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Research Paper

Anti-inflammatory effect of a *Nuphar lutea* partially purified leaf extract in murine models of septic shock

J. Ozer [a,*], T. Levi [a], A. Golan-Goldhirsh [c,1], J. Gopas [a,b,1]

[a] Department of Microbiology, Immunology and Genetics, Faculty of Health Sciences Ben-Gurion University of the Negev, Beer Sheva, Israel
[b] Department of Oncology, Soroka University Medical Center, Beer Sheva, Israel
[c] The Jacob Blaustein Institutes for Desert Research (BIDR), French Associates Institute for Agriculture and Biotechnology of Drylands, Sede Boqer Campus, 84990, Israel

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ABSTRACT

Ethnopharmacological relevance: Various plant organs of *Nuphar lutea* (L.) SM. (Nymphaeaceae) are used in traditional medicine for the treatment of arthritis, fever, aches, pains and inflammation. The main purpose of this study was to determine the anti-inflammatory effect of *Nuphar lutea* leaf extract (NUP) in two septic shock models: (1) Survival of mice challenged with a lethal dose of LPS, determination of pro-inflammatory and anti-inflammatory cytokines in serum, as well as in peritoneal macrophages in cell culture. (2) The effect of NUP in a murine model of fecal-induced peritonitis.

Materials and methods: NUP pre-treatment partially protected mice in two models of acute septic shock. We concluded that NUP is anti-inflammatory by inhibiting the NF-κB pathway, modulating cytokine production and ERK phosphorylation.

Results: A significant average survival rate (60%) of LPS lethally-challenged mice was achieved by pre-treatment with NUP. In addition, NUP pre-treatment reduced nuclear NF-κB translocation in peritoneal macrophages. The production of pro-inflammatory cytokines, TNF-α, IL-6 and IL-12, in the sera of LPS-treated mice or in the supernatants of peritoneal macrophages stimulated with LPS for 2–6 h was also decreased by NUP. Pre-treatment with NUP caused a significant increase in the anti-inflammatory cytokine IL-10. The NUP pre-treatment reduced and delayed mortality in mice with fecal-induced peritonitis. Our studies also revealed that NUP pre-treatment induced a dose-dependent phosphorylation of ERK in peritoneal macrophages. Since most of the reports about the anti-inflammatory effect of *Nuphar lutea* refer to rhizome and root powder and extracts, it is important to clarify the effectiveness of leaf extract as a source for such activity.

Conclusion: NUP pre-treatment partially protected mice in two models of acute septic shock. We concluded that NUP is anti-inflammatory by inhibiting the NF-κB pathway, modulating cytokine production and ERK phosphorylation.

1. Introduction

Natural products of *Nuphar lutea* (L.) SM. (Nymphaeaceae) have been widely used for treating inflammatory conditions in ethnic medicine. The use of *Nuphar lutea* leaf extracts for treating rheumatism is described in the traditional medicine of Lebanon (El Beyrouthy et al., 2008). In Japan, Jidabokuippo traditional medicine contains *Nuphar* rhizome powder, among other ingredients, which is used to treat contusion-induced swelling and pain (Nakae et al., 2012). In northwestern British Columbia, the local Gitksan ethnic group has used *Nuphar polysepalum* to treat tuberculosis, fractures, arthritis and other ailments (Johnson, 2006). The use of *Nuphar lutea* extracts for medicinal purposes by aboriginals of the Canadian boreal forest was reported by Uprety et al. (2012).

Scientific investigations on the medicinal properties of *Nuphar* extracts have indicated several potential applications, such as anti-leishmanial (El-On et al., 2009; Ozer et al., 2010), anti-bacterial (Turker et al., 2009) and anticancer (Matsuda et al., 2006; Yildirim et al., 2013).

In our laboratories, we have screened, over the years, a large number of plants used in ethnic pharmacopeia for biological activities in order to identify the active compounds (Sathiyamoorthy et al., 1997, 1999). As a result of our screening of Mediterranean plants, we

Abbreviations: NUP, *Nuphar lutea* leaf extract; LPS, Lipopolysaccharide; TNF-α, Tumor necrosis factor alpha; IL-6, Interleukin 6; IL-12, Interleukin 12; IL-10, Interleukin 10; MAPK, Mitogen-activated protein kinase; ERK, Extracellular signal-regulated kinase; NF-κB, Nuclear factor kappa B

* Corresponding author. Tel.: +972 8 6477253; fax: +972 8 6477626.
E-mail address: ozerj@post.bgu.ac.il (J. Ozer).

[1] AGG and JG contributed equally to this work.

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have identified Nuclear Factor κB (NF-κB) inhibitory activity in extracts of various plant parts of *Nuphar lutea*, in which the major components were sesquiterpene thioalkaloids (Ozer et al., 2009). It is important to investigate thoroughly the potential of leaf extracts for medicinal purposes, because leaf harvesting allows the maintenance of whole plant integrity and continuous production.

A plethora of natural products, including those derived from plants, have been reported to inhibit NF-κB and inflammation (Golan-Goldhirsh and Gopas, 2014).

NF-κB constitutes a family of transcription factors that are involved in many biological and pathological processes, especially in the pathways of the immune and inflammatory responses. A functional NF-κB molecule is a heterodimer composed of members of the Rel family of proteins, which includes p65 and p50. Translocation of NF-κB to the nucleus stimulates the transcription of a wide variety of genes (Hayden and Ghosh, 2004, 2012; Kumar et al., 2004). NF-κB induces interleukin-1 (IL-1) α and β, Tumor Necrosis Factor α (TNF-α) and other molecules. NF-κB is modulated by stimuli such as extracellular signal-regulated kinases (ERK) (Lee and Hung, 2008; Dhingra et al., 2009), pro-inflammatory cytokines and bacterial toxins (*e.g.*, lipopolysacharides (LPS)) (DiDonato et al., 2012).

LPS plays an important role in Gram-negative bacteria-induced sepsis and multiple organ dysfunction syndromes. It is one of the microbial products recognized early by monocytes and triggers cytokine release. Therefore, LPS is commonly used in inflammation models (Shao and Lin, 2008; Zhang et al., 2008).

Activated monocytes may release excessive amounts of pro-inflammatory cytokines (such as TNF-α, IL-6, IL-1α, etc.), which transform the beneficial role of inflammation into a harmful response that leads to host damage. Under normal conditions, the negative effects are balanced by the induction of anti-inflammatory cytokines, *e.g.*, IL-10 (Hotchkiss and Nicholson, 2006).

Therefore, it is very important to search for novel therapeutic approaches to modulate the balance between pro-inflammatory and anti-inflammatory responses and to restore immune homeostasis. In this paper, we investigated whether NUP pre-treatment can decrease the inflammatory response and play a protective role in toxic shock models.

2. Materials and methods

2.1. Preparation of Nuphar lutea extracts

Floating and submerged leaves of *Nuphar lutea* were collected from a cultivation pond at the Jacob Blaustein Institutes for Desert Research campus (BIDR), which was started with rhizomes harvested from the Yarkon River in Israel. The specimen voucher-Nuphar169, a sample is maintained at BIDR, the species was identified by Dr. Moshe Agami, director of the Botanical gardens of Tel Aviv University.

Preparation of the extract was performed as described by Ozer et al. (2009). Briefly, after harvesting, the leaves were washed in tap water and dried in an oven at 40 °C. The dry material was ground to a fine powder and used for extracting the active compounds.

Dried leaf powder was extracted in methanol at a ratio of 1:10 (w:v). The supernatant obtained after the extract was centrifuged at 4000 rpm and 4 °C for 30 min was rotary evaporated and re-dissolved in chloroform: 1 N HCl at a ratio of 1:1 (v:v). The mixture was separated in a separatory funnel into two phases. The lower chloroform phase was discarded, and the upper aqueous phase was saved and adjusted to pH 9 with 25% NH_4OH. The precipitate formed was separated by centrifugation at 4000 rpm and 4 °C for 30 min. The re-dissolved precipitate in methanol:water, 1:1 (v:v) was adjusted to pH 3 with HCl and was allowed to stand refrigerated overnight and then centrifuged as before; the supernatant that was then collected (named NUP) was used for the bioassays reported here.

The identities of the active components of the extract, as determined by NMR, were reported before (Ozer et al. 2009, Supplementary Figure S1 and Table S2, (http://www.landesbioscience.com/%20supplement/OzerCBT8–19-Sup.pdf)). *Nuphar* nupharidines were recently synthesized by Jansen and Shenvi (2013) and were shown by us to downregulate NF-κB activity (unpublished data).

2.2. Animals

Female BALB/c and C57BL/6 mice were obtained from Harlan Laboratories (Rehovot, Israel). All of the mice were maintained at the Animal Resource Center of Ben-Gurion University of the Negev and were used at 8–12 weeks of age. All animal experiments described in this work were approved by the Ben-Gurion University Committee for the Ethical Care and Use of Animals in Experiments (UCECUAE).

2.3. Induction of LPS-induced lethal toxic shock

Eight-week-old BALB/c female mice were used for this study. Ten mice per group were treated intra-peritoneally (i.p.) with 20 mg/kg of NUP or with a vehicle for two days prior to the i.p. injection of LPS, 50 mg/kg (Sigma-Aldrich, Rehovot, Israel). After the mice were injected with LPS, they were inspected every 24 h, to evaluate serious morbidity and mortality (Cao et al., 2011). UCECUAE approval number IL-67-11-2010.

2.4. Induction and determination of cytokine levels in serum

Seven BALB/c mice in each group were given NUP (20 mg/kg) or a vehicle i.p. two days prior to LPS treatment. Different concentrations of NUP were tested for toxicity. The experiments were performed with the highest effective non-lethal concentration in order to achieve optimal results. At the beginning of the experiment (day 0), all mice were injected with 50 mg/kg LPS.

Either four or six hours after LPS treatment, the mice were sacrificed by inhalation of CO_2, and blood was drained via the *vena cava*. The serum levels of IL-12, IL-6, TNF-α and IL-10 were measured by ELISA in triplicate using a commercial ELISA kit (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions.

2.5. Isolation of the peritoneal macrophages and cell culture

Selective isolation of peritoneal macrophages from BALB/c mice was achieved by pre-treatment of mice with an i.p. injection of thioglycolate (2 ml /mouse) (Hy-laboratories, Rehovot, Israel). After four days, the mice were sacrificed by inhalation of CO_2, and a peritoneal lavage was performed with 10 ml of sterile phosphate-buffered saline (PBS) using an 18-gauge needle; peritoneal lavage fluid (PLF) was collected in polypropylene tubes.

The cells were then cultured in six well plates in RPMI-1640 media supplemented with 10% FCS, 1% penicillin, and 1% streptomycin for 24 h at 37 °C in a humidified atmosphere of 5% CO_2/95% air incubator. Non-adherent cells were removed by replacing the medium after 24 h.

2.6. Induction and determination of cytokine levels in supernatants following LPS treatment

Peritoneal macrophages were treated *in vitro* with different combinations of LPS (3 μg/ml) either with or without NUP (0.75 μg/ml). After treatment, the macrophage supernatants were collected. The levels of IL-12, IL-6, TNF-α and IL-10 in the media were measured by ELISA in triplicate (BD Biosciences, Franklin Lakes, NJ, USA), according to the manufacturer's instructions.