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The Methanol Extract of *Azadirachta indica* A. Juss Leaf Protects Mice Against Lethal Endotoxemia and Sepsis

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Abstract

In the present study, the inhibitory effect of neem leaf extract (NLE) on lipopolysaccaride (LPS)-induced nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production was examined both *in vitro* and *in vivo*. *In vitro* study revealed that NLE treatment (100 μg/ml) inhibits LPS (100 ng/ml)-induced NO production by 96% and TNF-α production by 32%. The reduction in NO production is probably conferred by the complete suppression of inducible nitric oxide synthase (iNOS) expression. Interestingly, *in vivo* NLE significantly improved the survival rate of mice in an experimental sepsis model. Administration of NLE (100 mg/kg) 24 h before LPS treatment (20 mg/kg) improved the survival rate of mice by 60%. The inhibition of plasma NO and TNF-α production by NLE is likely to account for the improved survival of mice. Our results suggest that NLE may present a promising avenue in the development of therapeutic agents for the treatment of inflammatory diseases.

Key Words: Sepsis, *Azadirachta indica* A. Juss, Rutin, NO, TNF-α, LPS

INTRODUCTION

Sepsis is considered to be a serious problem in critically ill patients, despite medical progress and an improvement in our understanding of its pathophysiology. Victims of septic shock experience fever, falling blood pressure, myocardial suppression, dehydration, acute renal failure, and respiratory arrest (Tracey and Cerami, 1994). The morbidity and mortality associated with sepsis are mediated in part by bacterial endotoxins, which stimulate the release of pro-inflammatory cytokines from macrophages and monocytes (Ayala *et al.*, 2000). Lipopolysaccharide (LPS) in the outer membrane of gram-negative bacteria has been reported to activate macrophages and induce the subsequent release of massive amounts of pro-inflammatory cytokines and nitric oxide (NO) free radicals during endotoxic shock. In addition, bacterial LPS in the bloodstream induces overexpression of various inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β, nitric oxide (NO), and prostaglandin E_2 (PGE_2).

Many compounds such as microbial sensor kinase QseC inhibitors, LPS neutralizing agents, Toll-like receptor (TLR) antagonists, and inhibitors of the IκB kinase complex have been investigated for the treatment of sepsis, as they act by blocking microbe-initiated inflammatory signaling (Mora *et al.*, 2005; Ii *et al.*, 2006; Nguyen *et al.*, 2007; Sil *et al.*, 2007; Kim *et al.*, 2008; Rasko *et al.*, 2008). However, the clinical efficacy of these compounds has not yet been established (Hu *et al.*, 2011). Moreover, TAK-242, a TLR4 antagonist, has been proven to be ineffective in clinical trials (Hu *et al.*, 2011). Thus, to date, the only drug to be licensed by the FDA for the treatment of severe sepsis clinically is recombinant human activated protein C (Xigris; Eli Lilly) (Bernard *et al.*, 2001). However, this compound has been found to have no effect in patients with milder cases of sepsis (Toussaint and Gerlach, 2009). Therefore, the development of effective medicine for sepsis is still needed.

Since ancient times, many plants have been used medicinally to combat disease. *Azadirachta indica* A. Juss (neem) is one of these plants and has been used for more than two thousand years in India and neighboring countries. It has a wide

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spectrum of biological activity, and is one of the most versatile medicinal plants. Various parts of the neem tree have been used for food, medicine, and as insecticides. Several pharmacological actions and medicinal applications are known. In particular, the leaves of neem tree are traditionally used in medicinal preparations for their anti-inflammatory, antifungal, antibacterial, antiviral, antioxidant, hepatoprotective, and cardioprotective effects (Okpanyi and Ezeukwu, 1981; Rao *et al.*, 1998; Almas, 1999; Badam *et al.*, 1999; Yanpallewar *et al.*, 2003). Although these findings might have important implications for the role of methanol neem leaf extract (NLE) as an anti-inflammatory agent, the mechanism of action underlying the anti-inflammatory effect of NLE has not been intensively examined. Therefore, in this study, the function of NLE as an anti-inflammatory effector was investigated in a mouse model of sepsis.

Our results show the anti-inflammatory effect of NLE *in vitro* and *in vivo*, providing a new possible therapeutic strategy for the treatment of endotoxemia and sepsis.

MATERIALS AND METHODS

Cell culture

Murine RAW264.7 macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (w/v) fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in an incubator at 37°C with a humidified atmosphere of 95% air and 5% CO_2. All the reagents used in the cell culture were obtained from GIBCO (Grand Island, USA).

Preparation of methanol extract of neem leaves

Fresh *Azadirachta indica* A. Juss (neem) leaves were shade-dried for days at room temperature and powdered with a grinder. The dried powder of *Azadirachta indica* A. Juss (neem) was soaked in HPLC-grade methanol and 45°C. The mixture was filtrated, condensed using a rotary evaporator, and finally lyophilized (Modul Spin 40, Biotron Co.). This methanol extract of *Azadirachta indica* A. Juss (neem) leaves was obtained from the Plant Extract Bank (Daejeon, Korea) and was dissolved in dimethylsulfoxide (DMSO) for the subsequent studies.

Cell viability

Cell viability assays were carried out using the CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega (Madison, USA) according to the manufacturer's instructions. Briefly, after the RAW264.7 cells were seeded for 24 h, the medium was removed and replaced with fresh medium containing LPS (100 ng/ml) purchased from Sigma Chemical Co. (St. Louis, USA), and cells were incubated for 30 min. Next, the cells were cultured for 24 h with various concentrations of NLE (0-200 μg/ml), 20 μl of methanethiosulfonate/phenazine methosulfate solution (MTS) was added to each well, and the cells were incubated at 37°C for 1.5 h. The absorbance was read at a wavelength of 490 nm using a Tecan Infinite F200 microplate reader (Männedorf, Switzerland).

Determination of *in vitro* NO concentration

RAW264.7 cells (1×10^5 cells/well) were incubated in a 96-well plate for 24 h with 100 ng/ml of LPS and various concentrations of NLE (0, 25, 100, or 200 μg/ml). The concentration of nitrite (NO_2^-), the oxidized product of NO, was measured as an indicator of NO production using the nitric oxide detection kit obtained from iNtRON Biotechnology, Inc (Seoul, Korea). Culture supernatant (50 μl) was mixed with the same volume of Griess reagent [1% (w/v) sulfanilamide and 0.1% (w/v) *N*-[1-naphthyl]ethylenediamine dihydrochloride in 5% (w/v) phosphoric acid] for 10 min, and the absorbance was measured at 540 nm.

Measurement of *in vitro* TNF-α production

RAW264.7 cells (1×10^5 cells/well) were incubated in a 96-well plate for 24 h with 100 ng/ml of LPS and various concentrations of NLE (0, 25, 100, or 200 μg/ml). The culture supernatant was then collected, and TNF-α was detected using an ELISA kit according to the manufacturer's instructions, R&D Systems Inc (Minneapolis, USA).

Analysis of iNOS and COX-2 protein expression by western blotting

After treatment with NLE, proteins from RAW264.7 cells were obtained by lysing the cells in ice-cold radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate]. Whole cell lysates (50 μg) then underwent electrophoresis on 10% polyacrylamide gels, were transferred to nitrocellulose membranes, and probed. The membranes were preincubated for 1 h at room temperature in Tris-buffered saline (pH 7.6), containing 0.05% Tween 20 and 5% nonfat milk. The nitrocellulose membranes were then incubated with specific antibodies against iNOS, COX-2 and β-actin purchased from Santa Cruz Biotechnology (Santa Cruz, USA) and Sigma-Aldrich Chemical Co. (St. Louis, USA), respectively. Immunoreactive bands were then detected by incubating the membranes with anti-mouse IgG conjugated with horseradish peroxidase, followed by an enhanced chemiluminescence detection system, Amersham Biosciences (Piscataway, USA). β-actin was used as internal control. Quantification by densitometry was carried out using Image J. The absolute intensity of each sample band was divided by the absolute intensity of the standard (β-actin) to determine a relative intensity for each sample band.

Animals and the experimental design of the sepsis model

Specific pathogen-free (SPF) 5-week-old female C57BL/6 mice were purchased from Koatech (Pyeongtaek, Korea). The animals were maintained in an isolated SPF barrier room with regulated temperature (23 ± 1°C), humidity (50 ± 5%), and light/dark cycle (12/12 h). All the animals were allowed to acclimatize for 1 week before the experiments. All the studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. The C57BL/6 mice were divided into three groups consisting of five animals. NLE and saline were administered orally at a dose of 20 mg/kg or 100 mg/kg, 24 h before LPS administration. LPS was administered intraperitoneally at a dose of 20 mg/kg, and the survival of mice was monitored every 12 h for the 3 days following this injection. The survival curve was analyzed by using GraphPad Prism software to compare the survival rates of the different treatments.

Measurement of plasma NO and TNF-α

Mice were orally administered NLE (20 or 100 mg/kg) 24 h

before a challenge with LPS (20 mg/kg). For the measurement of plasma NO, whole blood samples were drawn 1 h and 8 h after treatment with LPS (Kim *et al.*, 2007), and plasma was isolated by centrifuging the blood sample at 3,000 rpm for 20 min, and was then stored at −70°C until analysis. Total NO (nitrite and nitrate) was determined using the Total NO/Nitrite/Nitrate Assay kit (R&D Systems). Briefly, on the day of analysis, the samples were filtered with a 10-kDa cut-off centrifugal filter device (Amicon). Samples were then added to a 96-well plate and nitrate reductase and nicotinamide adenine dinucleotide phosphate (NADP) were added to the samples and incubated at 37°C for 30 min to determine nitrate concentrations. Following incubation, the Greiss reagents (1 and 2) were added to the wells and incubated for 10 min at room temperature. Total NO was determined spectrophotometerically at 540 nm. Using a linear curve fit for the standard curve, plasma NO concentration (mM) was determined by relative comparison to the standard nitrite and nitrate solutions. For the measurement of plasma TNF-α, whole blood samples were drawn from mice 1 h and 8 h after LPS treatment (Sireci *et al.*, 2008), and plasma samples were prepared as described above. Plasma TNF-α concentration was measured using the same ELISA kit as was described for the *in vitro* experiment.

HPLC analysis

The chromatographic measurements were performed using a Agilent 1200 HPLC System (USA) equipped with Variable Wavelength UV Detector (Santa Clara, USA) (wavelength=254 nm). The dried extracts were dissolved in methanol to a concentration of 20 mg/ml and filtrated using Millex PTFE Syring filter (0.45 μm) (Millipore, USA) before injected into HPLC system. The sample was then analyzed using the column filled with a ZORBAX Eclipse Plus C18 (4.6×250 mm, 5 μm, Agilent) (Santa Clara, USA) and eluted using water/acetonitrile (10:90, v/v) at a rate of 1.0 ml/min. The column effluent was detected at 254 nm, with a run time of 30 min. The chromatographic peaks of the sample were confirmed by comparing their retention time and UV spectra to those of the reference standards. Rutin, Wako (Kyushu, Japan) and quercetin, Sigma-Aldrich Inc (St. Louis, USA) were used as the reference standards.

Statistical analysis

All the experiments were repeated a minimum of three times. Treatment groups were analyzed using Student's *t*-test and data with $p<0.05$ were considered statistically significant.

RESULTS

NLE treatment inhibits LPS-induced NO and TNF-α production

In order to determine the anti-inflammatory effect of NLE *in vitro*, cells were treated with NLE after LPS treatment. Firstly, the cytotoxic effect of NLE was examined. NLE doses of 25, 100, and 200 μg/ml were tested. No cytotoxic side-effects were observed for doses up to 100 μg/ml NLE (Fig. 1C). Thus, the 100 μg/ml dose of NLE was used as the maximum concentration for the subsequent *in vitro* assays.

LPS-induced NO and TNF-α production were significantly reduced by NLE treatment. As shown in Fig. 1A, NO was induced by 100 ng/ml LPS treatment (58.44 ± 2.8 μM) compared

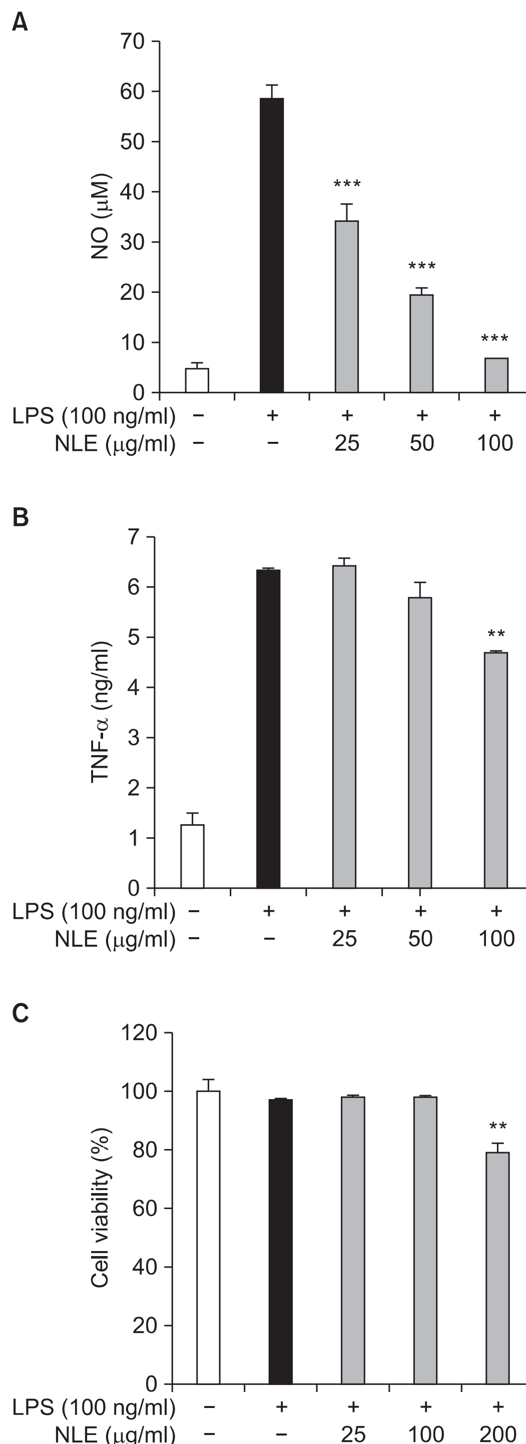
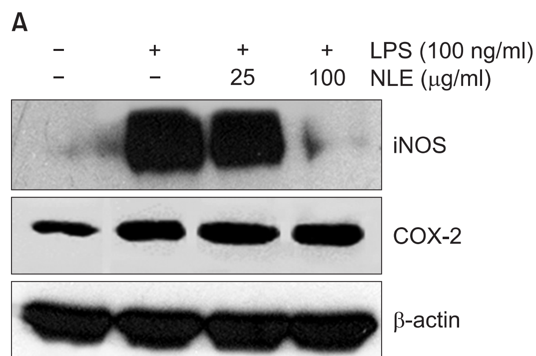
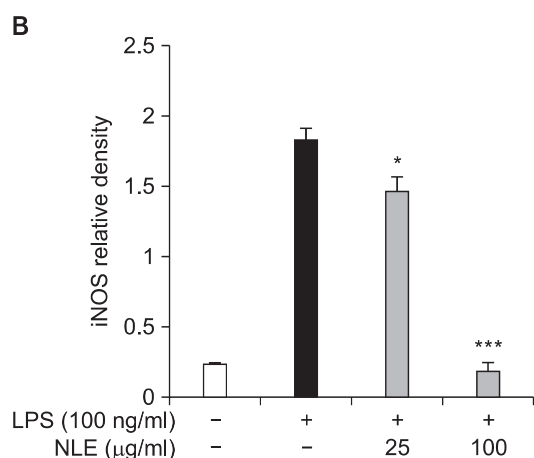


Fig. 1. NLE treatment reduces LPS-induced NO and TNF-α production in RAW264.7 cells. NO (A) and TNF-α (B) production were measured after treating the cells with LPS and various concentrations of NLE as indicated. Both NO and TNF-α production were significantly reduced by NLE treatment without any cytotoxic effects being observed in the cells (C) (**$p<0.01$; ***$p<0.001$).

to control levels (4.76 ± 1.2 μM). When cells were treated with NLE, LPS-induced NO production was significantly reduced in a dose dependent manner. NLE treatment at 25 and 50 μg/ml suppressed LPS-induced NO production by 45% and 72%, respectively. Strikingly, 100 μg/ml NLE treatment conferred a 96% suppression of LPS-induced NO production. TNF-α was also induced by 100 ng/ml LPS treatment (6.35 ± 0.1 ng/ml) compared to control levels (1.24 ± 0.5 ng/ml). The three doses of NLE were again evaluated. LPS-induced TNF-α production was not inhibited by 25 and 50 μg/ml NLE treatment (6.43 ± 0.3 ng/ml and 5.80 ± 0.5 ng/ml, respectively) but they were significantly suppressed by 100 μg/ml NLE treatment (4.69 ± 0.1 ng/ml) ($p<0.05$) (Fig. 1B). Taken together, our results suggest that NLE may function as an anti-inflammatory mediator *in vitro*.





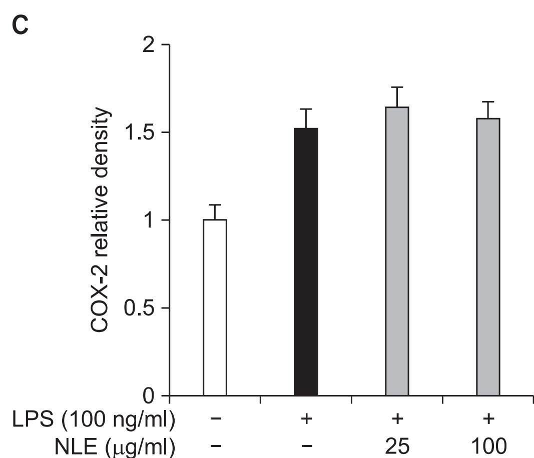


Fig. 2. Reduced NO production may be mediated by the NLE-induced inhibition of iNOS expression in LPS-stimulated RAW264.7 cells. iNOS expression levels were determined by western blot analysis using RAW264.7 cells that were treated with the indicated concentrations of NLE and LPS. Western blot (A) and corresponding densitometry analysis (B) strongly suggest that iNOS expression was inhibited by NLE *in vitro* (*$p<0.05$; ***$p<0.001$). However, COX-2 expression was not affected by NLE treatment (A, C).

NLE downregulates LPS-induced iNOS

To elucidate the mechanism of the reduction in NO induction by NLE in LPS treated macrophages, the effect of NLE on inducible nitric oxide synthase (iNOS) expression was determined by western blot analysis. As shown in Fig. 2A and B, iNOS expression was very low under control conditions (0.23 ± 0.02) but it was markedly increased by LPS treatment (1.83 ± 0.09). LPS-induced iNOS expression was significantly decreased by 25 μg/ml NLE treatment (1.47 ± 0.1). Interestingly, 100 μg/ml NLE treatment completely suppressed LPS-induced iNOS expression (0.19 ± 0.06). This suggests that NLE inhibits NO release in LPS-treated cells by decreasing the expression of iNOS.

In order to examine whether NLE affect the COX-2 expression, western blot analysis was carried out. As shown in Fig. 2A and C, COX-2 expression was significantly induced by LPS treatment (1.54 ± 0.09). However, no significant change of LPS-induced COX-2 expression was observed upon NLE treatment at both concentrations used (1.65 ± 0.11 by 25 μg/ml NLE and 1.56 ± 0.10 by 100 μg/ml NLE). This suggests that NLE may not be involved in the regulation of inflammatory response in COX II-PG pathway.

NLE improves the survival of C57BL/6 mice with LPS-induced sepsis

The *in vivo* anti-inflammatory effect of NLE was investi-

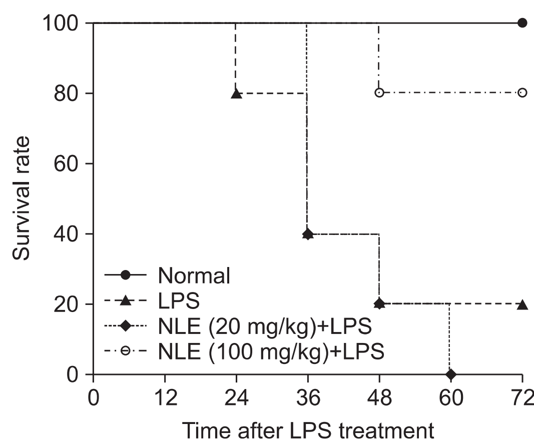


Fig. 3. NLE treatment improves the survival rate of mice treated with LPS. The survival rates of each experimental group, consisting of five animals, were measured. The control group survived throughout the experiment. The LPS-treated group showed a 20% survival rate on the last day of experiment. NLE treatment at a dose of 20 mg/kg displayed no effect on survival rate whereas NLE treatment at a dose of 100 mg/kg increased the survival rate up to 80%. $p<0.05$.

gated by observing its effect on mortality in a mouse sepsis model. C57BL/6 mice were injected with either LPS alone or LPS with NLE (20 or 100 mg/kg) and the survival rate of mice was examined for three days. As shown in Fig. 3, following the administration of LPS alone (20 mg/kg i.p.), only 20% of the mice survived for two days. However, the survival rate of mice increased to 80% when NLE (100 mg/kg) was co-administered with LPS. The lower dose of NLE (20 mg/kg) did not exert any effect on survival rate compared to LPS control group.

Next, the plasma concentration of NO and TNF-α was determined. NO has a very short half-life of a few seconds and hence the stable products of NO, nitrite and nitrate, were measured in the blood, the only source of which is from NO metabolism. Although previous studies suggest to examine the plasma concentration of NO and TNF-α at the specific time point (Kim *et al.*, 2007; Sireci *et al.*, 2008), the concentrations, in this study, were analyzed at two different time points of 1 h and 8 h after LPS challenge to clarify the critical time point of

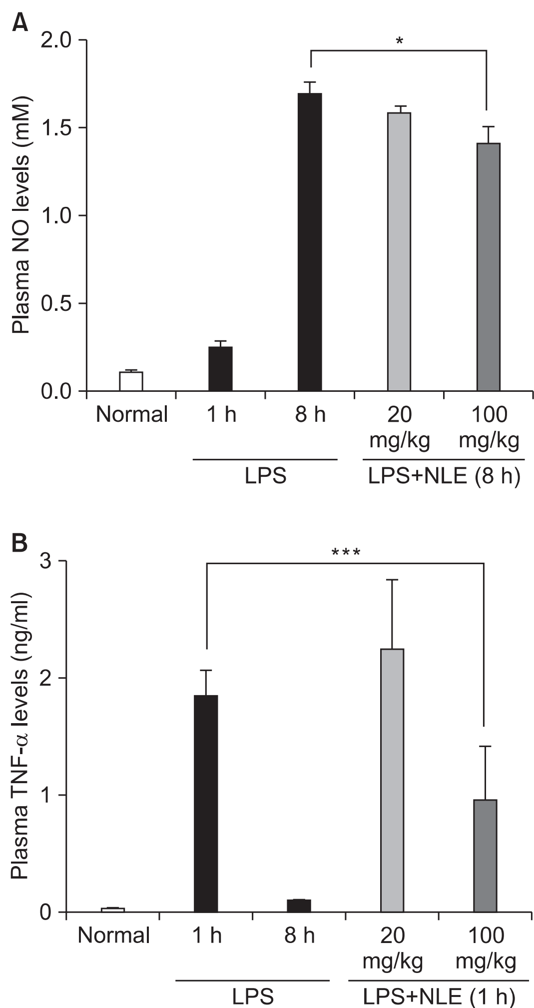


Fig. 4. NLE treatment reduced the plasma levels of TNF-α and NO in LPS-induced septic shock mice. Plasma levels of NO (A) and TNF-α (B) were measured after treating with LPS and NLE. (A) The measurement was carried out 1 h and 8 h after LPS challenge to test the critical time point for NO measurement. NLE was treated at a dose of 20 mg/kg and 100 mg/kg. (B) Plasma levels of TNF-α was measured 1 h and 8 h post-challenge of LPS (20 mg/kg), respectively. NLE was treated with LPS at a dose of 20 mg/kg and 100 mg/kg. Both NO and TNF-α levels in plasma were significantly reduced by NLE treatment at dose of 100 mg/kg (*p<0.05; ***p<0.001). The results are expressed as the mean ± SEM calculated from the values of four to six animals.

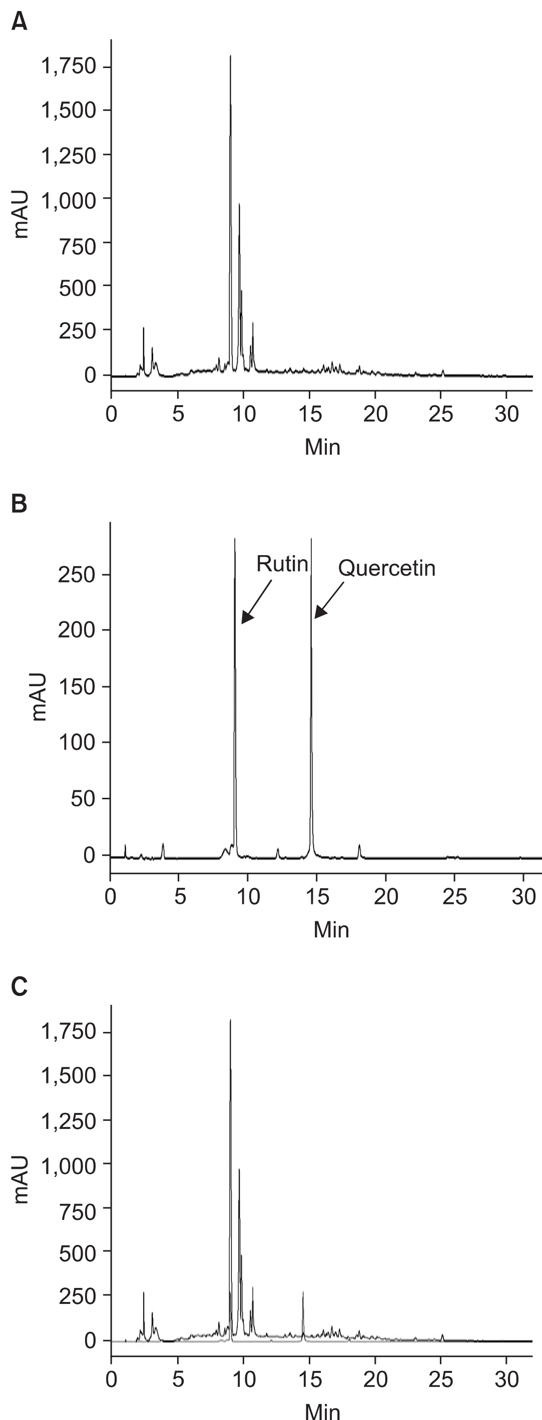


Fig. 5. HPLC chromatogram of NLE. NLE (A), reference standard (B) and the mixture of sample and standard (C) were analyzed by HPLC. The peaks in black indicate the NLE spectrum (C).

measurement. As shown in Fig. 4, NO and TNF-α concentration were dramatically increased by LPS positive control at 8 h and 1 h, respectively. Thus, each time point of 8 h and 1 h was considered to be critical time point to measure the concentration of NO and TNF-α in plasma.

Consistent with survival rate, the NO and TNF-α concentration in plasma were not affected in mice treated with 20 mg/kg NLE but were significantly reduced in mice treated with 100 mg/kg NLE compared to those treated with LPS alone (Fig. 4). As shown in Fig. 4A, the plasma NO level under control conditions was 0.1 ± 0.04 mM (n=4) but it was increased by LPS treatment (1.68 ± 0.18 mM, n=6). NLE treatment at the dose of 100 mg/kg significantly inhibited plasma NO production induced by LPS (1.40 ± 0.26 mM, n=6). However, 20 mg/kg NLE treatment did not inhibit NO production induced by LPS (1.57 ± 0.12 mM, n=6). Similarly, LPS induced the production of plasma TNF-α (1.83 ± 0.52 ng/ml, n=5) compared to that of control mice (0.04 ± 0.03 ng/ml, n=5). However, the increased plasma TNF-α level by LPS was prevented by NLE treatment at the dose of 100 mg/kg (0.94 ± 0.21 ng/ml, n=5) but was not by NLE treatment at the dose of 20 mg/kg (2.23 ± 0.60 ng/ml, n=5). Therefore, these results show that NLE (100 mg/kg) may function as an anti-inflammatory effector *in vivo*, which protects organisms against sepsis.

HPLC analysis shows significant presence of rutin in NLE

HPLC analysis was carried out to determine the composition of NLE. The chromatographs were compared between reference standards and samples (Fig. 5). The HPLC profile of NLE showed minimum two distinct peaks including the major peak at the retention time (min) of 8 (Fig. 5A). Comparison to the standard chromatograph indicated that the major peak in chromatograph of NLE is rutin, one of the flavonoids (Fig. 5B, C).

DISCUSSION

For the first time, we describe the function of *Azadirachta indica* A. Juss (neem) as an anti-inflammatory effector in an LPS-induced sepsis model. Neem is a member of the mahogany family, Meliaceae. Its main chemical constituents are three or four related compounds belonging to a class of natural products called triterpenes, or more specifically, limonoids. Azadirachtin, salannin, meliantriol, and nimbin are the best known compounds derived from neem and for now at least, seem to be the most significant. The leaves of the neem tree are traditionally considered to have anti-inflammatory, antifungal, antibacterial, antiviral, antioxidant, hepatoprotective, and cardioprotective effects (Okpanyi and Ezeukwu, 1981; Rao *et al.*, 1998; Almas, 1999; Badam *et al.*, 1999; Yanpallewar *et al.*, 2003). Although NLE has been suggested to have anti-inflammatory properties, it has not been studied to scientifically determine the mechanism of action of its anti-inflammatory effect in a sepsis model. Thus, the *in vitro* and *in vivo* anti-inflammatory function of NLE was investigated in an LPS-induced sepsis model.

To examine the anti-inflammatory function of NLE *in vitro*, the RAW264.7 macrophage-like cell line was used and treated with LPS to create an endotoxin-induced sepsis model. Pro-inflammatory molecules such as NO and TNF-α were examined to evaluate the anti-inflammatory effect of NLE because upon bacterial LPS stimulation, these molecules are known to induce oxidative stress, vascular anomalies such as disseminated intravascular coagulation, vascular perfusion, hypotension, multiple organ failure, and, in severe cases, septic shock and death (MacMicking *et al.*, 1995; Mayeux, 1997; Víctor *et al.*, 1998; Yazar *et al.*, 2010). In order to explore the *in vivo* effect of NLE, an LPS-induced mouse model of sepsis was established, using specific pathogen-free C57BL/6 mice. The dose of LPS used and the time course for the survival curve were selected in order to most accurately mimic the clinical condition in humans, where approximately 20-35% of patients with severe sepsis and 40-60% of patients with septic shock die within 30 days. Plasma NO and TNF-α levels were also examined along with the survival curve analysis of mice with sepsis.

In vitro results showed that NLE treatment significantly inhibited LPS-induced NO production by up to 96% in a dose-dependent manner and LPS-induced TNF-α production by up to 32%. We hypothesize that this reduction is the result of the complete suppression of iNOS expression by NLE. These results convincingly demonstrate a role for NLE as an anti-inflammatory effector. NO is known to be strongly associated with hypotension and hyporesponsiveness to vasoconstrictor stimuli in endotoxin-induced sepsis (Thiemermann, 1997; Titheradge, 1999). Moreover, mice lacking the inducible nitric oxide synthase (iNOS) gene were reported to be resistant to hypotension and death caused by LPS (MacMicking *et al.*, 1995; Wei *et al.*, 1995). TNF-α plays a critical role in the early phase of sepsis (Van Amersfoort *et al.*, 2003; Annane *et al.*, 2005; Carlson *et al.*, 2005; van Leeuwen *et al.*, 2005). Upon stimulation with LPS, TNF-α is synthesized and promotes the major alterations observed during septic shock, including vasodilatation and impaired coagulation (Tracey and Cerami, 1994; Annane *et al.*, 2005). Therefore, these results suggest that the inhibitory effect of NLE on TNF-α, NO, and iNOS production in the sepsis model might be very significant in the identification of a natural therapeutic agent for sepsis.

More intriguingly, the NLE treatment (100 mg/kg) improved the survival of LPS-induced sepsis mice by 60%. It is likely that the improved survival may be ascribed to the reduction of plasma NO and TNF-α production by NLE. The increased plasma NO and TNF-α level following LPS, was suppressed by NLE treatment by 17% and 49%, respectively. Thus, the results demonstrate that endotoxin-mediated death may be reversed by decreasing NO and TNF-α expression in animal models. Taken together, the results of this study showed that NLE inhibits LPS-induced NO and TNF-α production both *in vitro* and *in vivo*, thereby improving the survival of mice in a sepsis model.

Finally, the composition of NLE was analyzed by HPLC. The HPLC profile of NLE exhibited significant presence of rutin. Rutin is one of the flavonoid compounds. Previously, flavonoids have been implicated in various biological functions such as antioxidant, free radical scavenging, and apoptosis-inducing activity (Mora *et al.*, 1990; Chen *et al.*, 1999). Rutin, in particular, has been shown to inhibit LPS-induced NO and TNF-α production *in vitro* and/or *in vivo* (Takahashi *et al.*, 2001; Shen *et al.*, 2002). Thus, it is likely that rutin in NLE may be mainly responsible for the reduction of NO and TNF-α production in a LPS-induced model of sepsis in this study.

Sepsis is one of the leading death causes, particularly in ill patient. Our results open up the possibility of developing NLE

as a therapeutic agent for inflammatory diseases, including sepsis. However, further studies are needed to reveal the active compound (s) and to develop new therapeutic drugs for sepsis.

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REFERENCES

Almas, K. (1999) The antimicrobial effects of extracts of Azadirachta indica (Neem) and Salvadora persica (Arak) chewing sticks. *Indian. J. Dent. Res.* **10**, 3-6.

Annane, D., Bellissant, E. and Cavaillon, J. M. (2005) Septic shock. *Lancet.* **365**, 63-78.

Ayala, A., Song, G. Y., Chung, C. S., Redmond, K. M. and Chaudry, I. H. (2000) Immune depression in polymicrobial sepsis: the role of necrotic (injured) tissue and endotoxin. *Crit. Care Med.* **28**, 2949-2955.

Badam, L., Joshi, S. P. and Bedekar, S. S. (1999) 'In vitro' antiviral activity of neem (Azadirachta indica. A. Juss) leaf extract against group B coxsackieviruses. *J. Commun. Dis.* **31**, 79-90.

Bernard, G. R., Vincent, J. L., Laterre, P. F., LaRosa, S. P., Dhainaut, J. F., Lopez-Rodriguez, A., Steingrub, J. S., Garber, G. E., Helterbrand, J. D., Ely, E. W. and Fisher, C. J. Jr; Recombinant human protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study group (2001). Efficacy and safety of recombinant human activated protein C for severe sepsis. *N. Engl. J. Med.* **344**, 699-709.

Carlson, D. L., Willis, M S., White, D. J., Horton, J. W. and Giroir, B. P. (2005) Tumor necrosis factor-alpha-induced caspase activation mediates endotoxin-related cardiac dysfunction. *Crit. Care Med.* **33**, 1021-1028

Chen, Y. C., Liang, Y. C., Lin-Shiau, S. Y., Ho, C. T. and Lin, J. K. (1999) Inhibition of TPA-induced protein kinase C and transcription activator protein-1 binding activities by theaflavin-3,3'-digallate from black tea in NIH3T3 cells. *J. Agric. Food Chem.* **47**, 1416-1421.

Hu, Y., Xie, G. H., Chen, Q. X. and Fang, X. M. (2011) Small molecules in treatment of sepsis. *Curr. Drug Targets.* **12**, 256-262.

Ii, M., Matsunaga, N., Hazeki, K., Nakamura, K., Takashima, K., Seya, T., Hazeki, O., Kitazaki, T. and Iizawa, Y. (2006) A novel cyclohexene derivative, ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl] cyclohex-1-ene-1-carboxylate (TAK-242), selectively inhibits toll-like receptor 4-mediated cytokine production through suppression of intracellular signaling. *Mol. Pharmacol.* **69**, 88-95.

Kim, B. H., Roh, E., Lee, H. Y., Lee, I. J., Ahn, B., Jung, S. H., Leem H., Hanm S. B. and Kimm Y. (2008) Benzoxathiole derivative blocks lipopolysaccharide-induced nuclear factor-kappaB activation and nuclear factor-kappaB-regulated gene transcription through inactivating inhibitory kappaB kinase beta. *Mol. Pharmacol.* **73**, 1309-1318.

Kim, H. J., Tsoyi, K., Heo, J. M., Kang, Y. J., Park, M. K., Lee, Y. S., Lee, J. H., Seo, H. G., Yun-Choi, H. S. and Chang, K. C. (2007) Regulation of lipopolysaccharide-induced inducible nitric-oxide synthase expression through the nuclear factor-kappaB pathway and interferon-beta/tyrosine kinase 2/Janus tyrosine kinase 2-signal transducer and activator of transcription-1 signaling cascades by 2-naphthylethyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (THI 53), a new synthetic isoquinoline alkaloid. *J. Pharmacol. Exp. Ther.* **320**, 782-789.

MacMicking, J. D., Nathan, C., Hom, G., Chartrain, N., Fletcher, D. S., Trumbauer, M., Stevens, K., Xie, Q. W., Sokol, K., Hutchinson, N., et al. (1995) Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell.* **81**, 641-650.

Mayeux, P. R. (1997) Pathobiology of lipopolysaccharide. *J. Toxicol. Environ. Health.* **51**, 415-435.

Mora, A., Payá, M., Ríos, J. L. and Alcaraz, M. J. (1990) Structure-activity relationships of polymethoxyflavones and other flavonoids as inhibitors of non-enzymic lipid peroxidation. *Biochem. Pharmacol.* **40**, 793-797.

Mora, P., Masip, I., Cortés, N., Marquina, R., Merino, R., Merino, J., Carbonell, T., Mingarro, I., Messeguer, A. and Pérez-Payá, E. (2005) Identification from a positional scanning peptoid library of in vivo active compounds that neutralize bacterial endotoxins. *J. Med. Chem.* **48**, 1265-1268.

Nguyen, T. B., Adisechan, A. K., Suresh Kumar, E. V., Balakrishna, R., Kimbrell, M. R., Miller, K. A., Datta, A. and David, S. A. (2007) Protection from endotoxic shock by EVK-203, a novel alkylpolyamine sequestrant of lipopolysaccharide. *Bioorg. Med. Chem.* **15**, 5694-5709.

Okpanyi, S. N. and Ezeukwu, G. C. (1981) Anti-inflammatory and antipyretic activities of Azadirachta indica. *Planta. Med.* **41**, 34-39.

Rao, A. D., Devi, K. N. and Thyagaraju, K. (1998) Isolation of antioxidant principle from Azadirachta seed kernels: determination of its role on plant lipoxygenases. *J. Enzyme. Inhib.* **14**, 85-96.

Rasko, D. A., Moreira, C. G., Li de, R., Reading, N. C., Ritchie, J. M., Waldor, M. K., Williams, N., Taussig, R., Wei, S., Roth, M., Hughes, D. T., Huntley, J. F., Fina, M. W., Falck, J. R. and Sperandio, V. (2008) Targeting QseC signaling and virulence for antibiotic development. *Science.* **321**, 1078-1080.

Shen, S. C., Lee, W. R., Lin, H. Y., Huang, H. C., Ko, C. H., Yang, L. L. and Chen, Y. C. (2002) In vitro and in vivo inhibitory activities of rutin, wogonin, and quercetin on lipopolysaccharide-induced nitric oxide and prostaglandin E(2) production. *Eur. J. Pharmacol.* **446**, 187-194.

Sil, D., Shrestha, A., Kimbrell, M. R., Nguyen, T. B., Adisechan, A. K., Balakrishna, R., Abbo, B. G., Malladi, S., Miller, K. A., Short, S., Cromer, J. R., Arora, S., Datta, A. and David, S. A. (2007) Bound to shock: protection from lethal endotoxemic shock by a novel, non-toxic, alkylpolyamine lipopolysaccharide sequestrant. *Antimicrob. Agents. Chemother.* **51**, 2811-2819.

Sireci, G., La Manna, M. P., Di Liberto, D., Lo Dico, M., Taniguchi, M., Dieli, F. and Salerno, A. (2008) Prophylaxis of lipopolysaccharide-induced shock by alpha-galactosylceramide. *J. Leukoc. Biol.* **84**, 550-560.

Takahashi, K., Morikawa, A., Kato, Y., Sugiyama, T., Koide, N., Mu, M. M., Yoshida, T. and Yokochi, T. (2001) Flavonoids protect mice from two types of lethal shock induced by endotoxin. *FEMS. Immunol. Med. Microbiol.* **31**, 29-33, 2001.

Thiemermann, C. (1997) Nitric oxide and septic shock. Gen. *Pharmacol.* **29**, 159-166.

Titheradge, M. A. (1999) Nitric oxide in septic shock. *Biochim. Biophys. Acta.* **1411**, 437-455.

Toussaint, S., and Gerlach, H. (2009) Activated protein C for sepsis. *N. Engl. J. Med.* **361**, 2646-2652.

Tracey, K. J. and Cerami, A. (1994) Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu. Rev. Med.* **45**, 491-503.

Van Amersfoort, ES., Van Berkel, T. J. and Kuiper, J. (2003) Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. *Clin. Microbiol. Rev.* **16**, 379-414.

van Leeuwen, H. J., Van Der Tol, M., Van Strijp, J. A., Verhoef, J. and van Kessel, K. P. (2005) The role of tumour necrosis factor in the kinetics of lipopolysaccharide-mediated neutrophil priming in whole blood. *Clin. Exp. Immunol.* **140**, 65-72.

Víctor, V. M., Miñanom M., Guayerbasm N., Del Ríom M., Medinam S. and De la Fuente. M. (1998) Effects of endotoxic shock in several functions of murine peritoneal macrophages. *Mol. Cell Biochem.* **189**, 25-31.

Wei, X. Q., Charles, I. G., Smith, A., Ure, J., Feng, G. J., Huang, F. P., Xu, D., Muller, W., Moncada, S. and Liew, F. Y. (1995) Altered immune responses in mice lacking inducible nitric oxide synthase. *Nature.* **375**, 408-411.

Yanpallewar, S. U., Sen, S., Tapas, S., Kumar, M., Raju, S. S. and

Acharya, S. B. (2003) Effect of Azadirachta indica on paracetamol-induced hepatic damage in albino rats. *Phytomedicine.* **10**, 391-396.

Yazar, E., Bulbul, A., Avci, G. E., Er, A., Uney, K., Elmas, M. and Tras, B. (2010) Effects of enrofloxacin, flunixin meglumine and dexamethasone on disseminated intravascular coagulation, cytokine levels and adenosine deaminase activity in endotoxaemia in rats. *Acta. Vet. Hung.* **58**, 357-367.