In Vitro Antiinflammatory Activity of Kalopanaxsaponin A Isolated from *Kalopanax pictus* in Murine Macrophage RAW 264.7 Cells

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In the present study, effects of various hederagenin monodesmosides isolated from the stem bark of *Kalopanax pictus* NAKAI, such as hederagenin, δ -hederin, kalopanaxsaponin A, kalopanaxsaponin I, and sapindoside C, have been evaluated on lipopolysaccharide (LPS)-induced nitric oxide (NO), prostaglandin E₂ (PGE₂) and tumor necrosis factor- α (TNF- α) release by the macrophage cell line RAW 264.7. Among the tested monodesmosides, kalopanaxsaponin A was the most potent inhibitor of NO production, and it also significantly decreased PGE₂ and TNF- α release. Consistent with these observations, the expression level of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 enzyme was inhibited by kalopanaxsaponin A in a concentration-dependent manner. Thus, this study suggests that kalopanaxsaponin A-mediated inhibition of iNOS, COX-2 expression, and TNF- α release may be one of the mechanisms responsible for the anti-inflammatory effects of the stem bark of *Kalopanax pictus* NAKAI.

Key words hederagenin monodesmoside; macrophage; lipopolysaccharide; nitric oxide; prostaglandin E₂; COX-2; iNOS and TNF- α

The stem bark of *Kalopanax pictus* NAKAI (Araliaceae), a deciduous tree growing in East Asian countries,¹⁾ has been traditionally used for the treatment of rheumatoid arthritis, neurotic pain and diabetes mellitus. The constituents such as hederagenin glycosides, syringin, liriodendrin and coniferyl-aldehyde glucosides have been isolated from this plant.^{2,3)} Our previous reports on anti-diabetic,⁴⁾ cytotoxic⁵⁾ and anti-fungal assays⁶⁾ have shown that the active components should be hederagenin monodesmosides. In addition, we also demonstrated that hederagenin bisdesmoside could be converted to its monodesmosides and its serial saponins by human intestinal bacteria and that these metabolites showed anti-diabetic activity.⁷⁾ Although several pharmacological effects of hederagenin monodesmosides as well as its serial saponins are reported, there have been very few reports on the effects of hederagenin monodesmosides on the antiinflammatory role. During inflammatory processes, large amounts of pro-inflammatory mediators, nitric oxide (NO) and prostaglandin E₂ (PGE₂) are generated by the inducible isoforms of NO synthase (iNOS) and cyclooxygenase (COX-2), respectively.⁸⁾ In mammalian cells, three isoforms of NOS, types I, II, and III, have been identified on the basis of physical and biochemical characteristics of the purified enzymes. Type I (neuronal NOS, nNOS) and type III (endothelial NOS, eNOS) have been classified as constitutive NOS (cNOS) because these are continuously present in the cells, whereas type II, an iNOS, is expressed only after exposure to specific stimulants such as cytokines, bacterial endotoxic lipopolysaccharide (LPS), and calcium ionophore in some cells.^{9–11)} Cyclooxygenase (COX) is the enzyme which converts arachidonic acid to prostaglandins (PGs). Like NOS, COX has been found in two isoforms, and COX-2 is an inducible form responsible for the production of large amounts of proinflammatory PGs at the inflammatory site.¹²⁾

Our previous investigation concerning kalopanaxsaponin A demonstrated their anti-inflammatory syndromes in the rat induced by Freund's complete adjuvant reagent.¹³⁾ Thus, as a

prelude to reveal the underlying mechanisms for the anti-inflammatory effect of kalopanaxsaponin A, we evaluated and compared the effects of various hederagenin monodesmosides (Fig. 1) isolated from stem bark of *Kalopanax pictus* NAKAI on LPS-induced NO, PGE₂ and tumor necrosis factor- α (TNF- α) release by the macrophage cell line RAW 264.7 in the present study. Moreover, we also examined if these hederagenin monodesmosides reduced iNOS and COX-2 enzyme expression.

MATERIALS AND METHODS

Materials DMEM medium, fetal bovine serum (FBS),

Hederagenin monodesmoside	R
Hederagenin	H
δ -hederin	α -L-arabinopyranosyl
Kalopanaxsaponin A	α -L-rhamnopyranosyl(1-2)- α -L-arabinopyranosyl
Kalopanaxsaponin I	β -D-xylopyranosyl(1-3)- α -L-rhamnopyranosyl(1-2)- α -L-arabinopyranosyl
Sapindoside C	β -D-glucopyranosyl(1-4)- β -D-xylopyranosyl(1-3)- α -L-rhamnopyranosyl(1-2)- α -L-arabinopyranosyl

Fig. 1. Structure of Hederagenin and Its Glycosides Obtained from the Stem Bark of *K. pictus*

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penicillin, and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tertzolium bromide (MTT), sulfanilamide, aprotinin, leupeptin, phenylmethylsulfonyl fluoride, dithiothreitol, *N*-mono-methyl-arginine (NMA) and *E. coli* lipopolysaccharide (LPS) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). COX-2 and iNOS monoclonal antibodies and the peroxidase conjugated secondary antibody were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). EIA kit for PGE₂ and TNF- α were obtained from Amersham Pharmacia Biotech. (Piscataway, NJ, U.S.A.) and R&D systems (Minneapolis, MN, U.S.A.), respectively. Hederagenin monodesmosides used for this study were isolated from the stem bark of *Kalopanax pictus* as previously described.^{5,7)}

Cell Culture and Sample Treatment RAW 264.7 murine macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea). These cells were grown at 37°C in DMEM medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μ g/ml) in a humidified atmosphere of 5% CO₂. Cells were incubated with hederagenin monodesmosides at increasing concentrations (5–50 μ M) and stimulated with LPS 1 μ g/ml for 24 h.

MTT Assay for Cell Viability Cytotoxicity studies were performed in a 96-well plate. RAW 264.7 cells were mechanically scraped and plated 2×10^5 /well in 96-well plate containing 100 μ l of DMEM medium with 10% FBS and incubated overnight. Hederagenin monodesmosides were dissolved in dimethylsulfoxide (DMSO) and the DMSO concentrations in all assays did not exceed 0.1%. Twenty-four hours after seeding, 100 μ l new media or hederagenin monodesmosides was added, and the plates were incubated for 24 h. Cells were washed once before adding 50 μ l FBS-free medium containing 5 mg/ml MTT. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue, which formed in the cells, was dissolved in 50 μ l DMSO. The optical density was measured at 540 nm. The concentration required to reduce absorbance by 50% (IC₅₀) in comparison to control cells was determined.⁷⁾

Nitrite Assay Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by Griess reaction.¹⁴⁾ Briefly, 100 μ l of cell culture medium were mixed with 100 μ l of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphthylethylenediamine-HCl] and incubated at room temperature for 10 min, and then the absorbance at 550 nm was measured in a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was calculated from a sodium nitrite standard curve freshly prepared in culture medium.

PGE₂ and TNF- α Assay PGE₂ and TNF- α level in macrophage culture medium were quantified by EIA kits according to the manufacture's instructions.

Western Blot Assay Cellular proteins were extracted from control and kalopanaxsaponin A-treated RAW 264.7 cells. The washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES pH 7.0, 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM NaF, 0.5 mM Na orthovanadate) containing 5 μ g/ml each of leupeptin and aprotinin and

incubated with 15 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. Protein concentration was determined by Bio-Rad protein assay reagent according to the manufacture's instruction. 40–50 μ g of cellular proteins from treated and untreated cell extracts were electroblotted onto nitrocellulose membrane following separation on a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4°C, followed by incubation for 4 h with a 1 : 500 dilution of monoclonal anti-iNOS and COX-2 antibody (Santacruz, CA, U.S.A.). Blots were washed 2 times with PBS and incubated with a 1 : 1000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG secondary antibody (Santacruz, CA, U.S.A.) for 1 h at room temperature. Blots were again washed three times in tween 20/Tris-buffered saline (TTBS) and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).

Data Analysis Data are reported as mean $\pm$ S.E.M. values of three independent determinations. All experiments were done at least three times, each time with three or more independent observations. Statistical analysis was performed by analysis of variance (ANOVA) followed by Duncan's test.

RESULTS

Effect of Hederagenin Monodesmosides on Cell Viability Cytotoxic effect of all hederagenin monodesmosides (hederagenin, δ -hederin, kalopanaxsaponin A, kalopanaxsaponin I, and sapindoside C) was evaluated in the absence or presence of LPS. Hederagenin monodesmosides alone did not affect the cell viability at the concentrations used (10, 25, 50 μ M), whereas only kalopanaxsaponin A in the presence of LPS reduced cell viability 20% at the highest concentration (50 μ M).

Inhibition by Hederagenin Monodesmosides of LPS-Induced NO₂⁻ Production The LPS (1 μ g/ml) induced increased of NO₂⁻ production, however, was significantly suppressed by 1 mM positive inhibitor NMA (from 34.8 ± 1.7 μ M to 19.8 ± 0.6 μ M). All hederagenin monodesmosides used in this study showed an inhibitory effect on LPS-induced

Fig. 2. Evaluation of Nitrite Production by RAW 264.7 Cells Stimulated for 24h with LPS Alone or in Combination with Increasing Concentrations (5–10–25 μ M) of the Different Triterpenoids Used

●, δ -hederin; ■, hederagenin; ▲, kalopanaxsaponin I; —, kalopanaxsaponin C; ◆, kalopanaxsaponin A. The values are the means of at least 3 determinations $\pm$ S.E.M. ^a $p < 0.05$ vs. LPS-treated group; significance of difference between treated groups by ANOVA followed by Duncan's test.

Fig. 3. Time-Dependency of Inhibition of Nitrite Accumulation by Kalopanaxsaponin A

RAW 264.7 cells were stimulated with LPS. The values are means of 3 determinations $\pm$ S.E.M. ^a $p < 0.05$ vs. LPS-treated group; significance of difference between treated groups by ANOVA followed by Duncan's test. Control (■) and kalopanaxsaponin A 10 μ M (●).

Fig. 4. Effect of Kalopanaxsaponin A on PGE₂ Production by LPS-Induced RAW 264.7 Macrophage for 24 h

The values are means of 3 determinations $\pm$ S.E.M. ^a $p < 0.05$ vs. LPS-treated group, ^b $p < 0.05$ vs. 2.5 μ M kalopanaxsaponin A-treated group; ^c $p < 0.05$ vs. 5 μ M kalopanaxsaponin A-treated group; significance of difference between treated groups by ANOVA followed by Duncan's test.

Fig. 5. Effect of Kalopanaxsaponin A on LPS-Induced TNF- α Production by RAW 264.7 Cells

Cells were incubated with LPS for 24 h. The values, expressed as ng of TNF- α , are means of 3 determinations $\pm$ S.E.M. ^a $p < 0.05$ vs. LPS-treated group, ^b $p < 0.05$ vs. 2.5 μ M kalopanaxsaponin A-treated group; significance of difference between treated groups by ANOVA followed by Duncan's test.

NO production except δ -hederin (Fig. 2). Most active was kalopanaxsaponin A, and this effect was concentration-dependent. Although kalopanaxsaponins A reduced cell viability at 50 μ M, it still showed a statistically significant effect at lower and non-cytotoxic concentrations. In contrast, kalopanaxsaponin I, sapindoside C and hederagenin was only active at the highest concentration (Fig. 2). Additionally, all hederagenin monodesmosides tested in this study had no quenching effect on the Griess reagent at the concentration used. The inhibitory effect of kalopanaxsaponin A was also shown in a time-dependent manner (Fig. 3).

Fig. 6. Modulation by Kalopanaxsaponin A of LPS-Induced iNOS Expression in RAW 264.7 Cells

Lysates were prepared from control or 24 h-LPS (1 μ g/ml) stimulated cells alone or in combination with increasing concentrations (5-10-25 μ M) of kalopanaxsaponin A. All lanes contained 50 μ g of total proteins. A representative immunoblot from three separate experiments is shown.

Fig. 7. Modulation by Kalopanaxsaponin A of LPS-Induced COX-2 Expression in RAW 264.7 Cells

Lysates were prepared from control or 24 h-LPS (1 μ g/ml) stimulated cells alone or in combination with increasing concentrations (5-10-25 μ M) of kalopanaxsaponin A. All lanes contained 50 μ g of total proteins. A representative immunoblot from three separate experiments is shown.

Inhibition by Kalopanaxsaponin A of LPS-Induced PGE₂ and TNF- α Production LPS-induced production of PGE₂ and TNF- α was significantly inhibited only by kalopanaxsaponin A among the tested hederagenin monodesmosides (data not shown). This effect of kalopanaxsaponin A was also concentration-dependent (Figs. 4, 5), whereas hederagenin, kalopanaxsaponin I, and sapindoside C were nearly inactive.

Effect of Kalopanaxsaponin A on LPS-Induced iNOS and COX-2 Expression To determine if the inhibitory effect of kalopanaxsaponin A on these inflammatory mediators (NO and PGE₂) was related to a modulation of iNOS and COX-2 induction, we examined their expression levels by western blot analysis. In response to LPS, the expression level of iNOS was markedly induced, and kalopanaxsaponin A significantly inhibited the iNOS induction in a concentration-dependent manner (Fig. 6). A similar pattern was observed when the effect of kalopanaxsaponin A was examined on the LPS-induced COX-2 expression level; the densitomet-

ric analysis of three different experiments demonstrated that the COX-2 expression induced by LPS was inhibited $48.9 \pm 10\%$ in cells treated with $25 \mu\text{M}$ kalopanaxsaponin A (Fig. 7). However, at lower concentration, the effects were hardly detectable. In general, these results are consistent with the profile of the inhibitory effect of kalopanaxsaponin A on NO_2^- (Fig. 1) and PGE_2 release (Fig. 4).

DISCUSSION

Biological activities of active components of the stem bark of *K. pictus* have been presented haemolytic, antispasmodic, antifungal, and antiamebic activities.¹⁵⁾ Since 1996, we also have elucidated a potent hypoglycemic,⁴⁾ cytotoxic,⁵⁾ antimutagenic⁵⁾ and hepatotoxic¹⁶⁾ effects of hederagenin monodesmosides. Because the active component of this crude drug has never been reported on anti-inflammatory effect, we examined the effects of the various hederagenin monodesmosides on release of two inflammatory mediators (NO and PGE_2), $\text{TNF-}\alpha$, and the expression level of iNOS and COX-2 of the macrophages activated by LPS.

It is well known that macrophages play a crucial role in both non-specific and acquired immune responses, and macrophage activation by LPS leads to a functionally diverse series of responses including the production of pro-inflammatory cytokines (IL-1, $\text{TNF-}\alpha$ and IL-6), the activation of phospholipase A_2 which produces lipid metabolites of arachidonic acid such as PGs, and NO production.¹⁶⁾ In this study, we demonstrated exposure of RAW 264.7 macrophage to LPS for several hours was associated with an accumulation of nitrite in the medium, implying an enhanced NO production. This LPS-induced NO production was significantly inhibited by kalopanaxsaponin A in a time- and concentration-dependent manner without notable cytotoxicity. Results from western blotting analysis further indicated that LPS-induced iNOS expression in RAW 264.7 macrophages was significantly blocked by kalopanaxsaponin A. iNOS is responsible for long-lasting NO production, and it is strikingly induced by LPS as demonstrated in this study. Therefore, these results suggest that inhibition of LPS-induced NO production by kalopanaxsaponin A occurs mainly through the regulation of iNOS gene expression. However, we cannot rule out the possibility that kalopanaxsaponin A directly interferes with iNOS activity.

Mechanism of various anti-inflammatory drug action is at least shared by the inhibition of prostaglandin synthesis, which is mediated by COX.¹⁷⁾ COX exists in two isoforms, COX-1 and COX-2, each with distinct expression pattern in various cell types.¹⁸⁾ COX-1 has been suggested to provide a physiologic level of prostaglandins for normal platelet, stomach and kidney function. In contrast, COX-2 has been found to be highly induced at inflammatory sites in animals as well as patients with inflammatory diseases,^{19,20)} and thus it is considered to be responsible for pro-inflammatory prostaglandin formation. In addition to inhibition of NO release and iNOS induction, kalopanaxsaponin A also significantly inhibited PGE_2 production and COX-2 gene expression in LPS-treated RAW 264.7 macrophages. In addition, NF- κB response elements have been demonstrated to be on the promoter region of iNOS and COX-2, which are involved in inflammatory process by providing NO and

prostaglandin, respectively. Further investigation is needed to confirm whether kalopanaxsaponin A suppresses NF- κB activities and which components of NF- κB are suppressed.

Pro-inflammatory cytokines such as $\text{TNF-}\alpha$, IL-1 and IL-6 have been shown to control inflammation *in vitro* as well as *in vivo*,^{21,22)} and these cytokines are thought to be interlinked in a cascade, being produced serially by macrophages during an inflammatory response. Furthermore, the development of hyperalgesic states during inflammation is thought to be mediated by pro-inflammatory cytokines.²³⁾ Therefore, we investigated whether hederagenin monodesmoside would possibly influence the formation of $\text{TNF-}\alpha$ in an *in vitro* model. Among the tested compounds, only kalopanaxsaponin A affected the production of $\text{TNF-}\alpha$.

A saponin shows a broad spectrum of biological and pharmacological activities, and many of them have revealed an anti-inflammatory activity against several models of inflammation, *in vitro* and *in vivo*.²⁴⁾ Oh *et al.*,²⁵⁾ reported that the presence of free carboxylic acid of hederagenin monodesmoside is responsible for the anticomplement activity. In our study, the most active hederagenin monodesmoside among those examined was kalopanaxsaponin A although hederagenin, kalopanaxsaponin I and sapindoside C weakly reduced the production of NO induced by LPS. An active compound kalopanaxsaponin A has a disaccharide moiety of 3-*O*-rhamnosyl(1 $\rightarrow$ 2) arabinose (Fig. 1), and it seems that the arabinosyl moiety at C-3 blocks the activity of the hederagenin and that the position of the second sugar for glycoside linkage is important for anti-inflammatory activity. This suggestion indicated that attachment of these sugars at C-3 of the genins as well as the presence of carboxylic acid is essential.

In summary, the results of present study suggest that among hederagenin monodesmosides, kalopanaxsaponin A treatment inhibited LPS-induced iNOS and COX-2 protein expression and NO, PGE_2 and $\text{TNF-}\alpha$ production. Therefore, this compound may be useful in the prevention and treatment of disease accompanying an increased expression of iNOS and COX-2 such as in endotoxemia.

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