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Original Article

Inhibition of nitric oxide production by compounds from *Boesenbergia longiflora* using lipopolysaccharide-stimulated RAW264.7 macrophage cells

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Abstract

The inhibitory activity of extract and compounds isolated from *Boesenbergia longiflora* against nitric oxide (NO) was evaluated using RAW264.7 cells. Isolation of the chloroform extract of *B. longiflora* rhizomes afforded four known flavonoids, which were identified as kaempferol-3,7,4'-trimethyl ether (1), kaempferol-7,4'-dimethyl ether (2), rhamnazin (3), pinostrobin (4), together with four known diarylheptanoids, dihydrobisdemethoxycurcumin (5), curcumin (6), demethoxycurcumin (7) and bisdemethoxycurcumin (8), as well as one sterol, β-sitosterol-D-glucoside (9). Compound 6 exhibited the highest inhibitory activity against NO release with an IC_{50} value of 4.5 μM, followed by 7 (IC_{50} = 11.7 μM), 8 (IC_{50} = 15.7 μM), 5 (IC_{50} = 23.0 μM) and 1 (IC_{50} = 23.5 μM), respectively. This study demonstrated that diarylheptanoids and some methoxyflavonoids found in *B. longiflora* are responsible for anti-inflammatory activity and this is the first report the safety, chemical constituents and biological activity of this plant.

Keywords: Zingiberaceae, anti-inflammatory effect, diarylheptanoids, methoxyflavonoids

1. Introduction

The genus *Boesenbergia,* a member of the Zingiberaceae family, is distributed from India to Southeast Asia with approximately 80 species worldwide, where 19 species were previously accounted for Thailand (Delin and Larsen, 2000; Techaprasan *et al*., 2006). Many researchers have demonstrated several pharmacological properties of plants in the Zingiberaceae family, however, studies that describe the effects of *Boesenbergia* species are still limited. Only *Boesenbergia rotunda* has been extensively reported as having several pharmacological and phytochemical properties ranging from antimicrobial, antioxidant, anti-inflammatory, anticancer, and anti-HIV activities. Cyclohexenyl chalcone derivatives, 4-hydroxypanduratin A and panduratin A, the active constituents isolated from *B. rotunda,* exhibited inhibitory activities on dengue 2 virus NS3 protease (Kiat *et al*., 2006) and HIV-1 protease (Cheenpracha *et al*., 2006) as well as anti-inflamatory activity (Tuchinda *et al*., 2002; Tewtrakul *et al*., 2009). Due to these properties, *Boesenbergia* species have gained attention as important sources for medicinal treatment.

Nitric oxide (NO) is one of pro-inflammatory mediators released in response to pathogenic infections. During the inflammatory process, macrophages generate NO to eliminate foreign pathogens, recruit other cells to the infected area and subsequently resolve the inflammation. In general, NO is involved in vasodilation, platelet aggregation, host defense and a regulatory molecule with homeostatic activities (Korhonen *et al*., 2005; Min *et al*., 2009). However, excessive

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production of NO also attacks normal tissue surrounding the infected area by binding with other superoxide radicals and acts as a reactive radical which damages normal cell function. NO is synthesized from L-arginine via the catalytic action of the enzyme, the nitric oxide synthase (NOS). Inducible nitric oxide synthase (iNOS) is responsible for the over- production of NO which is known to be involved in various chronic inflammatory diseases, including cancer. Lipopolysaccharide (LPS) from Gram negative bacteria is particularly well known to increase iNOS expression and over-production of NO, leading to the initiation of an inflammatory response (Tewtrakul *et al*., 2009; Min *et al*., 2009). Thus, the inhibition of NO production is an important therapeutic consideration in the development of anti-inflammatory agents and treatment of inflammatory disorders.

Boesenbergia longiflora (Wall.) Kuntze is locally known in Thai as Wan priao. Its rhizome has been used in ethnomedical uses as tonic (Chuakul and Boonpleng, 2003). Neither phytochemical nor pharmacological studies have been previously reported for this plant species. In addition, the ethanol and water extracts from the rhizomes of *B. longiflora* exhibited anti-inflammatory activity in murine macrophage-like RAW264.7 cell line. We therefore, investigated the inhibitory activity of compounds isolated from *B. longiflora* on LPS-induced NO release using RAW264.7 cells.

2. Materials and Methods

2.1 Reagents

Lipopolysaccharide (LPS) from *Escherichia coli*, RPMI-1640 medium, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), ibuprofen, aspirin, L-nitro-arginine (L-NA), caffeic acid phenethyl ester (CAPE), indomethacin, phosphate buffer saline (PBS) and tween 80 were from Sigma Aldrich (Sigma Aldrich, Missouri, USA). Fetal calf serum (FCS) was from Gibco (Invitrogen, California, USA). Penicillin-streptomycin was from Invitrogen (Invitrogen, California, USA). 96-Well microplates were obtained from Nunc (Nunc, Birkrød, Denmark). Other chemicals were from Sigma Aldrich (Sigma–Aldrich, Missouri, USA).

2.2 Plant materials

The rhizomes of nine selected Zingiberaceous plants including *Kaempferia rotunda* L., *Kaempferia angustifolia* Roscoe, *Kaempferia marginata* Carey ex Roscoe, *Kaempferia* sp. (Prauh-Pa), *Boesenbergia thorelii* (Gagnep.) Loes., *Boesenbergia longiflora*, *Boesenbergia* sp. 1 (Kai-Dang) and *Boesenbergia* sp. 2 (Kai-Dam) were bought from Chatuchak weekend market in Bangkok, Thailand, whereas *Kaempferia pulchra* Ridl. rhizomes were collected in October 2010 in Suratthani province, Thailand. The voucher specimens are SKP 206111801, SKP 206110101, SKP206111301, SKP 2061100-101, SKP206022001, SKP2060200-101, SKP2060200-201, SKP2060200-301 and SKP206111601 respectively. The plant materials were identified by Dr. Charun Marknoi, Queen Sirikit Botanical Garden, Chiang Mai, Thailand. The voucher specimens are kept at the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. The plant material was cleaned with distilled water. Only fresh rhizomes, which did not have scars or spots of disease, were selected for further extraction. The rhizomes were cut into round slices in appropriate sizes of 3-5 mm transverse sections. The rhizomes were then dried in a hot air oven at 50°C until dryness. The dried rhizomes were pulverized to coarse powder using an electric blender.

2.3 Preparation of the plant extracts

Ten grams of each dried plant was extracted by reflux for 3 h with 200 ml of ethanol (EtOH) and water, separately. The solvents were removed under reduced pressure to give ethanolic and water extracts, respectively. The yields of ethanolic extracts of *K. rotunda*, *K. angustifolia*, *K. marginata*, *K. pulchra, Kaempferia* sp., *B. thorelii*, *B. longiflora*, *Boesenbergia* sp. 1 and *Boesenbergia* sp. 2 were found to be 18.5, 11.6, 31.7, 10.1, 21.6, 15.4, 14.9, 9.8 and 13.1 %w/w, respectively, whereas the water extracts were 44.9, 25.3, 14.1, 10.2, 16.3, 11.6, 20.5, 12.5 and 37.2 %w/w, respectively. Stock solutions (10 mg/ml) of the extracts were prepared in DMSO and stored at 4°C until use.

2.4 Extraction and isolation of compounds from *B. longiflora*

The fresh rhizomes of *B. longiflora* (20 kg, second lot) were sliced and oven-dried at 50°C. Five kilograms of pulverized rhizomes were macerated with EtOH at room temperature, four times (6 L × 4). The EtOH extract (416 g) was then concentrated and partitioned between 95% MeOH and hexane, and successively partitioned with chloroform and water. After that, the water layer was partitioned with ethyl acetate (EtOAc). Each partition was evaporated to dryness in vacuo to give residues of hexane (116.6 g), chloroform (285.6 g), EtOAc (2.3 g) and water fractions (23.3 g), respectively. The chloroform fraction (30.0 g), which possessed the highest NO inhibitory activity (IC_{50} = 5.5 µg/ml), was chromatographed on silica gel (800 g) using a stepwise gradient elution of hexane, hexane-chloroform (9:1, 8:2, 6:4, 4:6, 2:8), chloroform, chloroform-EtOAc (9:1, 8:2, 6:4, 4:6, 2:8), EtOAc and EtOAc-MeOH (9.5:0.5, 9:1), to give thirteen fractions (F1-F13).

Fraction F6 (11.6 g) was chromatographed on silica gel (400 g) using a gradient of hexane, hexane-chloroform (9:1, 8:2, 6:4, 4:6, 2:8), chloroform and chloroform-MeOH (9.5: 0.5, 9:1) solvent system, to give compound 1 (kaempferol-3,7, 4'-trimethyl ether, 24 mg), together with compound 2 (kaempferol-7,4'-dimethyl ether, 32 mg). Subfraction of F6 (12.7 mg) was chromatograped on preparative thin layer chromatographed using hexane-chloroform (1:1) as a developing solvent, to yield compound 4 (pinostrobin, 3 mg).

Fraction F8 (235 mg) was chromatographed, using the same solvent system as that of fraction 6, to give compound 3 (rhamnazin, 2 mg) and compound 5 (dihydrobisdemethoxycurcumin, 45 mg). Fraction F11 (250 mg) was chromatographed by gel per-meation chromatography on Sephadex LH-20 eluted with methanol. Subfraction F11/5 (96.2 mg) was chromatographed onsilica gel using chloroform and chloroform-MeOH as eluent, to yield compound 7 (demethoxycurcumin, 11 mg) and compound 8 (bisdemethoxycurcumin, 18 mg). Subfraction F11/1 (11 mg) was chromatographed on preparative thin layer chromatography using chloroform-MeOH (95:5) as a developing solvent, to obtain compound 6 (curcumin, 4 mg). Fraction F13 was recrystallized from chloroform-MeOH (9:1) as white powder of compound 9 (β-sitosterol-D-glucoside, 32.8 mg). The structures of 1-9 were elucidated by comparing the ^{1}H and ^{13}C NMR spectral data with those reported (Dong and Chen, 1998; El-Mousellami *et al*., 2000; Piccinelli *et al*., 2004; Park *et al*., 2005; Ching *et al*., 2007; Sutthanut *et al*., 2007; Li *et al*., 2009; Rahmana *et al*., 2009).

2.5 Acute toxicity test of *B. longiflora* extract in mice

All experimental protocols were carried out in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Animal Ethic Committee, Prince of Songkla University (MOE 0521.11/303). Both male and female Swiss albino mice (30–40 g) were used in the experiments. All animals obtained from the Southern Laboratory Animal Facility, Prince of Songkla University, Hat Yai, Songkhla, Thailand. Mice were housed in a standard environmental conditions with a 12-hour light/dark cycle. They were provided *ad libitum* with standard rodent diet and water.

The 50% lethal dose (LD_{50}) of the ethanol and chloroform extracts of *B. longiflora* rhizomes were estimated by the up-and-down method in mice (Bruce, 1985). The animals were fasted for 6 h prior to dosing. Doses were adjusted by a constant multiplicative factor (viz., 1.5) for this experiment. The dose for each successive animal was adjusted up or down depending on the previous outcome. The crude extracts were dissolved in cosolvent solution (Tween 80: DMSO: water = 2.5:2.5:95) and orally administered in a single dose by gavage using a stomach tube to both groups of male and female mice. Animal behaviors were observed individually at least once during the first 30 minutes after administration, periodically during the first 8 hours and daily thereafter, for a total of 7 days. The signs of toxicity, including tremor, convulsion, hyperactivity, sedation, grooming, loss of righting reflex, respiratory depression and coma, were observed.

2.6 Cell culture and test sample treatments

The murine macrophage-like RAW264.7 cell line (purchased from Cell lines Services) was cultured in RPMI medium supplemented with 0.1% sodium bicarbonate and 2 mM glutamine, penicillin G (100 units/ml), streptomycin (100 µg/ml) and 10% FCS in 75-cm^2 culture flasks at 37°C in a humidified atmosphere containing 5% CO_2. Cells were harvested with trypsin-EDTA and diluted to a suspension in a fresh medium. The cells were seeded in 96-well plates with 1×10^5 cells/well and allowed to adhere for 1 h at 37°C in a humidified atmosphere containing 5% CO_2. After that the medium was replaced with a fresh medium containing 25 µg/ml of LPS together with the test samples at various concentrations (1-100 µg/ml for crude extracts and 3-100 µM for pure compounds) and was then incubated for 48 h. NO synthase inhibitor (L-NA), NF-κB inhibitor (CAPE), and non-steroidal anti-inflammatory drugs, NSAIDs (indomethacin, ibuprofen and aspirin), were used as positive controls. The stock solution of each test sample was dissolved in DMSO, and the solution was added to the medium RPMI (final DMSO is 1%).

2.7 Measurement of NO production from RAW264.7 cells

NO production by RAW264.7 cells was determined by measuring the accumulation of nitrite in the culture supernatant using the Griess reagent as previously described (Tewtrakul *et al*., 2009). After 48 h of incubation at 37°C in a humidified atmosphere containing 5% CO_2, the supernatant (100 µl) was collected and reacted with Griess reagent (100 µl). NO production was measured spectrophotometrically at 570 nm using a microplate reader, Power Wave X from Bio-Tex Inc. The % inhibition was calculated based on the following equation and IC_{50} values were determined graphically ($n = 4$):

$$\text{Inhibition (\%)} = [(A - B) / (A - C)] \times 100$$

A-C: NO_2^- concentration (µM) [A: LPS (+), sample (-); B: LPS (+), sample (+); C: LPS (-), sample (-)].

2.8 Assay of cell viability

Viability of RAW264.7 cells was assayed using a MTT colorimetric method. This method requires active mitochondria of living cells to reduce MTT, a pale yellow substrate, to yield a dark blue formazan product. Briefly, after 48 h incubation with the test samples at 37°C in a humidified atmosphere containing 5% CO_2, MTT solution (10 µl, 5 mg/ml) was added to the wells and further incubated for 4 h at 37°C. Thereafter, the medium was removed, the formazan products resulting from dye reduction by viable macrophage cells were dissolved using isopropanol containing 0.04 N HCl, and the optical density was measured with a microplate reader at a wavelength of 570 nm. The test compounds were considered to be cytotoxic when the optical density of the sample-treated group was less than 80% of that in the control (vehicle-treated) group.

2.9 Statistics

For statistical analysis, all data values were expressed

as mean ± S.E.M of four determinations. The IC_{50} values were calculated using the Microsoft Excel program. The data analysis was performed by one-way analysis of variance (ANOVA), followed by Dunnett's test. The p values < 0.05 and < 0.01 were considered to be significant.

3. Results and Discussion

3.1 *In vitro* activity of *B. longiflora* extracts and isolated compounds

Our previous investigation demonstrated the anti-inflammatory effect of the Zingiberaceous plants such as *Kaempferia parviflora* and *B. rotunda* through the inhibition of NO releases (Tewtrakul *et al*., 2009). Therefore, other Zingiberaceous plants in the genus of *Kaempferia* and *Boesenbergia* including *K. rotunda*, *K. angustifolia*, *K. marginata*, *K. pulchra*, *Kaempferia* sp., *B. thorelii*, *B. longiflora*, *Boesenbergia* sp. 1 and *Boesenbergia* sp. 2 were investigated for the inhibitory effect of NO production using murine macrophage-like RAW264.7 cells. The ethanolic extracts of these plants showed activity against NO release with IC_{50} values of 34.7, 22.1, 10.9, 17.7, 12.4, >100, 6.0, 41.1 and 5.4 μg/ml, respectively, whereas those of the water extracts were >100, 73.7, 94.2, >100, 94.9, >100, 72.2, 69.5 and 87.9 μg/ml, respectively (Table 1). This result may support the use of some Zingiberaceous plants for anti-inflammation through the inhibition of NO release.

Since *B. longiflora* showed good activity against NO release (IC_{50} 6.0 μg/ml), this work aimed to determine the pharmacological properties, bioactive constituents and the safety of this plant. From bioassay-guided fractionation of *B. longiflora* (Table 2), $CHCl_3$ fraction significantly suppressed LPS-induced NO production in a dose-dependent manner with an IC_{50} value of 5.5 μg/ml, while hexane-, EtOAc- and water fractions showed moderate effects with IC_{50} values of 40.8, 41.1 and 60.9 μg/ml respectively. The $CHCl_3$ fraction was then chromatographed further to obtain nine compounds (Figure 1). These isolated compounds could be classified into three groups, flavonoids (1, 2, 3 and 4), diarylheptanoids (5, 6, 7 and 8) and a sterol (9), and were tested for their NO inhibitory effects (Table 3). Among these, 6 exhibited the most potent inhibitory effect with an IC_{50} value of 4.5 μM, followed by 7 (IC_{50} = 11.7 μM), 8 (IC_{50} = 15.7 μM), 5 (IC_{50} = 23.0 μM) and 1 (IC_{50} = 23.5 μM), respectively. This is the first report of compound 5, a diarylheptanoid derivative, on anti-inflammatory effect. This compound has also been reported to show anti-platelet activity (Dong and Chen, 1998). Compounds 1, 5, 6, 7 and 8 exhibited higher effect than positive controls, L-NA (NO synthase inhibitor, IC_{50} = 59.4 μM), indomethacin (IC_{50} = 29.0 μM), ibuprofen (IC_{50} = 55.0 μM) and aspirin (IC_{50} = 71.7 μM), the clinically used non-steroidal anti-inflammatory drugs. Moreover, compound 6 (IC_{50} = 4.5 μM) also possessed higher activity than CAPE, an NF-κB inhibitor

Table 1. Inhibitory effects on NO production in RAW264.7 cells of some selected plants in the Zingiberaceae family.

Plant species	% inhibition at various concentrations (μg/ml)						IC_{50} (μg/ml)
	0	1	3	10	30	100	
Kaempferia rotunda L. (EtOH)	0.0±2.0	-	-	25.0±2.9**	42.8±1.2**	75.0±3.3**	34.7
K. rotunda L. (H_2O)	0.0±2.0	-	-	9.2±4.5	15.2±0.6*	46.2±2.6**	>100
K. angustifolia Roscoe (EtOH)	0.0±2.0	-	-	33.9±3.4**	53.4±4.5**	85.6±0.4**	22.1
K. angustifolia Roscoe (H_2O)	0.0±2.7	-	-	10.8±2.4	21.5±1.5**	66.8±3.9**	73.7
K. marginata Carey ex Roscoe (EtOH)	0.0±2.0	23.6±3.9**	28.4±2.6**	52.7±3.0**	96.2±1.2^{**,a}	99.3±1.2^{**,a}	10.9
K. marginata Carey ex Roscoe (H_2O)	0.0±1.4	-	-	9.9±1.1	23.7±1.2**	52.0±6.6**	94.2
K. pulchra Ridl. (EtOH)	0.0±1.4	11.3±3.2	12.9±1.7	27.7±5.0**	60.0±4.2**	97.1±1.0^{**,a}	17.7
K. pulchra Ridl. (H_2O)	0.0±1.4	-	-	8.5±1.1*	20.0±2.9*	37.9±0.4**	>100
Kaempferia sp. (EtOH)	0.0±1.4	22.6±3.9**	29.4±2.6**	48.9±3.0**	87.0±1.7**	94.5±1.2^{**,a}	12.4
Kaempferia sp. (H_2O)	0.0±1.4	-	-	11.0±1.7*	25.7±2.0**	52.6±3.9**	94.9
Boesenbergia thorelii (Gagnep.) Loes. (EtOH)	0.0±1.4	-	-	6.9±1.4	9.0±3.1*	51.8±0.8**	>100
B. thorelii (Gagnep.) Loes. (H_2O)	0.0±1.4	-	-	14.9±1.1**	16.7±1.7**	47.2±1.6**	>100
B. longiflora (Wall.) Kuntze (EtOH)	0.0±2.7	17.2±2.9**	24.8±1.5**	71.2±1.50**	86.0±2.9**	98.0±2.7^{**,a}	6.0
B. longiflora (Wall.) Kuntze (H_2O)	0.0±2.0	-	0.9±2.0	2.2±1.2	25.1±2.2**	68.6±2.4**	72.2
Boesenbergia sp. 1 (EtOH)	0.0±1.4	-	-	19.5±7.7*	36.0±4.2**	74.4±1.4**	41.1
Boesenbergia sp. 1 (H_2O)	0.0±1.4	-	-	4.9±4.3	20.2±1.9**	67.3±1.7**	69.5
Boesenbergia sp. 2 (EtOH)	0.0±2.7	10.8±3.3	23.6±3.7**	95.6±1.8^{**,a}	98.8±2.0^{**,a}	98.0±1.8^{**,a}	5.4
Boesenbergia sp. 2 (H_2O)	0.0±2.0	-	-	-1.3±2.3	13.9±2.3**	57.8±2.6**	87.5

Value represents mean ± S.E.M. (N=4). Significantly different from the control (0 μg/ml), $^{*}p$<0.05, $^{**}p$<0.01
[a]Cytotoxic effect was observed. (-) = not determined.

Table 2. Inhibition of NO production by EtOH extract and fractions from *Boesenbergia longiflora* rhizomes.

Sample	% inhibition at various concentrations (μg/ml)						IC_{50} (μg/ml)
	0	1	3	10	30	100	
EtOH extract	0.0±0.3	33.6±4.2**	38.3±3.7**	74.8±2.9**	98.4±0.5^{**,a}	102.9±2.4^{**,a}	3.1
Hexane fraction	0.0±0.3	17.4±2.1**	21.5±1.9**	26.3±1.3**	39.5±2.2**	97.5±0.9^{**,a}	40.8
$CHCl_3$ fraction	0.0±1.9	21.8±2.8**	26.1±3.4**	62.7±3.3**	97.0±0.5^{**,a}	97.9±1.0^{**,a}	5.5
EtOAc fraction	0.0±1.6	-	26.3±3.0**	30.6±0.9**	34.6±2.1**	93.5±2.4**	41.1
H_2O fraction	0.0±1.6	-	24.7±1.5**	30.8±1.8**	33.9±1.9**	67.4±1.0**	60.9

Value represents mean ± S.E.M. (N=4). Significantly different from the control (0 μg/ml), $^{*}p<0.05$, $^{**}p<0.01$
[a]Cytotoxic effect was observed. (-) = not determined.

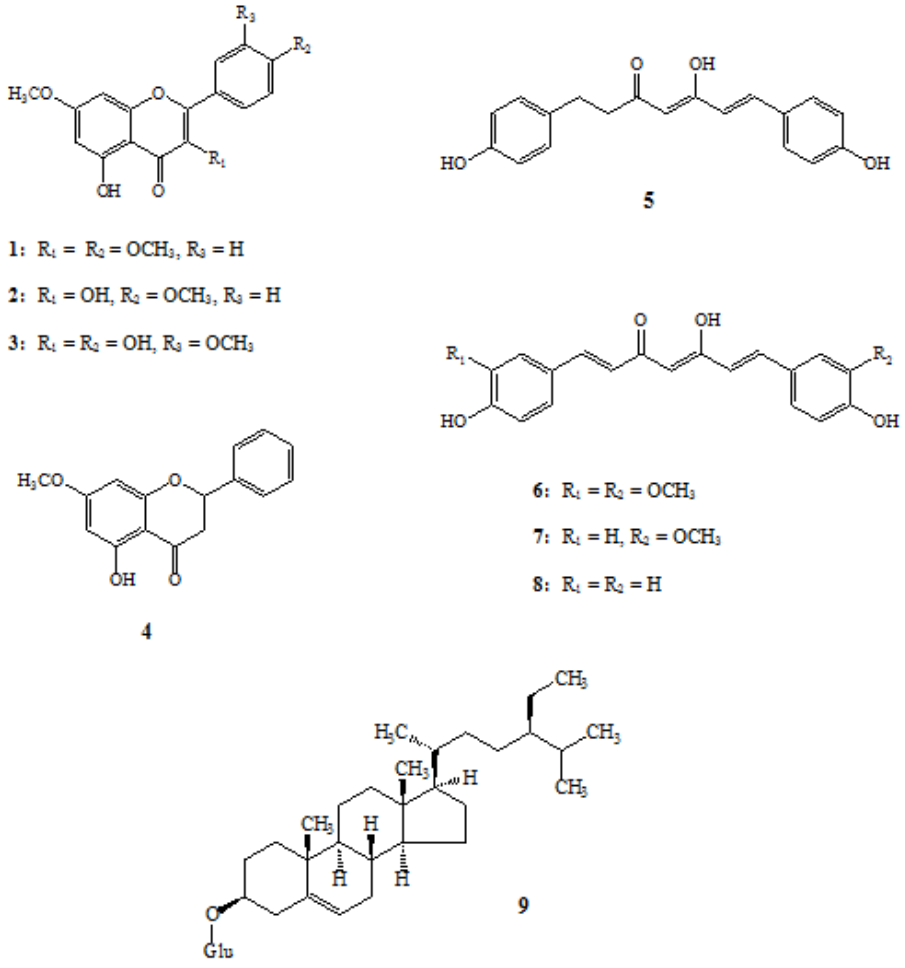

Figure 1. Chemical structures of 1-9 isolated from the rhizomes of *Boesenbergia longiflora*

(IC_{50} = 5.6 μM).

The present study showed that four diarylheptanoids [5-8] significantly decreased the levels of NO production from LPS-stimulated RAW264.7 cells. This result is concurrent with the previous study on the NO inhibitory effects of curcuminoids from *Curcuma longa* (Brouet and Ohshima, 1995; Guo *et al*., 2008). Recently, many reports have provided scientific data that curcuminoids produces anti-inflammatory effects through multiple mechanisms in other *in vivo* and *in vitro* inflammatory models (Maheshwari *et al*., 2006; Itokawa *et al*., 2008; Aggarwal and Harikumar, 2009).

3.2 *In vivo* acute toxicity test of *B. longiflora* extracts in mice

In the acute toxicity test, the results demonstrated that oral administration of ethanol and chloroform extracts of *B. longiflora* rhizomes as a single dose up to 2,000 mg/kg to

Table 3. Inhibitory effects on NO production of compounds isolated from *Boesenbergia longiflora* rhizomes.

Compound	% inhibition at various concentrations (μM)					IC_{50} (μM)
	0	3	10	30	100	
Kaempferol-3,7, 4'-trimethyl ether (1)	0.0±3.3	-	28.9±1.8**	51.6±3.7**	92.6±2.3^{**,a}	23.5
Kaempferol-7,4'-dimethyl ether (2)	0.0±3.3	-	12.9±2.6^{a}	14.5±1.4^{*,a}	46.1±4.1^{**,a}	>100
Rhamnazin (3)	0.0±2.5	-	18.2±3.4**	43.6±2.3**	60.1±4.3**	53.4
Pinostrobin (4)	0.0±3.3	-	9.7±2.6	25.8±1.4**	82.9±1.8**	43.2
Dihydrobisdemethoxycurcumin (5)	0.0±3.3	-	17.4±4.1**	62.9±4.1**	103.2±2.6^{**,a}	23.0
Curcumin (6)	0.0±1.9	36.1±2.7**	69.7±3.8**	100.0±2.0**	105.3±1.8^{**,a}	4.5
Demethoxycurcumin (7)	0.0±1.9	-	38.9±2.4**	88.9±3.0**	102.9±3.5^{**,a}	11.7
Bisdemethoxycurcumin (8)	0.0±1.9	-	23.7±1.0**	93.2±2.0**	101.1±3.6^{**,a}	15.7
β-Sitosterol-D-glucoside (9)	0.0±2.0	-	23.6±3.6**	26.1±3.5**	50.6±2.3**	>100
L-NA	0.0±2.7	-	19.1±2.5**	30.5±3.1**	75.8±2.4**	59.4
Ibuprofen	0.0±3.4	-	5.2±1.4	22.3±2.0**	72.2±4.9**	55.0
Aspirin	0.0±4.7	-	11.5±1.5**	20.6±1.0**	68.9±3.9**	71.7
Indomethacin	0.0±1.8	2.6±2.0	18.7±2.1**	39.1±1.4**	89.1±0.3**	29.0
CAPE	0.0±2.7	22.9±2.6**	81.2±1.2^{**,a}	90.6±3.6^{**,a}	103.6±2.4^{**,a}	5.6

Value represents mean ± S.E.M. (N=4). Significantly different from the control (0 μM), $^{*}p<0.05$, $^{**}p<0.01$
^{a}Cytotoxic effect was observed. (-) = not determined.

male and female mice were not associated with any mortality. No significant signs of toxicity at 2,000 mg/kg, including tremor, convulsion, hyperactivity, sedation, grooming, loss of righting reflex, respiratory depression or coma, were observed. This result may indicate that *B. longiflora* extract does not have any acute toxicity.

4. Conclusions

This is the first report of the biological property, bioactive constituents and the safety of *B. longiflora*. The study revealed that isolated compounds from *B. longiflora* rhizomes exert a strong anti-inflammatory effect through the inhibition of NO release. It is suggested that diarylheptanoids and some methoxyflavonoids isolated from this plant are responsible for the activity and might involve the suppression of iNOS gene. Further investigation on anti-inflammatory mechanism of the active compounds and the isolation of other constituents from *B. longiflora* is now in progress.

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