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; Li 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...
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 (Oporny and Ezekwu, 1981; Rao et al., 1998; Almas, 1999; Badam et al., 1999; Yanpallawar 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% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, in an incubator at 37 °C with a humidified atmosphere of 95% air and 5% CO2. 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 filtered, 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 the cells were incubated for 24 h. The mixture was added to each well, and the absorbance was read at a wavelength of 540 nm using a Tecxan 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 INIRON 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 acclimate 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...