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Antiplatelet and Antithrombotic Activities of *Lindera obtusiloba* Extract *in vitro* and *in vivo*

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Abstract − Several studies have shown that plant-derived polyphenols reduce cardiovascular accidents in high-risk patients and the inhibition of platelet function may be responsible for part of this benefit. *Lindera obtusiloba* is widely used in traditional herbal medicine for the treatment of cardiovascular and inflammatory diseases. Therefore, the antiplatelet and antithrombotic activities of *Lindera obtusiloba* Extracts (LOE) on *in vitro* platelet aggregation, radical scavenging activity and *in vivo* murine pulmonary thrombosis were examined. LOE was able to directly scavenge the stable DPPH radical in a concentration-dependent manner and its IC_{50} value was 3.9 ± 0.1 μg/ml. LOE significantly inhibited collagen- and ADP-induced platelet aggregation in a concentration-dependent manner and its IC_{50} value is 0.9 ± 0.1 mg/ml and 0.4 ± 0.1 mg/ml respectively. The inhibitory effect of LOE was comparable to aspirin (IC_{50} values were 1.0 ± 0.5 and 1.0 ± 0.7 mg/ml, respectively). Furthermore, oral administration of LOE suppressed the death of mice with pulmonary thrombosis induced by intravenous injection of collagen plus epinephrine. Taken together, our results suggest LOE may be a promising candidate for antithrombotic agent, and the antithrombotic effect of LOE may be due to, at least in part, antiplatelet activity.

Keywords: *L.obtusiloba,* Platelet, Thrombosis, Antioxidant

INTRODUCTION

Platelets are essential in the maintenance of vascular integrity and the control of bleeding through forming blood clot, but they are also implicated in pathogenesis of thrombosis including their adherence to the sites of vessel injury, aggregation to form hemostatic plugs or thrombi, and acceleration of the coagulation cascades leading to the formation of thrombosis (May *et al.,* 2008). Only activated platelets can change shape, aggregate and release the contents of their intracellular granules via several intracellular biochemical pathways (Kahner *et al.,* 2006). The interactions between platelets and blood vessel walls are important in the development of thrombosis and cardiovascular disease such as myocardial infarction, stroke, and atherosclerosis (Mayr and Jilma*,* 2006). Therefore, the regulation of platelet function can be a promising target for the prevention or treatment of thrombosis.

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However, commonly used antithrombotic agents such as aspirin, recently developed drugs like the ADP receptor antagonist such as ticlopidine and its thienopyridine derivative clopidogrel and antagonists of the glycoprotein IIb/IIIa such as abciximab, eptifibatide (Harder *et al.,* 2001) that inhibit platelet aggregation and thromboxane A2 synthetase, have side effects including internal bleeding, prolonged bleeding time, and palpitation gastrointestinal symptoms and hemorrhage (Francescone and Halperin*,* 2008; Johnson*,* 2008). Therefore, development of antithrombotic agent from medicinal plants with fewer side effects has attracted much interest (Allman-Farinelli and Dawson*,* 2005; Vitseva *et al.,* 2005).

During our previous research aimed at the discovery of new cardiovascular protective agents, about three hundred different medicinal plants used in the oriental medicine were screened by evaluating antithrombotic activity of their solvent extract. Among them, the extract from *Lindera obtusiloba* was found to exhibit high potency.

L. obtusiloba, which is distributed in many provinces of Korea and China, is widely used in traditional herbal medi-

cine for the treatment of bruise, extravasation and inflammation (Yook, 1989). Chemically, bioactive components isolated from the Japanese spicebush *L. obtusiloba* belong to the groups of lignans, butanolides, and several phytosterols, and they were shown to exert antitumor activity and antioxidant activity (Kwon *et al.,* 2000; Lee *et al.,* 2009; Ruehl *et al.,* 2009).

This study investigated whether the LOE has antithrombotic properties using *in vitro* and *in vivo* experimental model.

MATERIALS AND METHODS

Extract and polyphenol contents

The *L. obtusiloba* were collected at the vicinity of Hongcheon, Republic of Korea and the voucher specimen (No. YJP-14) was deposited at the Herbarium of KIST Gangneung Institute, Republic of Korea. *L. obtusiloba* was identified and provided by Dr. Chul Young Kim, KIST Gangneung Institute, Gangneung, Korea. Dried small branches were cut into small pieces and ground using a commercial food mixer. The *L. obtusiloba* (15.8 kg) was extracted four times with hot 50% EtOH for 4 h. This residue was evaporated in vacuo to yield the total extract (1.7 kg, 7.0 % w/w). The amount of polyphenols contained in the LOE were determined according to the Folin-Ciocalteu colorimetic method. The solid was stored at −70°C until use.

Animals

Male ICR mice were purchased from Orient BIO Inc., Korea and were used in these studies. Animals were housed in colony cages, under standard laboratory conditions (12:12 h light/dark cycle) and have free access to standard commercial diet and water. The study conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and has been approved by the Institutional Animal Care and Utilization Committee for Gyeonggi BioCener, Suwon, Republic of Korea.

DPPH radical scavenging effect

The antioxidant properties of test materials were evaluated by 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activity. Ethanol solutions of the test samples at various concentrations (0.1-100 μg/ml) were added to a solution of DPPH in methanol (0.2 mM) in 96 well plates. After storing these mixtures for 30 minutes at room temperature, the remaining amount of DPPH was colorimetrically determined at the wavelength of 520 nm using a microplate reader (Yoshida *et al.,* 1989). The scavenging activity was determined by comparing the absorbance with that of ascorbic acid (1.5 μg/ml) as a positive control. % scavenging activity=[(Absorbance of blank−Absorbance of samples)/Absorbance of blank]×100. The mean values were obtained from minimum triplicated experiments.

Platelet aggregation

Platelet aggregation was measured by Born's method (Born *et al.,* 1971) using a dual channel automatic optical aggregometer (560, Chrono-log Co.). Rat platelet rich plasma (PRP) was incubated at 37°C for 2 min in the aggregometer with stirring at 1,000 rpm and exposed to various concentrations (0.1, 0.3, or 1 mg/ml) of LOE for 3 min. After incubation, platelet aggregation was induced by addition of collagen (20 μg/ml) or ADP (20 μM). The resulting aggregation was measured by the change in light transmission and recorded for 10 min. The inhibition extent of platelet aggregation is expressed as % inhibition (X) using the following equation: X=[(A−B)/A]×100, where A is the maximal aggregation rate of vehicle-treated platelets, and B is the maximal aggregation rate of LOE-treated platelets.

Thromboembolism mouse model

The antithrombotic effect of LOE was measured by mouse pulmonary thrombosis test as DiMinno's method (DiMinno and Silver, 1983) described. In brief, LOE (400 mg/kg) and aspirin (50 mg/kg) as a positive control were orally administered once per day for 5 days to ICR mice weighing 25-30 g. 24 hr after final oral administration of the samples, a mixture solution of collagen (20 μg/mouse) plus epinephrine (2 μg/mouse) was injected into the tail vein to induce pulmonary thrombosis (Jin *et al.,* 2004). The collagen solution containing native collagen fibrils from equine tendons was used (385, Chrono-log Co.). The experiments were triplicated. The number of dead mice was counted for 15 min, and the survival rate (%) was calculated with the following equation: [1−(dead or paralyzed mice)/total mice tested]×100.

Statistical analysis

The experimental results were expressed as the mean ± S.E.M. Fisher's Exact Test for the thromboembolism mouse model, and unpaired Student's *t* test for the sample-tested groups and control groups were performed. The data were considered significant with a probability less than 0.05.

RESULTS

Polyphenol concentration and antioxidant property of LOE

Antioxidant activities are assumed to be useful for the prevention of oxidative damage in aging and age-related disorders. The concentration of polyphenols in *L. obtusiloba* Extracts (LOE) measured by analyzing for total phenol by the Folin-Ciocalteau procedure was 23.7%. The free radical scavenging activity of LOE was examined to bleach a stable radical DPPH, which provided information on the reactivity of LOE with free radicals. LOE was able to directly scavenge the stable DPPH radical in a concentration-dependent manner (Fig. 1) and the free radical scavenging activity of LOE (IC_{50} value was 3.9 ± 0.1 μg/ml) was potent although it was slightly less effective than that of ascorbic acid (IC_{50} value was 1.4 ug/ml).

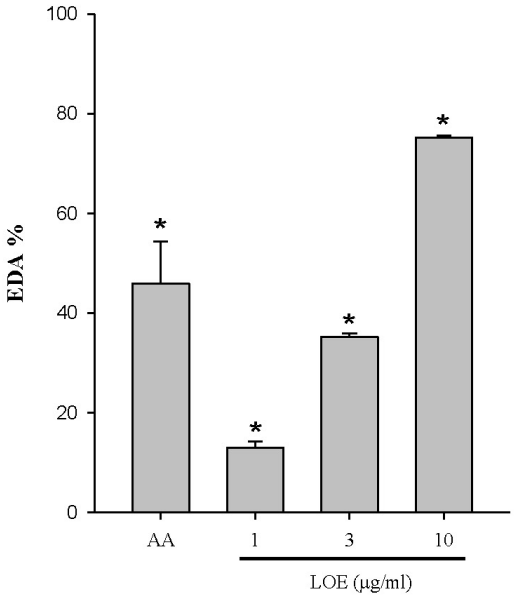


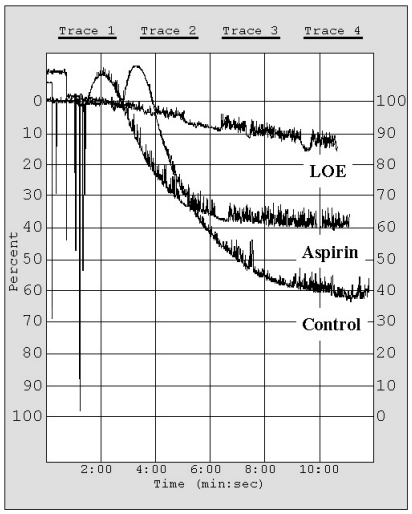
Fig. 1. DPPH free radical scavenging activity of *L. obtusiloba* Extracts (LOE). LOE were incubated with 30 μM of DPPH ethanol solution for 30 min. 1.5 μg/ml of ascorbic acid was used as positive control. All data represent means ± S.D. (n=3). *p < 0.05, compared with control.

Effect of LOE on aggregation of agonist-stimulated platelets

Rat platelets were pre-incubated with various concentrations of LOE (0.1, 0.3, or 1 mg/ml), and then exposed to collagen (20 μg/ml) or ADP (2 μM). As shown in Fig. 2 and 3, LOE strongly inhibited the platelet aggregation induced by collagen at 1 mg/ml and IC_{50} value was 0.9 ± 0.1 mg/ml. The inhibitory effect on ADP-induced aggregation was more pronounced (IC_{50} value was 0.4 ± 0.1 mg/ml) and shown dose-dependent manner. In addition, the inhibitory effect of LOE on collagen- and ADP-induced platelet aggregation was comparable to aspirin (IC_{50} values were 1.0 ± 0.5 and 1.0 ± 0.7 mg/ml, respectively).

Effect of LOE on pulmonary thromboembolism in mice

The above *in vitro* assays supported antithrombotic activity of LOE. We therefore tested the *in vivo* antithrombotic effect using a pulmonary thromboembolism model induced by intravenous injection of collagen (20 μg/mouse) plus epinephrine (2 μg/mouse). The lethal effect of these aggregating agonists in mice was known to be caused by



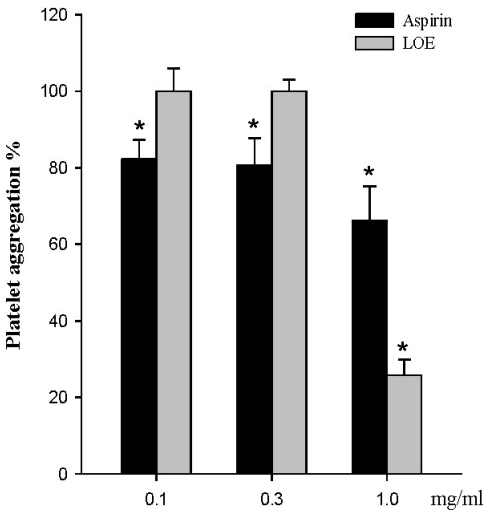
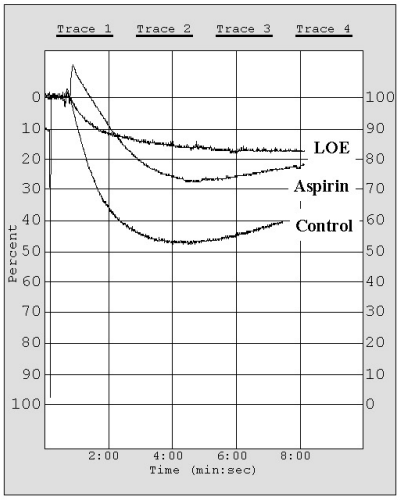


Fig. 2. Effect of LOE on platelet aggregation by collagen. Representive platelet aggregation profiles in collagen treated platelets in absence or presence of aspirin (1 mg/ml), LOE (1 mg/ml) (A) and cumulative result in dose dependent manner (B). All data represent means ± S.D. (n=3). *p < 0.05, compared with untreated control.



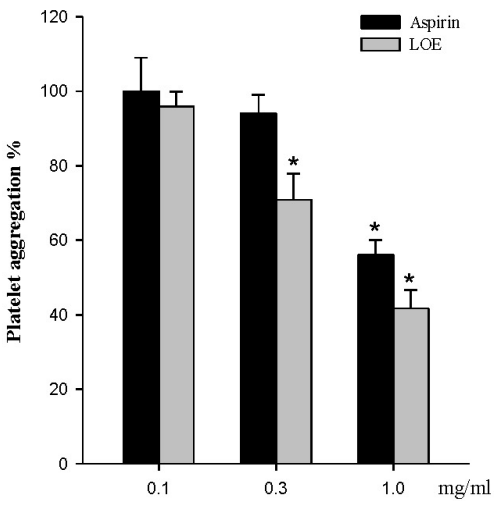


Fig. 3. Effect of LOE on platelet aggregation by ADP. Representative platelet aggregation profile in collagen treated platelets in absence or presence of aspirin (1 mg/ml), LOE (1 mg/ml) (A) and cumulative result in dose dependent manner (B). All data represent means ± S.D. (n=3). *$p < 0.05$, compared with untreated control.

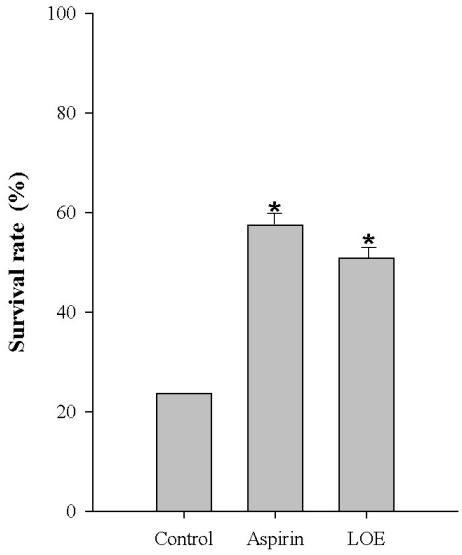


Fig. 4. Effect of LOE on pulmonary thrombosis in mice. LOE (400 mg/kg) was orally administered for 5 days before the intravenous injection of epinephrine (2 μg/mouse) plus collagen (20 μg/mouse). *$p < 0.05$ by Fisher's Exact Test, compared with control. n=54-57.

massive occlusion of the microcirculation of the lungs via platelet thromboembolism. Vehicle or LOE (400 mg/kg) was orally administered to mice once a day for 5 days before the thromboembolism induction. The number of dead mice was counted 15 min after the thromboembolism induction. As shown in Fig. 4, oral administration of LOE to mice resulted in a significant protection from the death due to thromboembolism.

DISCUSSION

Thrombosis is the formation of a blood clot, which is known as thrombus, inside a blood vessel, obstructing the flow of blood through the circulatory system. Pathologically, thrombosis may occur if the hemostatic stimulus is unregulated, either at the level of stimulatory or inhibitory pathways. The balance between stimulatory and inhibitory pathways is also well established for platelets. Platelets are activated by various endogenous factors and a platelet-rich thrombus is formed subsequently in the lumen of the injured vessel. Platelet deposition by aggregation at these sites seems to be an important event leading to thrombotic disorders. In particular, coronary thrombosis is the underlying mechanism in transition from stable to unstable coronary diseases (Eisenberg and Ghigliotti, 1999; Furie and Furie, 2008). Also, thrombosis is an important factor in the pathophysiology of unstable angina and myocardial infarction. Therefore, platelet aggregation has been identified as a promising target for development of antithrombotic agents (Meyer, 1998). However, existing antithrombotic drugs, including aspirin, ticlopidine and clopidogrel, have side effects such as gastrointestinal diseases and bleeding (Cannon *et al.,* 1998). Therefore, in the present study, we focused on the *in vitro* and *in vivo* experimental evaluation of LOE, one of medicinal plants used in Korean folk remedy with little side effects.

Recently, the consumption of polyphenol-rich foods, including vegetables and fruits, was reported to reduce the risk for acute coronary disease incidence, which was closely associated with thrombosis (Ozben *et al.,* 2006; Park *et al.,* 2008). Therefore, antithrombotic agent development from medicinal plants with little side effects has at-

tracted much interest (Vitseva *et al.,* 2005). Several medicinal plants offer potential for the prevention and treatment of cardiovascular diseases. (Ernst*,* 2005; Ihm *et al.,* 2009) The best-selling herbal remedies in the USA and world-wide are formulations of Ginkgo biloba. It is reported that Ginkgo biloba extract is effective in the inhibition of platelet aggregation and thus it could be potentially used as an effective oral anti-platelet therapeutic agent.

In this work, we provide the first experimental evidence for antiplatelet/antithrombotic properties of *L. obtusiloba* Extracts (LOE). LOE strongly inhibited collagen, ADP-induced platelet aggregation in a concentration dependent manner, suggesting LOE can be a platelet aggregation inhibitor. This hypothesis was further investigated using *in vivo* pulmonary thromboembolism model, which the most noteworthy data came from. Oral administration of LOE (400 mg/kg) resulted in increase of survival rates of mice with pulmonary thromboembolism, indicating strongly that LOE exerts *in vivo* antithrombotic activity as predicted by our *in vitro* platelet aggregation assays.

The exact mechanism of anti-platelet aggregation properties should be determined by using different experimental model. One possible explanation of inhibitory effect of LOE on platelet aggregation is its strong anti-oxidant property. A previous study demonstrated that polyphenols inhibit platelet function by enhancing redox status (Freedman *et al.,* 2001). The balance between oxidative stress and platelet production of NO plays a key role in the process of platelet recruitment, which is an important phase of platelet activation at the site of vascular injury (Freedman *et al.,* 1997). It is recently reported that reactive oxygen species (ROS) influence platelet function and coagulation, and are associated with the pathogenesis of cardiovascular disease (Krotz *et al.,* 2004; Gorlach*,* 2005; Arthur *et al.,* 2008). In addition, polyphenols exert an antioxidant effect via inhibition of O_2^- (superoxide) generation and suggest that this effect could result in enhanced NO bioavailability and inhibit platelet recruitment (Pignatelli *et al.,* 2006). It is thought that these results may lead to extend further the wide range of effects described for LOE, and encourage other studies to elucidate the mechanisms of action involved in the antithrombotic action.

In summary, LOE strongly inhibited platelet aggregation in collagen and ADP activated platelets and its inhibitory effect is comparable to aspirin. In addition, LOE effectively decreased the mortality rate of mice from thromboembolism. Our results suggest LOE could be very promising candidate for cardiovascular protective agent.

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