Arch Pharm Res Vol 32, No 11, 1509-1514, 2009
DOI 10.1007/s12272-009-2102-4

Archives of Pharmacal Research
www.springer.com/12272

(-)-Nyasol (cis-hinokiresinol), a Norneolignan from the Rhizomes of *Anemarrhena asphodeloides*, is a Broad Spectrum Inhibitor of Eicosanoid and Nitric Oxide Production

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(Received February 21, 2009/Revised July 22, 2009/Accepted July 25, 2009)

To assess the anti-inflammatory activity of constituents from the rhizomes of *Anemarrhena asphodeloides*, (-)-nyasol {*cis*-hinokiresinol, 4,4-[1Z,3R]-3-ethenyl-1-propene-1,3-diyl]bisphenol} was isolated and its anti-inflammatory activity was examined in lipopolysaccharide (LPS)-treated RAW 264.7 cells and A23187-treated RBL-1 cells. *In vivo* activity was measured using carrageenan-induced paw edema assay. At > 1 μM, (-)-nyasol significantly inhibited cyclooxygenase-2 (COX-2)-mediated PGE_2 production and inducible nitric oxide synthase (iNOS)-mediated NO production in LPS-treated RAW 264.7 cells, a mouse macrophage-like cell line, but did not affect the expression levels of COX-2 and iNOS. (-)-Nyasol also inhibited 5-lipoxygenase (5-LOX)-mediated leukotriene production in A23187-treated RBL-1 cells. Furthermore, (-)-nyasol potently inhibited carrageenan-induced paw edema in mice (28.6-77.1% inhibition at 24-120 mg/kg). Therefore, (-)-nyasol is a potential new lead compound and may contribute to the anti-inflammatory action of *A. asphodeloides*, possibly by inhibiting COX-2, iNOS and 5-LOX.

Key words: Nyasol, Hinokiresinol, *Anemarrhena asphodeloides* bunge (Liliaceae), Cyclooxygenase, Nitric oxide synthase, Lipoxygenase

INTRODUCTION

Numerous molecules are involved in the inflammatory response. Among them, eicosanoids [prostaglandins (PG) and leukotrienes (LT)] and nitric oxide (NO) are important chemical mediators (Gallin and Snyderman, 1999). PGs are synthesized by cyclooxygenases (COX), which exist as at least two isoforms, COX-1 and the inducible COX-2. LTs are synthesized from arachidonic acid by 5-lipoxygenase (5-LOX). NO is synthesized from arginine by nitric oxide synthases, among which the inducible isoform of nitric oxide synthase (iNOS) is mainly responsible for producing large amounts of NO in inflammatory lesions. Therefore, inhibition of these enzymes may exert anti-inflammatory effects.

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The rhizomes of *Anemarrhena asphodeloides* Bunge (Liliaceae) have been used to treat infectious, pyretic and inflammatory disorders in Chinese medicine (Kawasaki and Yamauchi, 1963; Park et al., 2003). Various constituents have previously been isolated from the same plant material, including mangiferin, timosaponins and (-)-nyasol (*cis*-hinokiresinol) (Bae et al., 2007; Lee et al., 1995; Kim et al., 2006). Among these constituents, the anti-inflammatory actions of mangiferin were previously demonstrated. For example, mangiferin isolated from another plant extract, *Mangifera indica* L., inhibited production of PGE_2 and LTB_4 from J774 cells (Garrido et al., 2006) and exhibited antiallergic properties, including inhibition of IgE production, histamine release and lymphocyte proliferation (Rivera et al., 2006). However, studies of the anti-inflammatory activity of other constituents have been very limited. Thus, it is necessary to explore the anti-inflammatory principles further in order to establish the anti-inflammatory action of *A. asphodel-*

oides and to examine the therapeutic value of other constituents. In our preliminary experiments, several constituents were isolated and their anti-inflammatory potential was elucidated, leading to the identification of (-)-nyasol as an anti-inflammatory principle. In this study, the *in vitro* and *in vivo* anti-inflammatory activities of (-)-nyasol (*cis*-hinokiresinol) were examined and (-)-nyasol proved to be a broad spectrum enzyme inhibitor of eicosanoid and NO metabolism.

MATERIALS AND METHODS

Chemicals

N-[2-Cyclohexyloxy-4-nitrophenyl]methane sulfonamide (NS-398) was obtained from Biomol (Plymouth Meeting, PA). 2-Amino-5,6-dihydro-6-methyl-4*H*-1,3-thiazine hydrochloride (AMT) was purchased from Tocris Cookson Ltd. Arachidonic acid (AA, 99%), nordihydroguaiaretic acid (NDGA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), prednisolone and lipopolysaccharide (LPS, *Escherichia coli* 0127:B8) were purchased from Sigma. DMEM and other cell culture reagents, including FBS, were products of Gibco BRL. The protein assay kit was purchased from Bio-Rad Lab.

Isolation of (-)-nyasol (cis-hinokiresinol)

The rhizomes of *Anemarrhena asphodeloides* Bunge (Liliaceae) were purchased from Omni Herb. The plant material was authenticated by Prof. J. H. Lee at Dongkuk University. A voucher specimen (No. EA249) was deposited at the Natural Product Chemistry Lab, College of Pharmacy, Ewha Womans University. (-)-Nyasol (*cis*-hinokiresinol, Fig. 1) was isolated from the EtOAc extracts of the rhizomes of *A. asphodeloides* and structurally identified as previously described (Bae et al., 2007). The purity was checked by HPLC {99.9%, t_R.21 min, 1mL/min, UV 265 nm, (0.1% formic acid)-[CAN-MeOH-0.1%FA (45:45:10)] = 85:15, nanospace SI-1, capcellpak C18 (4.6 × 250 mm, 3 μm)}.

RAW 264.7 cell culture and measurement of NO and PGE_2 concentrations

RAW 264.7 cells obtained from American Type Culture Collection (ATCC) were cultured with DMEM supplemented with 10% FBS and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) in a 5% CO_2 atmosphere at 37°C. The cells were activated with LPS as previously described (Chi et al., 2001). Briefly, cells were plated in 96-well plates (2×10^5 cells/well). After pre-incubation for 2 h, test compounds and LPS (1 μg/mL) were added and incubated for 24 h unless otherwise specified. Test compounds dissolved in DMSO were diluted to appropriate concentrations with serum-free DMEM. The final concentration of DMSO was adjusted to 0.1% (v/v). Cell viability was assessed using MTT assay as described previously (Mossman, 1983). PGE_2 concentration in the medium was measured using an ELISA kit for PGE_2 (Cayman Chem. Co.) according to the manufacturer's recommendations. To assess NO production, the stable conversion product of NO, nitrite (NO_2^-), was measured using Griess reagent and the optical density was determined at 550 nm. The direct inhibitory activity of (-)-nyasol against COX-2 and iNOS was also examined. RAW 264.7 cells were treated with LPS for 24 h in the absence of test compound to fully induce COX-2 and iNOS. After washing completely with serum-free media, the cells were further incubated with AA (100 μM) and (-)-nyasol for 15 min in order to avoid phospholipase A_2 inhibitory action. To measure the iNOS inhibitory action, the washed cells were incubated with (-)-nyasol for 24 h. PGE_2 and NO concentrations in the media were measured as described above.

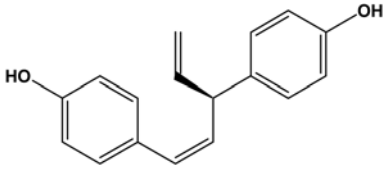

Fig. 1. Chemical structure of (-)-nyasol

Western blot analysis and electrophoretic mobility shift assay (EMSA)

To measure the protein levels of iNOS and COX-2, Western blotting technique were used (Chi et al., 2001). RAW 264.7 cells were cultured in 6-well plates (5×10^6 cells/well) in the presence or absence of LPS (1 mg/mL) with/without test compounds for 16-20 h. After cell homogenates were prepared, the supernatant was obtained by centrifugation at 15,000 g for 30 min. Using Tris-glycine gels (8%), electrophoresis was carried out and bands were blotted to PVDF membranes. The membranes were incubated with COX-2 antibody (No-160116, Cayman Chem.) and iNOS antibody (N32030, Transduction Lab.) and the bands were visualized by chemiluminescent reagent.

For EMSA, RAW cells were treated with LPS and various concentrations of test compounds for 3 h. To prepare nuclear fractions, the cells were washed with PBS, harvested and resuspended in 400 μL of buffer A (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, pH 7.9) for 15 min on ice. After

10% NP-40 (25 μL) was added, the tubes were vortexed vigorously for 10 s. The nuclei were collected by centrifugation at 5,000 rpm for 3 min and the supernatant was saved as the cytosolic fraction. The nuclei were lysed in buffer B (20 mM HEPES, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, pH 7.9). Nuclear transcription factor-κB (NF-κB) consensus oligonucleotide (Promega) was phosphorylated using T4 polynucleotide kinase (10 units) with 10 μCi of [γ-^{32}P] ATP (3,000 Ci/mmol) at 37°C for 10 min. Unincorporated oligonucleotides were removed with a Microspin G-25 column . Nuclear extract containing 5 μg protein was incubated with ^{32}P-labeled NF-κB consensus oligonucleotide in gel shift binding buffer at room temperature for 20 min. The incubation mixture was subjected to electrophoresis on a 4% polyacrylamide gel in TBE buffer (0.5X) at 350V. The gel was dried and exposed to X-ray film overnight at -70°C.

COX-1 enzyme assay

The COX-1 assay kit (Cayman Chem.) was used to measure the COX-1 inhibitory activity according to the manufacturer's recommendations in the presence/absence of (-)-nyasol.

Effects on 5-LOX

In order to evaluate the 5-LOX inhibitory activity, rat basophilic leukemia cells (RBL-1) purchased from ATCC were cultured in RPMI 1640 with 10% FBS, 2 mM glutamine and 1% antibiotics in a 5% CO_2 atmosphere at 37°C. The cells were plated in 96-well plates for 2 h. The cells were preincubated with the test compounds for 10 min. 3 μM A-23187 (ionophore) was then added to activate 5-LOX and the cells were incubated for 15 min as previously described with slight modification (Tries et al., 2002). Media was collected and the concentration of 5-LOX products, cysteinyl leukotrienes ($LTC_4/D_4/E_4$), was measured using an ELISA kit (Cayman Chem.) as recommended by the manufacturer.

λ-Carrageenan (CGN)-induced paw edema in mice

To examine the *in vivo* anti-inflammatory activity, the mouse CGN-induced paw edema assay by Winter et al. (1962) was used with slight modification. Specific pathogen-free male ICR mice were purchased from Orient Bio and acclimatized in the animal facility for at least 7 days prior to the experiment with lab chow and water ad libitum. Nyasol or reference compound dissolved in DMSO (0.05 mL/mouse) was administered intraperitoneally to mice. One hour later, 1% CGN (w/v) dissolved in pyrogen-free sterile saline solution (0.05 mL/paw) was injected into the right hind paw and paw volume was measured using plethysmometer (Ugo Basil) 5 h later. A paw volume increase from the initial non-treated paw volume was

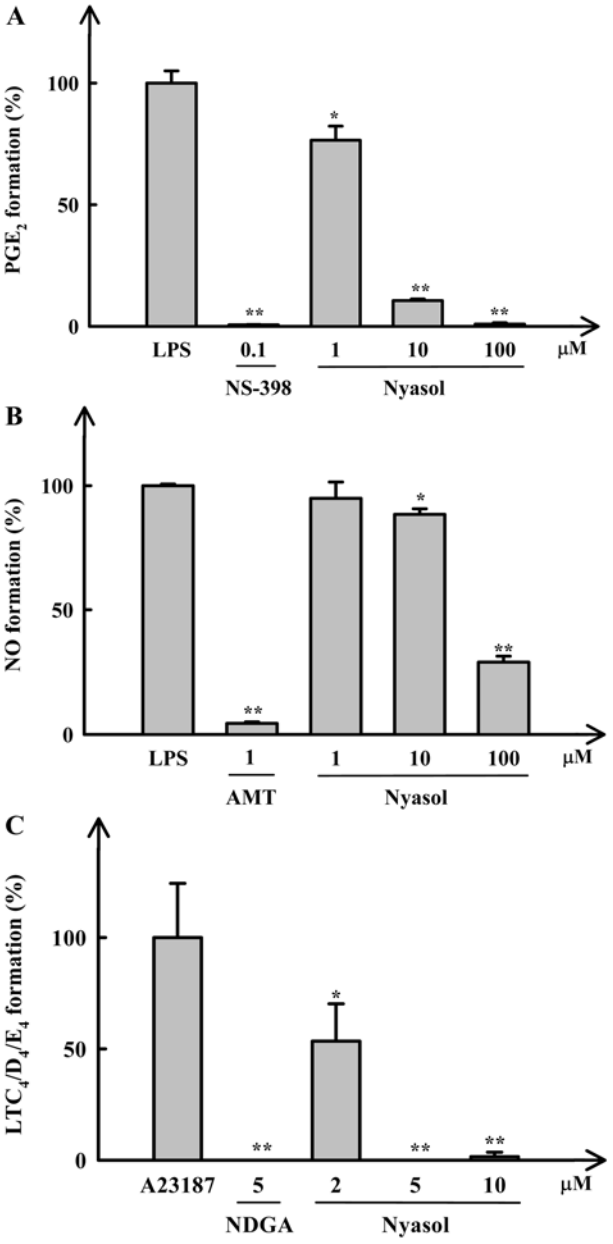

Fig. 2. Effects of (-)-nyasol on eicosanoid and NO production. (**A**) Inhibition of COX-2-catalyzed PGE_2 production from LPS-treated RAW 264.7 cells, (**B**) Inhibition of iNOS-catalyzed NO production from LPS-treated RAW 264.7 cells, LPS and the test compounds were simultaneously added and the cells were incubated for 24 h. (n = 3), (**C**) Inhibition of 5-LOX-catalyzed LT production from A23187-treated RBL-1 cells, The cells were incubated for 15 min after adding A23187 and the test compounds (n = 4). *p < 0.05, **p < 0.001, significantly different from the LPS-treated or A23187-treated control group.

regarded as edema.

Statistical analysis

Experimental values were presented as arithmetic mean ± S.D. The unpaired Student's *t*-test was used to determine the statistical significance.

RESULTS

COX-2 and iNOS are highly induced in RAW 264.7 cells following treatment with LPS for 24 h. These enzymes produce large amounts of PGE_2 and NO, respectively. Under this condition, (-)-nyasol strongly inhibited COX-2-mediated PGE_2 production, but was less active against iNOS-mediated NO production (Fig. 2A and 2B). As expected, NS-398 (COX-2 inhibitor) and AMT (iNOS inhibitor) potently inhibited PGE_2 and NO production with 98.5% and 95.2% inhibition, respectively. On the other hand, RBL-1 cells produce cysteinyl LTs by 5-LOX when stimulated with A23187 (calcium ionophore). In this experiment, NDGA (LOX inhibitor) potently inhibited $LTC_4/D_4/E_4$ production (99.9% inhibition) as shown in Fig. 2C. (-)-Nyasol also inhibited 5-LOX-mediated LT production.

To elucidate the inhibitory mechanism of (-)-nyasol

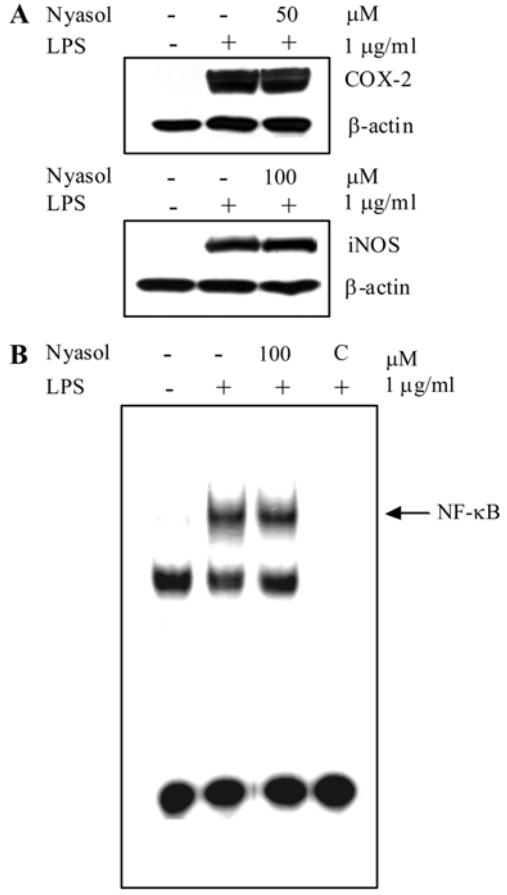

Fig. 3. Effects of (-)-nyasol on expression of COX-2 and iNOS and NF-κB activation. (**A**) Western blotting analysis, (**B**) EMSA, C: competitor.

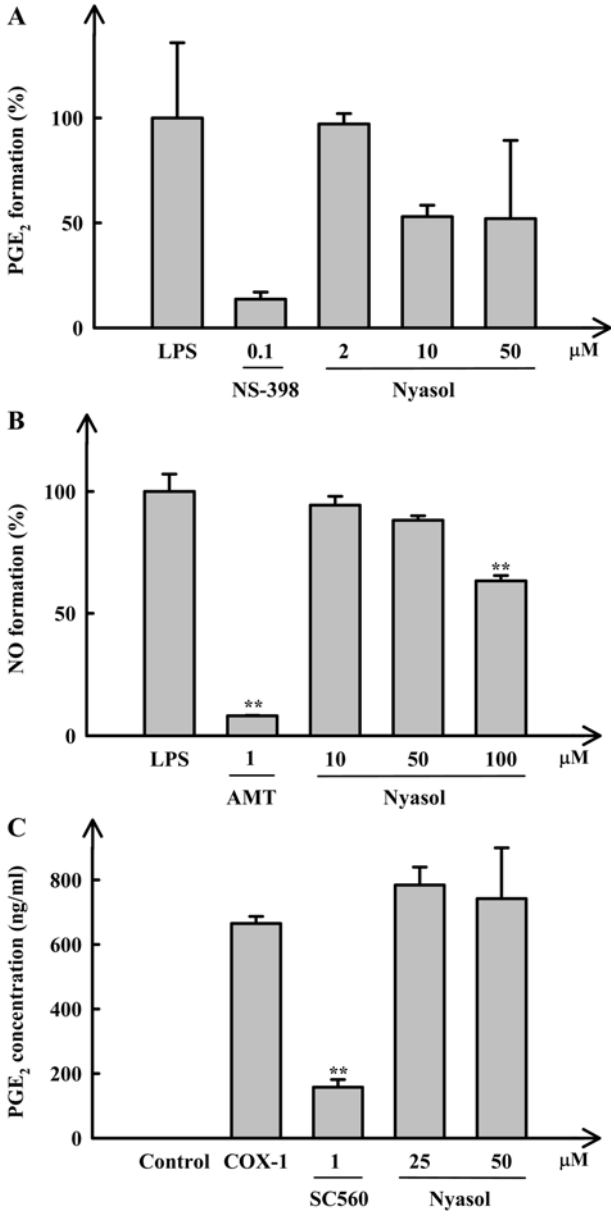

Fig. 4. Effects of (-)-nyasol on COX-1, COX-2 and iNOS activity. (**A**) Inhibition of pre-induced COX-2 from LPS-treated RAW 264.7 cells. The cells were pretreated with LPS for 24 h. To the completely washed cells, the test compounds and AA were added and the cells were further incubated for 15 min. (**B**) Inhibition of pre-induced iNOS from LPS-treated RAW 264.7 cells. The cells were pretreated with LPS for 24 h. To the completely washed cells, the test compounds were added and the cells were further incubated for 24 h. (**C**) Inhibition of recombinant COX-1, The compounds were incubated with recombinant COX-1 for 15 min according to the manufacturer's recommendation. ($n = 3$), *$p < 0.05$, **$p < 0.001$, significantly different from the control group.

Table I. Inhibition of λ-carrageenan (CGN)-induced paw edema in mice by (-)-nyasol

Compounds	Dose (mg/kg)	Increased paw volume (mL)	% inhibition
CGN	-	0.140 ± 0.012	-
Prednisolone	10.0	0.085 ± 0.031*	39.3
(-)-Nyasol	4.8	0.148 ± 0.015	-
	24.0	0.100 ± 0.032*	28.6
	72.0	0.062 ± 0.036*	56.0
	120.0	0.032 ± 0.036**	77.1

All compounds were intraperitoneally administered. $n = 5$, the paw volume of the untreated control group was 0.130 ± 0.008 mL. *$p < 0.05$, **$p < 0.01$, significantly different from the CGN-treated group by unpaired Student t-test.

against PGE_2 and NO production, Western blotting and EMSA techniques were employed. However, (-)-nyasol did not affect the levels of COX-2 and iNOS expression and NF-κB activation (Fig. 3). These results suggest that (-)-nyasol may inhibit PGE_2 and NO production through COX-2 and iNOS inhibition, without down-regulating COX-2 and iNOS expression. To prove a direct effect against COX-2 and iNOS activity, RAW cells in which COX-2 and iNOS were preinduced by LPS treatment were incubated with (-)-nyasol. In this experiment, (-)-nyasol inhibited PGE_2 and NO production considerably (Fig. 4A and 4B), indicating that it directly inhibits COX-2 and iNOS. To assess the selectivity against COXs, COX-1 inhibitory activity was examined using COX-1 assay kit. However, (-)-nyasol did not inhibit COX-1 activity at concentrations of up to 50 μM, while SC560 (COX-1 inhibitor) strongly inhibited at 1 uM as expected (Fig. 4C). Thus, (-)-nyasol appears to be a broad spectrum inhibitor of COX-2, iNOS and 5-LOX, but not COX-1.

In addition, the *in vivo* anti-inflammatory activity of (-)-nyasol was examined. By intraperitoneal injection, (-)-nyasol strongly and dose-dependently inhibited CGN-induced paw edema in mice (Table I). In particular, (-)-nyasol inhibited paw edema by more than 70% at 120 mg/kg, i.p. Considering that (-)-nyasol is a natural product, its potency is promising for its development as a new anti-inflammatory agent.

DISCUSSION

The present investigation has clearly shown that (-)-nyasol possesses anti-inflammatory activity *in vitro* and *in vivo*. Its anti-inflammatory action is due, at least in part, to the inhibition of COX-2, iNOS and 5-LOX. In contrast, (-)-nyasol does not affect the expression levels of COX-2 and iNOS. Also, it does not inhibit NF-κB activation. Thus (-)-nyasol is a broad spectrum inhibitor of eicosanoid and NO metabolism without the capacity to down-regulate COX-2 and iNOS.

There are two stereochemical isomers of hinokiresinol, *cis*- and *trans*-hinokiresinol (referred to as hinokiresinol). (-)-Nyasol is *cis*-hinokiresinol. Some biological activities of (-)-nyasol have been described previously. For instance, inhibition of angiogenesis and hyaluronidase inhibitory activity were demonstrated (Jeong et al., 1999; 2003). Anti-oomycete and antiviral activities of (-)-nyasol were also investigated (Park et al., 2003; Bae et al., 2007). Antioxidant and anti-artherogenic activities were also described (Song et al., 2007). Moreover, antiallergic activity, such as LTB_4 receptor antagonistic activity and inhibition of passive cutaneous anaphylaxis, was previously reported (Lee and Ryu, 1999; Bae et al., 2006). In contrast, anti-inflammatory activity of (-)-nyasol has not been described to date. This is the first report to demonstrate anti-inflammatory activity of (-)-nyasol.

COX inhibitors have been widely used clinically. In recent years, several COX-2 selective inhibitors have been developed in order to reduce severe gastric side effects. However, two COX-2 inhibitors were withdrawn from the market mainly due to the higher incidence of cardiovascular failure (Roumie et al., 2008). It is thought that dual inhibitors of COX/LOX or broad spectrum inhibitors may be more desirable as new anti-inflammatory agents (Kulkarni and Singh, 2007). In this respect, (-)-nyasol has potential as a new anti-inflammatory agent.

In conclusion, the present investigation shows that (-)-nyasol has anti-inflammatory activity *in vitro* and *in vivo*. In addition to mangiferin, (-)-nyasol may also contribute to the anti-inflammatory action of *A. asphodeloides,* probably by reducing eicosanoid and NO concentrations in inflammatory lesions.

ACKNOWLEDGEMENTS

This study was financially supported by the research fund of Studies on the Identification of the Efficacy of Biologically Active Components from Oriental Herbal Medicines from Korean Food and Drug Administration (2007) and post-BK21 project from Ministry of Education and Science Technology, Korea.

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