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Anti-angiogenic, antinociceptive and anti-inflammatory activities of *Lonicera japonica* extract

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Abstract

This study aimed to elucidate some novel pharmacological activities of *Lonicera japonica* (Caprifoliaceae), which is widely used in Oriental folk medicine. The ethanolic extract of *L. japonica* (LJ) dose dependently inhibited chick chorioallantoic membrane angiogenesis. The antinociceptive activity of LJ was assessed using the acetic acid-induced constriction model in mice. LJ showed anti-inflammatory activity in two in-vivo models: the vascular permeability and air pouch models. LJ suppressed the production of nitric oxide via down-regulation of inducible nitric oxide synthase in lipopolysaccharide-stimulated RAW264.7 macrophage cells. However, LJ was unable to suppress induction of cyclooxygenase-2 in the stimulated macrophage cells. LJ decreased the reactive oxygen species level in the stimulated macrophage cells. In brief, the flowers of *L. japonica* possess potent anti-angiogenic and antinociceptive activities, in addition to anti-inflammatory activity, which partly supports its therapeutic efficacy.

Introduction

Angiogenesis, the formation of new blood capillaries from pre-existing capillaries and post-capillary venules, is associated with embryonic development and pathological conditions. It is tightly modulated through a balance of positive and negative regulatory factors, and is triggered by pro-angiogenic growth factors, which in turn induce activation of their respective receptors on the surface of endothelial cells (Risau 1995). Diverse pathological conditions, such as diabetic retinopathy, atherosclerosis, inflammatory diseases, tumour growth and metastasis, are driven by undesirable angiogenesis (Folkman 1995). Accordingly, the inhibition of angiogenesis, or anti-angiogenesis, is one promising approach to the treatment of such conditions. Disruption of the signal pathway of angiogenesis can give rise to the obstruction of angiogenesis. Some anti-angiogenic principles are known to inhibit aminopeptidase N, suppress receptor phosphorylation, antagonize vascular endothelial growth factor-mediated anti-apoptosis, and disrupt endothelial tube formation. In recent years, there have been a variety of anti-angiogenic components isolated from natural products, such as psammaplin A from marine sponges *Poecillastra* sp. and *Jaspis* sp. (Shim et al 2004), erianin from *Dendrobium chrysotoxum* (Gong et al 2004), shiraiachrome A and 11,11′-dideoxyverticillin from *Shiraia bambusicola* (Tong et al 2004; Chen et al 2005), epigallocatechin-3-gallate from dried tea leaves (Fassina et al 2004), pseudolarix acid B from *Pseudolarix kaempferi* (Tan et al 2004), withaferin A from *Withania somnifera* (Mohan et al 2004), and geniposide from the gardenia *Gardenia jasminoides* (Koo et al 2004a).

Lonicera japonica Thunb. (Caprifoliaceae) has long been used in Oriental medicine for the treatment of fever, inflammatory diseases, including arthritis, and infectious diseases. The anti-inflammatory properties of *L. japonica* extract were previously investigated in proteinase-activated receptor 2-mediated mouse paw oedema (Tae et al 2003), activated RAW264.7 macrophage cells (Park et al 2005), mouse ear oedema (Kwak et al 2003) and lipopolysaccharide (LPS)-induced rat liver sepsis (Lee et al 2001). It has also been reported that an aqueous extract of *L. japonica* flowers shows anti-inflammatory activity via direct inhibition of cyclooxygenase isoenzymes as well as down-regulation of cyclooxygenase-2 (COX-2) mRNA and protein in A549 lung carcinoma cells (Xu et al 2007). In the present study, an ethanol extract prepared from *L. japonica* flowers was tested for novel pharmacological activity.

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Materials and Methods

Chemicals and fertilized eggs

Retinoic acid, Evans blue, indometacin, dexamethasone, carboxymethyl cellulose (CMC), *Escherichia coli* LPS, 2′,7′-dichlorofluorescein diacetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Griess reagent were purchased from Sigma Chemical Co. (St Louis, MO, USA). Fertilized brown Leghorn eggs were obtained from Pulmuone Food Co., Seoul, Korea. All other chemicals used were of reagent grade or better.

Plant material

The dried flowers of *L. japonica* were purchased at a local market (Cheju, Korea) in March 2006, and authenticated by Professor Ki-Oug Yoo, Division of Life Sciences, Kangwon National University, Chuncheon, Korea. A voucher specimen of the plant material was deposited in the herbarium of the Division of Life Sciences, College of Natural Sciences, Kangwon National University under the acquisition number KWNU56521.

Experimental animals

Male ICR mice (~25 g) were obtained from Samtaco Animal Farm (Osan, Korea). The animal room was maintained at 23 ± 2°C with a 12-h light–dark cycle. Food and tap water were freely available. At least seven mice were used in each experimental group. The ethical guidelines described in the NIH Guide for Care and Use of Laboratory Animals were followed throughout the experiments. Animal experiments performed in this work were approved by the Ethical Committee, Kangwon National University, Chuncheon, Korea.

Preparation of the ethanol extract

The dried flowers of *L. japonica* were ground under liquid nitrogen and extracted for 1 week with 70% ethanol at room temperature. The liquid extract was evaporated in-vacuo to generate dried powder (LJ). The yield was measured to be 24.6%. Using high-performance liquid chromatography (HPLC) analysis, LJ was found to contain two known *L. japonica* constituents (Choi et al 2007): caffeic acid (2.30 g kg^{-1} extract) and luteolin (0.17 g kg^{-1} extract). However, LJ does not contain chrysin, myricetin and quercetin, which were previously identified in *L. japonica* (Kumar et al 2005; Qian et al 2008). For animal experiments, LJ was dissolved in 1% CMC in distilled water; for other experiments, it was dissolved in 70% ethanol.

Cell culture

The RAW264.7 cells, a murine macrophage cell line, were obtained from the American Type Culture Collection (Manassas, VA, USA). The mammalian cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum, 25 mM HEPES (pH 7.5), 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The RAW264.7 cells were plated at a density of 1×10^6 and pre-incubated for 24 h at 37°C, and maintained in a humidified atmosphere containing 5% CO_2. For all experiments, the cells were grown to 80–90% confluence and subjected to no more than 20 cell passages.

Chorioallantoic membrane (CAM) assay

The anti-angiogenic activity of LJ was measured using the CAM assay as previously described (Song et al 2004). The fertilized chicken eggs used in this work were kept in a humidified egg incubator at 37°C. After a 3.5-day incubation, approximately 2 mL of albumen was aspirated from the eggs through a small hole drilled at the narrow end of the eggs, allowing the CAM and yolk sac to drop away from the shell membrane. The shell covering the air sac was punched out and removed by forceps, and the shell membrane on the floor of the air sac was peeled away. A sample-loaded Thermanox coverslip was applied to the CAM surface of the 4.5-day-old chick embryo. At 2 days after returning the chick embryo to the incubator, an appropriate volume of 10% fat emulsion (Intralipose, 10%) was injected into the 6.5-day-old embryo chorioallantois. The eggs were then observed under a microscope. The branching pattern of each egg was graded as 0, 1+ or 2+. Convergence of a few vessels toward the CAM surface was denoted as 1+, and 2+ reflected an increased density and length of vessels toward the CAM face.

Acetic acid-induced vascular permeability

The acetic acid-induced vascular permeability test was performed according to a modification of the method of Whittle (1964). At 30 min after oral administration of vehicle (1% CMC), LJ (30, 100 or 300 mg kg^{-1}), or indometacin (5 mg kg^{-1}) as a positive control, each mouse was injected intravenously with 0.1 mL/10 g bodyweight of 1% Evans blue solution. At 30 min later, 0.1 mL/10 g bodyweight of 0.7% acetic acid in saline was intraperitoneally injected. At 20 min after the administration of acetic acid, the mice were killed by cervical dislocation. After 10 mL of saline was injected into the peritoneal cavity, the washing solutions were collected in test tubes. Concentrations of Evans blue leaked into the peritoneal cavities were measured by the absorbance at 630 nm. The vascular permeability was represented in terms of the absorbance (A_{630}).

Acetic acid-induced abdominal constriction response

The antinociceptive activity of LJ was detected as previously described (Olajide et al 2000). Nociception was induced by intraperitoneal injection of 0.7% acetic acid solution at a dose of 0.1 mL/10 g bodyweight. Each experimental group of mice was treated orally with vehicle (1% CMC), LJ (30, 100 or 300 mg kg^{-1}) or indometacin (5 mg kg^{-1}) as a positive control. At 1 h after the oral administration, 0.7% acetic acid solution was injected. From 5 min later, the number of abdominal constrictions during the following 10 min period was counted.

Carrageenan-induced inflammation in the air pouch

λ-Carrageenan-induced air pouch inflammation was carried out according to a modification of the procedure of Ghosh et al (2000). At 6 days before drug treatment, the air pouch was formed in the intrascapular region of mice by initial subcutaneous injection of 4 mL sterile air and successive injections of 2 mL sterile air every 3 days to sustain its patency. On Day 0, vehicle (1% CMC), LJ (0.03, 0.1 or 0.3 mg/pouch) or dexamethasone (0.01 mg/pouch) was administered into the pouch immediately after injection of λ-carrageenan (1 mL of 2.0% solution). After 16 h, the pouch cavity was opened and the exudates were collected. The exudate volumes were measured using a graduated tube. Aliquots were diluted with Turk solution and the polymorphonuclear leucocytes were counted in a standard haemocytometer chamber.

Nitrite analysis

Accumulated nitrite (NO_2^-) in the media obtained from the cell cultures and the exudates prepared from the carrageenan-induced air pouches was determined using colorimetric assay based on the Griess reaction (Sherman et al 1993). The samples (100 μL) were reacted with 100 μL Griess reagent (6 mg mL^{-1}) at room temperature for 10 min, and then the NO_2^- concentration was determined by measuring the absorbance at 540 nm. The standard curve was constructed using known concentrations of sodium nitrite.

Western blotting

The RAW264.7 cells were incubated with LPS (1 μg mL^{-1}) in the presence or absence of LJ for 24 h and then washed twice with ice-cold phosphate-buffered saline. The cells were lysed in a buffer containing 20 mM HEPES (pH 7.9), 0.1 M KCl, 0.3 M NaCl, 10 mM EDTA, 1% sodium dodecyl sulfate, 1 mM PMSF, 1 μg mL^{-1} leupeptin and 1 μg mL^{-1} pepstatin. For immunoblotting, anti-inducible nitric oxide synthase (Transduction Laboratories, Lexington, KY, USA), anti-COX-2 (Transduction Laboratories) and anti-β-actin (Sigma-Aldrich, St Louis, MO, USA) antibodies were used.

Determination of intracellular reactive oxygen species (ROS)

For analysis of intracellular ROS, the redox-sensitive fluorescent probe DCFH-DA was used as previously described (Royall & Ischiropoulos 1993). After preincubation with varying concentrations of LJ for 1 h, the 1×10^6 RAW264.7 cells were treated with LPS for 24 h. Then, they were incubated with 5 μM DCFH-DA for 30 min at 37°C. The harvested cells were immediately analysed by flow cytometry.

MTT reduction assay

Cell viability was quantified by the MTT assay (Freshney 1994). Briefly, 1×10^5 cells incubated with various concentrations of LJ were treated with 10 μL of MTT solution (5 mg mL^{-1}) for 2 h. The cells were then lysed with isopropyl alcohol, and the absorbance was read at a wavelength of 540 nm.

HPLC analysis

Constituents of LJ were analysed using an Agilent instrument with an 1100 series quaternary pump, an autosampler and a diode array detector linked to an Agilent ChemStation data handling system. Reverse-phase separations were carried out using a Hypersil BDS C18 column (4.6 × 250 mm i.d., 5 μm; Hewlett-Packard, Germany). Reverse phase HPLC was performed by using water/methanol/acetic acid (60/75/5) as a mobile phase, and the column temperature was maintained at 25°C. The flow rate was 1 mL min^{-1} and the injection volume was 10 μL. The eluted components were identified based on the retention time in comparison with the used reference standards. The identity of constituents was also confirmed with a photodiode array detector by comparison with UV spectra of standards over the wavelength range 190–400 nm.

Statistical analysis

The results are expressed as mean ± s.e. Comparison between experimental groups was performed by analysis of variance followed by Tukey's multiple range tests. P values less than 0.05 were considered to be significant. The dose required for half-maximal inhibition (IC50) was calculated from the dose–response linear regression plots.

Results and Discussion

Anti-angiogenic activity

Angiogenesis contributes to the development and progression of various pathological conditions, including tumour growth and metastasis, cardiovascular diseases, inflammatory disease and psoriasis. Down-regulation of angiogenesis has been considered to be advantageous for the prevention of neoplastic growth and inflammation. Some anti-angiogenic substances were identified to be effective in animal models of arthritis, and several antirheumatic drugs, such as indometacin, methotrexate and corticosteroids, have anti-angiogenic activity (Tong et al 2004). Currently, anti-angiogenic strategies are based on inhibition of endothelial cell proliferation, interference with endothelial cell adhesion and migration, and interference with metalloproteinases (Griffioen & Molema 2000). Many researchers have attempted to screen novel anti-angiogenic principles from various natural products. Angiogenesis inhibitors such as fumagillin and minocycline have been isolated from microbial sources (Ingber et al 1990; Tamargo et al 1991). Recently, anti-angiogenic activity has been identified from *G. jasminoides* fruits (Park et al 2003), fruiting bodies of the medicinal mushrooms *Phellinus linteus* (Song et al 2003), *Ganoderma lucidum* (Song et al 2004) and *Cordyceps militaris* (Won & Park 2005). Genipin, the aglycone of the iridoid glycoside, geniposide, was also shown to be an anti-angiogenic principle in *G. jasminoides* (Koo et al 2004b).

The chick CAM is an extra-embryonic membrane commonly used in-vivo to measure both angiogenesis and anti-angiogenesis. It was used to examine the inhibitory activity of LJ on vascular development; retinoic acid was used as a positive control for the assay. Retinoic acid inhibits angiogenesis by down-regulating the expression and release of pro-angiogenic factors (Iurlaro et al 1998). The disc did not give rise to changes in vascular density, indicating that it was unable to affect the growth of blood vessels in the CAM assay (data not shown). After the 2-day treatment, retinoic acid at 1 μg/egg produced an inhibition of ~80.0% in the branching patterns of blood vessels (Figure 1). When 0.1, 0.3, 1.0, 3.0 and 10.0 μg/egg of LJ was applied in the CAM assay, the inhibition of CAM angiogenesis was found to be 4.9%, 13.7%, 32.9%, 45.1% and 58.1%, respectively (Figure 1). This clearly indicates that LJ contains anti-angiogenic activity in a dose-dependent manner. The IC50 of LJ was determined to be 7.14 μg/egg. Taken together, *L. japonica* flowers possess potent anti-angiogencic activity, which might provide pharmacological support for its traditional use for the treatment of inflammatory diseases.

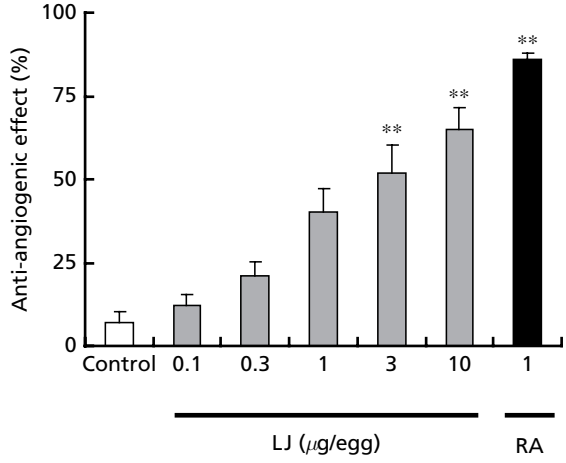

Figure 1 Dose-dependent anti-angiogenic activity of an ethanolic extract of *Lonicera japonica* (LJ) in the chick embryo chorioallantoic membrane assay. Retinoic acid (RA, 1 μg/egg) was used as a positive control. Each group contained at least 20 eggs. Each column represents the mean ± s.e. of the three independent experiments. $^{**}P < 0.01$, significantly different compared with the control group.

Antinociceptive activity

The antinociceptive activity of LJ was evaluated by the acetic acid-induced abdominal constriction response. Although the acetic acid-induced abdominal constriction test is not a very specific nociception model, it is believed to reveal a general antinociceptive activity of the sample under study. Accordingly, the abdominal constriction response of mice to an intraperitoneal injection of acetic acid has been used to screen for antinociceptive activity. Acetic acid is known to cause pain by liberating endogenous substances that excite pain nerve endings. The effect of LJ at the tested doses on the abdominal constriction response in mice is shown in Table 1. It was found that LJ inhibited the abdominal constriction response induced by acetic acid. Its antinociceptive activity was dose-dependent. Indometacin (5 mg kg^{-1}), used as a positive control, significantly inhibited the abdominal constriction response (Table 1). The findings suggest that LJ possesses antinociceptive activity.

In-vivo evaluation of anti-inflammatory activity

Although the anti-inflammatory activity of *L. japonica* has been suggested in several different experimental models, its in-vivo anti-inflammatory activity has not been clearly assessed to date. The present study was designed to clarify the anti-inflammatory activity of LJ using two in-vivo models: the vascular permeability and air pouch models.

In the vascular permeability model, a model typical of the first stage inflammatory reactions, mediators of inflammation released following stimulation lead to dilation of arterioles and venules and increased vascular permeability (Vogel & Vogel 1997). LJ at oral doses of 30, 100 and 300 mg kg^{-1} showed inhibition of 7.9%, 18.8% and 27.2% in the vascular permeability assay, respectively (Table 1). This result implies that the anti-inflammatory activity of LJ arises partly from its ability to prevent the release of inflammatory mediators at the first stage.

Carrageenan-induced inflammation in the air pouch is known to be an excellent acute inflammatory model in which fluid extravasation, leucocyte migration and biochemical parameters in the exudate can be easily detected. The injection of carrageenan into a subcutaneous air pouch on the dorsal surface of rats initiates an inflammatory process. In the carrageenan-induced inflammation in the air pouch model,

Table 1 Inhibitory effects of an ethanolic extract of *Lonicera japonica* (LJ) in acetic acid-induced vascular permeability and abdominal constriction response in mice

Group	Dose (mg kg^{-1})	Evans blue absorbance (inhibition %)	Number of abdominal constrictions (inhibition %)
Control	–	1.11 ± 0.06	23.50 ± 1.28
LJ 30	30	1.03 ± 0.06 (7.9)	20.33 ± 1.09 (13.5)
LJ 100	100	0.90 ± 0.04* (18.8)	15.50 ± 0.68** (34.0)
LJ 300	300	0.81 ± 0.05** (27.2)	12.00 ± 1.06** (48.9)
Indometacin	5	0.80 ± 0.03** (28.0)	2.33 ± 0.71** (90.1)

Indometacin (5 mg kg^{-1}) was used as a positive control. LJ (30, 100 and 300 mg kg^{-1}) was administered orally. Vascular permeability was represented by the absorbance at 630 nm. The results are expressed as mean ± s.e., with seven mice in each group. The experiment was performed in triplicate. Numbers in parentheses indicate inhibition % with respect to the control group treated only with 1% carboxymethyl cellulose. $^{*}P < 0.05$, $^{**}P < 0.01$, significantly different compared with the control group.

Table 2 Effects of an ethanolic extract of *Lonicera japonica* (LJ) in the carrageenan-induced air pouch model in mice

Group	Dose (mg/pouch)	Volume of exudate (mL) (inhibition %)	Number of total leucocytes ($\times 10^6$ cells) (inhibition %)	Content of nitrite (μM) (inhibition %)
Control	–	1.62 ± 0.09	43.63 ± 5.08	12.13 ± 1.65
LJ 0.03	0.03	1.52 ± 0.10 (6.2)	28.78 ± 3.91* (34.0)	11.22 ± 2.24 (7.5)
LJ 0.1	0.1	1.38 ± 0.19 (14.4)	19.12 ± 2.96** (56.2)	4.35 ± 0.42** (64.1)
LJ 0.3	0.3	1.27 ± 0.16* (21.6)	16.44 ± 1.75** (62.3)	4.18 ± 0.42** (65.6)
Dexamethasone	0.01	1.20 ± 0.05** (25.8)	15.08 ± 2.23** (65.0)	2.61 ± 0.23** (78.5)

The results are expressed as mean ± s.e., n = 8. Figures in parentheses indicate inhibitory percentages with respect to the corresponding control. Dexamethasone was used as a positive control. The control group was treated only with 1% carboxymethyl cellulose. The experiment was performed in triplicate. *$P < 0.05$, **$P < 0.01$, significantly different compared with the control group.

dexamethasone (0.01 mg/pouch), a non-selective cyclooxygenase inhibitor, reduced the volume of exudates by 25.8% (Table 2). Treatment with LJ at 0.03, 0.1 and 0.3 mg/pouch gave rise to an inhibition of 6.2%, 14.4% and 21.6%, respectively, with respect to control exudate volume (Table 2). Total numbers of polymorphonuclear leucocytes in the air pouches were also decreased by treatment with LJ at 0.03, 0.1 and 0.3 mg/pouch, the percentage inhibition being 34.0%, 56.2% and 62.3%, respectively (Table 2). Taken together, LJ has acute anti-inflammatory activity, which confirms the in-vivo anti-inflammatory activity of *L. japonica*.

Inhibitory effect on nitric oxide (NO) production

Inducible nitric oxide synthase (iNOS) plays a regulatory role in the expression of pro-inflammatory mediators in inflammation. iNOS-derived NO is involved in various pathological conditions, such as inflammation and autoimmune diseases, and leads to cellular injury (Singh et al 2000). Suppression of NO production is believed to be closely linked with anti-inflammatory action. The inhibitory effect of LJ was evaluated on LPS-induced NO expression in RAW264.7 macrophages (Figure 2). The accumulated nitrite in the medium, determined by the Griess method, was used as an index of the NO level. When the macrophage cells were treated with LPS, the nitrite content increased ~11-fold (Figure 2). When the macrophage cells were pretreated with 0.1, 0.5 and 1.0 mg mL^{-1} LJ, the NO production induced by LPS was significantly suppressed in a concentration-dependent manner (Figure 2), which agrees with the previous findings of Park et al (2005). A suppressive effect of LJ on the production of NO was also confirmed in the in-vivo air pouch model. As shown in Table 2, LJ gave rise to a decrease in the content of nitrite in the exudates obtained from the carrageenan-induced inflammation in the air pouch, which corresponds with the in-vitro results obtained using the macrophages (Figure 2). LJ was also found to suppress iNOS induced in LPS-activated RAW264.7 macrophage cells (Figure 3). However, LJ was unable to suppress COX-2 induced in LPS-activated RAW264.7 macrophage cells (Figure 3). This finding differs from the previous finding that an aqueous extract of *L. japonica* can cause down-regulation of COX-2 (Xu et al 2007). Accordingly, different constituents in aqueous and ethanolic extracts are estimated to be responsible for an anti-inflammatory activity of each extract. Taken together, LJ suppresses NO production by

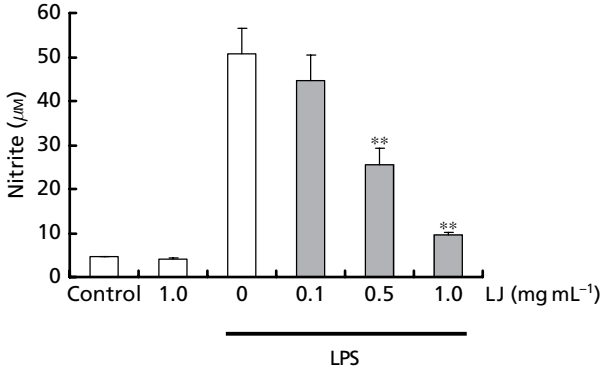

Figure 2 Inhibitory effect of an ethanolic extract of *Lonicera japonica* (LJ) on lipopolysaccharide (LPS)-induced nitric oxide production in RAW264.7 macrophage cells. The mammalian cells were incubated for 24 h with LPS (1 μg mL^{-1}) in the presence or absence of the indicated concentrations of LJ. The values are mean ± s.e. of three independent experiments. **$P < 0.01$, significantly different compared with LPS only.

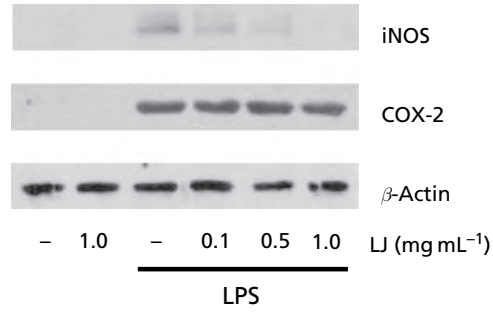

Figure 3 Effects of *Lonicera japonica* extract (LJ) on lipopolysaccharide (LPS)-induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in RAW264.7 macrophage cells. The mammalian cells were incubated for 24 h with LPS (1 μg mL^{-1}) in the presence or absence of indicated concentrations of LJ. After 24-h incubation, the cell lysates (30 μg protein) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane and blotted with appropriate antibodies. β-Actin was used as an internal control.

inhibiting induction of iNOS in the activated macrophages, which might support its anti-inflammatory and antinociceptive activities.

Suppressive effect on the ROS level

ROS, at the physiological concentrations required for normal cellular function, are involved in intracellular signalling and redox regulation (Nordberg & Arner 2001). Excessive levels of ROS cause oxidative stress, which threatens the integrity of various biomolecules and is involved in ageing. ROS are known to initiate a variety of toxic oxidative reactions, including lipid peroxidation, direct inhibition of mitochondrial respiratory chain enzymes, inactivation of glyceraldehyde-3-phosphate dehydrogenase, inhibition of membrane sodium/potassium ATPase activity, inactivation of membrane sodium channels, and other oxidative modification of proteins (Cuzzocrea 2006). All these reactions are considered to play a role in inflammatory processes (Cuzzocrea 2006). A pathway leading to an activation of transcription factor NF-κB, a regulator of inflammation, is also under ROS-mediated control (Bubici et al 2006). As shown in Table 3, when the macrophage cells were treated with LPS only, the intracellular ROS level was increased ~7-fold. However, LJ was able to suppress ROS generation that was elevated by LPS in the macrophages. Its suppressing ability was weak but significant (Table 3). This finding may suggest that LJ has in-vivo antioxidant activity.

Cytotoxicity

This study demonstrates that LJ contains some novel pharmacological activities, such as anti-angiogenic and antinociceptive activities. LJ was also found to reduce the production of NO via suppression of iNOS expression in LPS-activated RAW264.7 macrophage cells. No cytotoxicity on the macrophages was observed at the concentrations of LJ used, as determined by the MTT assay (Table 4). LJ, up to 1.0 mg mL^{-1}, was unable to affect cell viability (Table 4). This result seems to correspond with the previous findings of Thanabhorn et al (2006). The single oral dose of an ethanol extract of *L. japonica* did not produce mortality or significant changes in the general behaviour and gross appearance of the internal organs of rats (Thanabhorn et al 2006). In the sub-acute toxicity test, there were no significant differences in the bodyweight and organ weights between the control and the treated groups (Thanabhorn et al 2006).

There have been several reports on the constituents of *L. japonica*. Loniceroside A, a triterpenoid saponin, was isolated from the aerial parts of *L. japonica*, and showed in-vivo anti-inflammatory activity against mouse ear oedema provoked by croton oil (Kwak et al 2003). Luteolin (3′,4′,5,7-tetrahydroxyflavone) was also identified as an active constituent of *L. japonica*, which produced anti-tumour activity (Leung et al 2006). Ochnaflavone, isolated from *L. japonica*, was found to inhibit COX-2 and 5-lipoxygenase in mouse bone marrow-derived mast cells (Son et al 2006). Various antioxidant constituents, such as caffeic acid, 5-hydroxymethyl-2-furfural, luteolin 7-*O*-β-D-glucopyranoside and kaempferol 3-*O*-β-D-glucopyranoside, were also isolated from *L. japonica* (Choi et al 2007). The ethanolic extract used in this study was found to contain caffeic acid and luteolin using HPLC analysis; other constituents of the ethanolic extract remain elusive at present.

Table 3 Effects of an ethanolic extract of *Lonicera japonica* (LJ) on the reactive oxygen species (ROS) level in RAW264.7 macrophage cells

Group	LPS treatment	LJ concentration (mg mL^{-1})	DCF fluorescence
Control 1	–	–	22.6 ± 1.7
Control 2	–	1.0	29.1 ± 7.3
LPS only	+	–	157.5 ± 9.7 (100)
LJ 0.1	+	0.1	142.5 ± 6.8 (90.5)
LJ 0.5	+	0.5	130.7 ± 5.9** (83.0)
LJ 1.0	+	1.0	124.3 ± 2.9** (78.9)

The mammalian cells were incubated for 24 h with 1 μg mL^{-1} lipopolysaccharide (LPS) in the presence or absence of the indicated concentrations of LJ. DCF fluorescence is an index of ROS levels. Data represent the mean ± s.e. of three independent experiments. The figures in the parentheses indicate relative values compared to the value of the LPS only. **$P < 0.01$, significantly different compared with the LPS only.

Table 4 Effects of an ethanolic extract of *Lonicera japonica* (LJ) on the cellular viability in RAW264.7 macrophage cells

Group	Concentration (mg mL^{-1})	Relative viability (%)
Control	–	100.0 ± 11.8
LJ 0.1	0.1	107.6 ± 8.2
LJ 0.5	0.5	112.1 ± 2.7
LJ 1.0	1.0	118.0 ± 14.2
LPS	0.001	97.2 ± 13.3

The mammalian cells were incubated for 24 h with 1 μg mL^{-1} lipopolysaccharide (LPS) or in the presence or absence of the indicated concentrations of LJ. Cell viability was evaluated by the MTT assay. Data represent the mean ± s.e. of three independent experiments.

Conclusion

LJ has anti-angiogenic activity and also significant antinociceptive activity. LJ was confirmed to have in-vivo anti-inflammatory activity, which further supports the in-vitro anti-inflammatory activity of *L. japonica*. LJ exhibited an in-vivo suppressive effect on the production of NO in the air pouch model. LJ suppressed production of NO via inhibition of iNOS induction in LPS-stimulated RAW264.7 macrophage cells. LJ was able to suppress the enhanced ROS level in the LPS-stimulated RAW264.7 macrophage cells. These findings provide additional pharmacological information regarding the therapeutic efficacy of *L. japonica*.

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