards and by obtained by NMR analysis (data not shown). The contents of sweroside and loganin in SKLJI were estimated to be 23.1% and 16.7%, respectively.

To confirm that the two major constituents of SKLJI, loganin and sweroside, account for the anti-inflammatory and analgesic activities of SKLJI, croton oil-induced and arachidonic acid-induced mouse ear edema tests and acetic acid-induced writhing tests were done with loganin and sweroside after i.v. administration. As shown in Fig. 5, loganin and sweroside significantly reduced the ear swelling and number of writhing responses, indicating that loganin and sweroside are the active ingredients in the anti-inflammatory and analgesic effects of SKLJI.

**Discussion**

In the present study, we found that SKLJI, the injectable herbal phytomedicine purified from *L. japonica*, can manifest anti-inflammatory and analgesic efficacy in vivo without hemolysis or protein precipitation. The active ingredients were determined to be sweroside and loganin, and additionally, we found that suppression of inflammatory enzymes such as COX-2, iNOS, and 5-LO can mediate the anti-inflammatory activities of SKLJI.

We tried to develop a new injectable herbal analgesic and anti-inflammatory drug with *Lonicera japonica*. Lee et al.\(^\text{11}\) reported that an *n*-butanol (BuOH) fraction of *Lonicera japonica* at oral doses of 100–400 mg/kg showed anti-inflammatory effects in several in vivo animal models, but the low solubility and the saponin and tannin components of the BuOH fraction limit its therapeutic use via the intravenous route, because saponin and tannin can induce protein precipitation and hemolysis.\(^\text{12,13}\)
Therefore, removal of these harmful ingredients and purification of the active portion are required to allow intravenous application. In the present study, we purified the \( n \)-butanol extract through two-step column purification methods and obtained a highly purified, water-soluble extract that can be injected intravenously without induction of hemolysis or protein precipitation. SKLJI was found to be free of saponins and tannins, and had a solubility above 1,000 mg/ml in water (data not shown). In another study of croton oil-induced ear edema, the BuOH fraction showed about 31% inhibition at 400 mg/kg, and SKLJI showed comparable activity, about 42% inhibition at 10 mg/kg. These results indicate that through purification, the potency and safety of herbal drugs can be greatly enhanced.

In this study, SKLJI showed anti-inflammatory and analgesic effects in \textit{in vivo} animal models with intravenous treatment. Major ingredients of SKLJI, loganin and sweroside, showed good anti-inflammatory and analgesic effects through the intravenous route. Several lines of evidence indicate that these ingredients might be one of the active principles in the activities of SKLJI although contributions from other iridoids and flavonoids of \textit{Lonicera japonica}\textsuperscript{20} cannot be ruled out. Loganin through the oral route can inhibit croton oil-and arachidonic acid-induced ear edema at a dose of 100 mg/kg,\textsuperscript{10} and can suppress prostaglandin production in macrophages.\textsuperscript{21} Sweroside is known to have hepatoprotective and antibacterial effects.\textsuperscript{22,23} In this study, loganin and sweroside showed stronger anti-inflammatory and analgesic activity when parenterally administered, indicating that stronger activities can be anticipated \textit{via} the intravenous route than the oral.

The anti-inflammatory activities of SKLJI were found to be due to the inhibition of COX-2, iNOS, and 5-LO. In RAW264.7 cells, SKLJI inhibited COX-2, iNOS, and 5-LO in a dose-dependent manner. In RAW264.7 cells, SKLJI inhibited COX-2 and iNOS in a dose-dependent manner. In RAW264.7 cells, SKLJI inhibited COX-2 and iNOS in a dose-dependent manner. In RAW264.7 cells, SKLJI inhibited COX-2 and iNOS in a dose-dependent manner.
production of inflammatory mediators, including prostaglandins, NO, leukotrienes, and cytokines.\(^{24,25}\) This finding is in line with the mechanism underlying the inflammatory disease models employed in the current study. Croton oil-induced ear edema has been reported to be related to increases of phospholipase A\(_2\) (PLA\(_2\)) activity\(^{26}\) and PLA\(_2\) activation results in the release of free arachidonic acid and the subsequent biosynthesis of prostaglandins and leukotrienes through COX-2 and lipoxygenase.\(^{27-29}\) In addition, croton oil induced COX-2 mRNA expression in a RAW264.7 cell line.\(^{30}\) Arachidonic acid-induced ear edema is also related to the formation of prostaglandins and leukotrienes, such as PGE\(_2\), leukotriene C\(_4\), LTB\(_4\) and leukotriene D\(_4\), which indicates the involvement of COX-2 and 5-LO of macrophages in arachidonic acid-induced inflammation.\(^{31-34}\) In a carrageenan-injection model, edema was associated with marked accumulation of COX-2 mRNA and a selective inhibitor of COX-2 inhibited edema.\(^{35}\) It has also been suggested that nitric oxide (NO) is involved in the inflammatory process in these disease models. Excessive, prolonged iNOS-mediated NO generation can act as a cytotoxic agent in inflammatory disorders, and inhibition of iNOS is beneficial in the treatment and alleviation of inflammatory responses.\(^{36,37}\) Further confirming this, blockade of COX-2, iNOS, and 5-LO was shown to be efficacious in these \(in\) \(vivo\) models as well as in RAW264.7 cell models.\(^{38-42}\)

In summary, we found that SKLJI intravenous injection potently inhibits croton oil- and arachidonic acid-induced mouse ear edema. It reduced acetic acid-induced writhing numbers and carrageenan-induced hindpaw hyperalgesia at low doses. These effects were mediated, at least in part, by inhibition of COX-2 induction and activity of 5-LO and iNOS. All these results indicate that SKLJI, a highly purified extract from \(Lonicera\) \(japonica\), is a candidate for a novel anti-inflammatory and analgesic botanical drug applicable via the parenteral route.

**Authorship**

Contributions: K.H.R. designed the experiments, analyzed the data, and wrote the manuscript. H.I.R., J.H.K., H.Y., B.Y.L., and K.A.U. performed \(in\) \(vitro\) and \(in\) \(vivo\) experiments and analyzed the data. K.K., J.Y.N., and K.M.L. analyzed the data and edited the manuscript. J.H.C. supervised the study.

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