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Abstract

The current *in vitro* study was designed to investigate the anti-inflammatory, cytotoxic and antioxidant activities of boesenbergin A (BA), a chalcone derivative of known structure isolated from *Boesenbergia rotunda*. Human hepatocellular carcinoma (HepG2), colon adenocarcinoma (HT-29), non-small cell lung cancer (A549), prostate adenocarcinoma (PC3), and normal hepatic cells (WRL-68) were used to evaluate the cytotoxicity of BA using the MTT assay. The antioxidant activity of BA was assessed by the ORAC assay and compared to quercetin as a standard reference antioxidant. ORAC results are reported as the equivalent concentration of Trolox that produces the same level of antioxidant activity as the sample tested at 20 µg/mL. The toxic effect of BA on different cell types, reported as IC_{50}, yielded 20.22 ± 3.15, 10.69 ± 2.64, 20.31 ± 1.34, 94.10 ± 1.19, and 9.324 ± 0.24 µg/mL for A549, PC3, HepG2, HT-29, and WRL-68, respectively. BA displayed considerable antioxidant activity, when the results of ORAC assay were reported as Trolox equivalents. BA (20 µg/mL) and quercetin (5 µg/mL) were equivalent to a Trolox concentration of 11.91 ± 0.23 and 160.32 ± 2.75 µM, respectively. Moreover, the anti-inflammatory activity of BA was significant at 12.5 to 50 µg/mL and without any significant cytotoxicity for the murine macrophage cell line RAW 264.7 at 50 µg/mL. The significant biological activities observed in this study indicated that BA may be one of the agents responsible for the reported biological activities of *B. rotunda* crude extract.

Key words: *Boesenbergia rotunda*; Boesenbergin A; Cytotoxicity; Antioxidation; Anti-inflammatory

Introduction

Traditional Asian folk medicine practitioners utilize numerous herbal plants belonging to the Zingiberaceae family. They have been studied extensively for the last decade to identify its potential phytochemicals and their mechanism of action. Some of these herbs include *Renealmia alpinia* (1), *Zingiber zerumbet* (2) and *Kaempferia parviflora* (3). All of these plants have been subjected to rigorous bioassay-guided isolation and some of their compounds are currently used in clinical practice. *Boesenbergia rotunda* (L.) is a perennial herb locally known as "Krachai" in Thailand and "Temu Kunci" in Malaysia, which belongs to the family Zingiberaceae.

B. rotunda is the most abundant *Boesenbergia* species in Malaysia (4,5), which is botanically described as a small herbaceous plant with short, fleshy or slender rhizomes. The fresh rhizomes have a characteristic aroma and a slightly pungent taste (6,7) and are commonly used in Southeast Asia as food spices or condiments (8), as well as in folk medicine. Traditional healers have been using this plant for therapeutic purposes against inflammation, aphthous ulcer, dry mouth, stomach discomfort, dysentery, leukorrhea, oral diseases, cancers, and kidney disorders (9). The

rhizomes are given as tonics to women in mixtures after childbirth, are added to lotions for rheumatism and muscular pains, and to pastes for application to the body after confinement. In addition to being used for primary health care, the rhizomes have been reported to have aphrodisiac properties (4-7,10).

Regarding biological activity, *B. rotunda* exhibits antimutagenic, antibacterial, antifungal, analgesic, antipyretic, antispasmodic, anti-inflammatory, and insecticidal properties. The isolation of pinostrobin, 1,5-cineol, pinocembrin, chromene, panduratin C, panduratin A, uvangoletin, cardamonin, and alpinetin has been reported from the rhizomes of *B. rotunda* (5,11).

Chalcones are aromatic ketones that form the central core of a variety of important biological compounds. They contain two aromatic rings with an unsaturated chain. They show antibacterial, antifungal, chemopreventive, antiviral, antiprotozoal, insecticidal, anticancer, and anti-

Figure 1. Structure of boesenbergin A {(±)-(*E*)-1-[7'-hydroxy-5'-methoxy-2'-methyl-2'-(4''-methylpent-3''-enyl)-2'*H*-chromen-8'-yl]-3-phenylprop-2-enone} (top) and quercetin {2-(3,4-dihydroxy-phenyl)-3,5,7-trihydroxy-4*H*-chromen-4-one} (bottom).

inflammatory properties (10,12,13). Boesenbergin A (BA; (±)-(*E*)-1-[7'-hydroxy-5'-methoxy-2'-methyl-2'-(4''-methyl-pent-3''-enyl)-2'*H*-chromen-8'-yl]-3-phenylprop-2-enone) is a chalcone derivative, which was first isolated from *B. rotunda* by Jaipetch in 1982. The same investigator has also suggested a simple method for the synthesis of BA (11). Even though other chalcones have been described in terms of their various medicinal uses, BA has not been studied extensively. Thus, the current study was designed to characterize the anti-inflammatory, cytotoxic and antioxidant activities of BA.

Material and Methods

Isolation of BA

The rhizomes of *B. rotunda* were purchased from Puchong Market, Selangor Darul Ehsan, Malaysia. The material was identified by a botanist at the Faculty of Science, University Putra Malaysia, where a voucher specimen was deposited (BR-R11-01). One kilogram of finely ground air-dried rhizomes of *B. rotunda* was extracted three times with hexane for 72 h each at room temperature. The extract was subjected to flash column chromatography separation and eluted stepwise with various ratios of hexane, hexane/ethyl acetate, ethyl acetate, ethyl acetate/methanol and methanol, yielding 60 fractions. Fraction 19 yielded BA (Figure 1) as orange needle-shaped crystals ($C_{26}H_{28}O_4$) using hexane and ethyl acetate.

Characterization of BA was initially obtained as orange-colored oil and orange needle-shaped crystals were obtained after recrystallization in methanol. IR max (cm^{-1}, KBr disc): 3442 (OH), 2962, 1636 (C=O), 1590, 1546, 1446, 1344, 1152 (C-O). ^{1}H NMR (400 MHz, MeOD): d 14.23 (^{1}H, *s*, 7'-OH), 8.18 (^{1}H, *d*, *J* = 15.60 Hz, H-2), 7.78 (^{1}H, *d*, *J* = 8.00 Hz, H-3), 7.59 (^{2}H, *m*, H-2''', H-6'''), 7.39 (^{3}H, *m*, H-3''', H-4''', H-5'''), 6.62 (^{1}H, *d*, *J* = 10.12 Hz, H-4'), 6.05 (^{1}H, *s*, H-6'), 5.42 (^{1}H, *d*, *J* = 10.12 Hz, H-3'), 5.08 (^{1}H, *t*, *J* = 6.40 Hz, H-3''), 3.86 (^{3}H, *s*, 5'-OMe), 1.72-2.23 (^{4}H, *m*, H-1'', H-2''), 1.63 (^{3}H, *s*, 2'- Me), 1.46 (^{6}H, *d*, *J* = 7.32 Hz, 4''-Me, H-5''). ^{13}C NMR (100 MHz, MeOD): 192.8 (C-1), 167.4 (C-5'), 161.2 (C-8a'), 155.9 (C-7'), 142.2 (C-3), 135.6 (C-1'''), 132.1 (C-4''), 130.0 (C-4'''), 128.9 (C-3''', C-5'''), 128.3 (C-2''', C-6'''), 127.5 (C-2), 123.6 (C-3''), 123.4 (C-3'), 117.2 (C-4'), 106.1 (C-8'), 102.8 (C-4a'), 92.5 (C-6'), 77.3 (C-2'), 55.7 (C-5'-OMe), 41.4 (C-1''), 26.6 (C-2'-Me), 25.6 (C-4''-Me), 23.0 (C-2''), 17.5 (C-5''). MS (*m/z*, rel. int.): *m/z* 404 ([M$^+$], 20%), 389 ([M - CH$_3$]$^+$, 4), 321 ([M - C$_6$H$_5$]$^+$, 100), 217 (99), 77 (10), 55 (6).

The structure of BA was established on the basis of its spectral data using distortionless enhancement by polarization transfer, correlation spectroscopy, heteronuclear single quantum coherence, and heteronuclear multiple bond correlation in combination with ^{1}H and ^{13}C NMR spectroscopic data (data not shown). The purity of the compound was analyzed by UFLC using a Shimadzu UFLC system
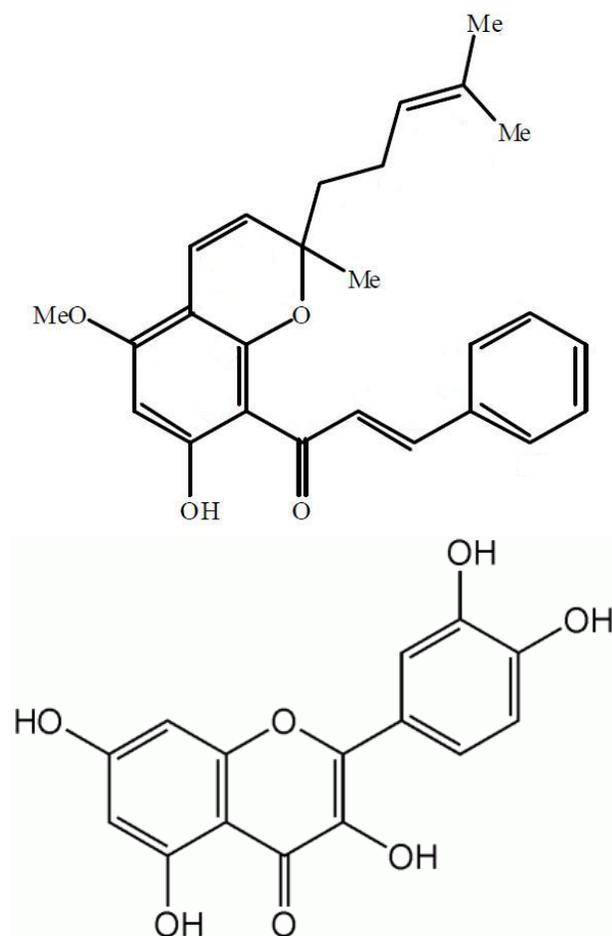
equipped with a PDA UV detector and Ion Trap TOF mass spectrometer. Column: Waters Xbridge 50 x 2.1 mm 2.5 μM; mobile phase: H_2O (0.1% formic acid):MeCN (0.1% formic acid); flow rate: 0.50 mL/min; column temperature: 40°C; Figure 2), and the compound was also identified by comparison of its spectral data to those reported in the literature (5,11).

MTT assay

Cell culture. All cells used in the present study were obtained from the American Type Cell Collection (ATCC) and maintained in an incubator at 37°C with 5% CO_2 saturation. Human hepatocellular carcinoma cells (HepG2), colon adenocarcinoma cells (HT-29), and normal hepatic cells (WRL-68) were maintained in Dulbecco's modified Eagle's medium (DMEM), whereas non-small cell lung cancer cells (A549) and prostate adenocarcinoma cells (PC3) were maintained in RPMI-1640 medium. Both media were supplemented with 10% fetal calf serum (FCS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

Cell viability. The different cell lines mentioned above were used to determine the inhibitory effect of BA on cell growth using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as previously described (14). This colorimetric assay is based on the conversion of the yellow tetrazolium bromide (MTT) to the purple formazan derivatives by mitochondrial succinate dehydrogenase in viable cells. For measurement of cell viability, cells were seeded at a density of 1 x 10^5 cells/mL in a 96-well plate and incubated for 24 h at 37°C and 5% CO_2. Cells were treated with BA, incubated for 24 h and the MTT solution at 2 mg/mL was then added for 1 h. Absorbance was measured at 570 nm. Data are reported as percent of control thus providing percent cell viability after 24-h exposure to the test agent. The potency of cell growth inhibition by BA is reported as an IC_{50} value, defined as the concentration that caused a 50% loss of cell growth. Viability was defined as the ratio (expressed as percent) of absorbance of treated cells to untreated cells.

ORAC antioxidant activity assay

The oxygen radical absorbance capacity (ORAC) assay was carried out by the procedure described by Cao et al. (15). The ORAC assay is the only method that takes free radical action to completion and uses an area under the fluorescein decay curve (AUC) technique for quantitation, thus combining percent inhibition and the time of inhibition of free radical action by antioxidants into a single quantity (16). Briefly, 175 μL of the sample/blank was dissolved in PBS at concentrations of 160 μg/mL (pH 7.4, 75 mM) and serial dilutions for the Trolox standards were prepared accordingly. The ORAC assay was performed in a 96-well black microplate with 25 μL samples/standard/positive control and 150 μL fluorescence sodium salt solution, followed by 25 μL 2,20-azobis (2-aminopropane) dihydrochloride
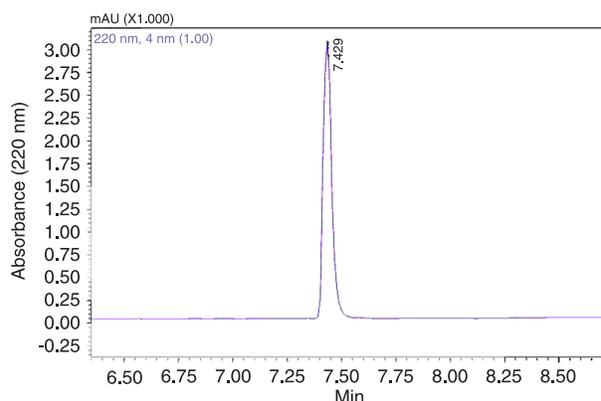
Figure 2. Chromatogram of boesenbergin A at 220 nm. Analysis was performed using a Shimadzu UFLC system equipped with a PDA UV detector and Ion Trap TOF mass spectrometer. Column: Waters Xbridge 50 x 2.1 mm 2.5 μM. Mobile phase: H_2O (0.1% formic acid):MeCN (0.1% formic acid). Flow rate: 0.50 mL/min. Column temperature: 40°C. Gradient: 10-100% MeCN over 7 min.

(AAPH) solution after a 45-min incubation at 37°C (200 μL total well volume). Fluorescence was recorded until a zero value was obtained (excitation wavelength at 485 nm, while emission wavelength at 535 nm) in a fluorescence spectrophotometer (Perkin-Elmer LS 55), equipped with an automatic thermostatic autocell holder at 37°C. The positive control was quercetin and the negative control was blank solvent/PBS. Data were collected every 2 min for a period of 2 h. Results were calculated using the differences of the AUC between the blank and the sample and are reported as Trolox equivalents.

Anti-inflammatory assay

Chemicals. The following reagents were obtained commercially: antibiotics (5000 U/mL penicillin and 5000 μg/mL streptomycin) and DMEM from Flowlab™, Australia; fetal bovine serum (FBS) from iDNA Technologies Inc., Singapore; recombinant mouse interferon-γ (IFN-γ) from eBioscience Inc., USA; lipopolysaccharide (LPS) from *Escherichia coli* (strain 055:B5), sulfanylamide, N-(1-naphthyl)-ethylenediamine and diphenylpicrylhydrazine from Sigma, USA; MTT from Fluka Chemie GmbH, Switzerland.

Cell culture and stimulation. The murine monocytic macrophage cell line (RAW 264.7) was purchased from the European Collection of Cell Cultures (Porton Down, UK) and maintained in DMEM supplemented with 10% FBS, 4.5 g/L glucose, 1 mM sodium piruvate, 2 mM L-glutamine, 50 μg/mL streptomycin, and 50 U/mL penicillin at 37°C and 5% CO_2. Cells at 80-90% confluence were centrifuged at 120 *g* at 4°C for 10 min and cell concentration was adjusted to 2 x 10^6 cells/mL, with cell viability being always more than 90%, as determined by the Trypan blue exclusion assay. Fifty microliters of cell suspension was seeded in a tissue

culture grade 96-well plate (4 x 10^5 cells/well) and incubated for 2 h at 37°C and 5% CO_2 for cell attachment. The cells were then stimulated using 100 U/mL IFN-γ and 5 µg/mL LPS with or without the presence of BA at a final volume of 100 µL/well. DMSO was used as vehicle to increase the solubility of the sample tested in the culture medium. The final concentration of DMSO was maintained at 0.1% in all cultures. Cells were further incubated at 37°C and 5% CO_2 for 17-20 h. The culture supernatant was subjected to the Griess assay for nitrite determination and the cells remaining in the well were tested for cell viability using the MTT reagent. L-NAME as a positive control (Sigma Aldrich, USA) was used at a concentration of 250 µM.

Nitrite determination using the Griess assay. To evaluate the inhibitory activity of BA on nitric oxide (NO) production, the culture medium was assayed by the Griess reaction. Briefly, an equal volume of Griess reagent (1% sulfanylamide and 0.1% N-(l-naphthyl)-ethylenediamine dihydrochloride dissolved in 2.5% H_3PO_4) was mixed with the culture supernatant and color development was measured at 550 nm using a microplate reader (SpectraMax Plus, Molecular Devices Inc., USA). The amount of nitrite in the culture supernatant was calculated from a standard curve (0-100 µM) of sodium nitrite freshly prepared in deionized water. Percent NO inhibition was calculated using the nitrate level of the IFN-γ/LPS-induced group as control.

RAW 264.7 cell viability. The cytotoxicity of BA on the macrophage cell line RAW 264.7 was determined by assaying the reduction of MTT reagents to formazan salts. After removing the supernatant, the MTT reagents (2 mg/mL dissolved in sterile PBS, pH 7.0) were added to each

well. The remaining cells were incubated at 37°C for 4 h and the formazan salts formed were dissolved by adding 100 µL 100% DMSO to each well. The absorbance was then measured at 570 nm using a SpectraMax Plus microplate reader (Molecular Devices). Percent cell viability was calculated using the cell viability of the IFN-γ/LPS-induced group as control.

Statistical analysis

Data are reported as means ± SD of three replicates. The independent *t*-test and ANOVA were used for comparisons with $P < 0.05$ considered to be significant. All statistical analyses were performed using the SPSS software (Release 18).

## Results

Cytotoxic activity

Cell viability was determined by the MTT assay. BA induced cytotoxicity in a concentration-dependent manner, as shown in Figure 3. These concentration titration curves allowed us to determine the IC_{50} of BA towards different cell lines (Table 1). The results of the MTT assay showed that BA had different degrees of cytotoxicity on the tested cells, with IC_{50} values ranging from 9.32 to 94.10 µg/mL (Table 1). PC3 and WRL-68 were the most sensitive cells to BA. These results indicate that cell lines differ in their sensitivity to BA. Paclitaxel (Sigma-Aldrich) was used as positive control and presented an IC_{50} value as low as 1% of that obtained with BA. DMSO (0.1%) was used as vehicle control and did not show any sign of toxicity.

ORAC antioxidant activity assay

The antioxidant capacity of BA was measured by ORAC assay and the potency of the natural compound was compared with that of the positive control, quercetin. The AUC was calculated for BA, Trolox and quercetin. The ORAC results are shown in Table 2. BA displayed considerable antioxidant activity. At 20 µg/mL, this compound was equivalent to a 11.91 ± 0.23 µM Trolox. Quercetin at 5 µg/mL is equivalent to a Trolox concentration of 160.32 ± 2.75 µM.

Effect of BA on NO_2^- production and RAW cell viability

The induction of RAW 264.7 cells into an inflammatory state by treatment with LPS/IFN-γ caused synthesis and secretion of NO. The breakdown product of secreted NO, namely NO_2^-, was detected in the medium at a mean concentration of 36.68 ± 2.95 µM. Cells that were not induced released trace amounts of NO. The results clearly showed a concentration-dependent decrease in NO production in the induced cells, with a significantly low level being evident even at 12.5, 25, and 50 µM concentrations, with a level of 30.90 ± 3.1, 29.69 ± 2.5, and 25.69 ± 2.0, respectively (Figure 4). The cell viability assay carried out on the mouse
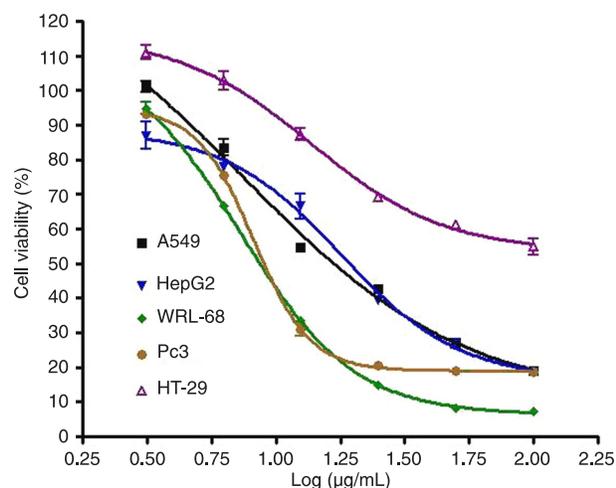
Figure 3. Dose-response curves of the cytotoxicity of boesenbergin A. Each value represents the mean ± SD. Human hepatocellular carcinoma (HepG2), colon adenocarcinoma (HT-29), non-small cell lung cancer (A549), prostate adenocarcinoma (PC3), and normal hepatic cells (WRL-68) were used to evaluate the cytotoxicity of boesenbergin A by the MTT assay.

leukemic monocyte macrophage cell line (RAW 264.7) showed no sign of toxicity up to the maximum concentration (50 µM) used in this study. L-NAME (250 µM) induced a significant decrease in NO production.

Discussion

The results revealed that BA had different degrees of cytotoxicity (Table 1). It has been reported that panduratin A, a chalcone derivative isolated from *B. pandurata*, inhibited the growth of MCF-7 and HT-29 cancer cells with an IC_{50} of 3.75 and 6.56 µg/mL, respectively (17). In contrast, BA exhibited high toxicity against normal hepatic cells (WRL-68) with an IC_{50} value of 9.3 µM, this being the main disadvantage of the compound. A major problem of the use of chemopreventing agents in cancer treatment is the potential toxicity of these drugs to normal cells (18). Another normal cell line (RAW 264.7) employed in the present study did not exhibit any significant signs of toxic effects. The rate of drug metabolism is higher in liver cells than in other organs (19). The variation of the toxicity profile of BA in normal cell lines may be due to the characteristics of the WRL cell line, which, as a liver cell line, may metabolize the active compound to more toxic metabolites. On the other hand, BA showed remarkable anti-tumor activity against PC3, non-small cell lung cancer cells, with an IC_{50} value of 10.69 ± 2.64 µg/mL.

The ORAC assay is unique in that it is an ROS generator, and AAPH produces a peroxyl-free radical upon thermal decomposition that is commonly found in the body, making the reaction biological relevant. Furthermore, since AAPH reacts with both water- and lipid-soluble substances it can be used to measure the total antioxidant potential. Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid), a vitamin E analogue and a known antioxidant, is used as the standard to which all unknown antioxidants are compared. The antioxidant activity of BA and quercetin was tested using the ORAC assay and the results were reported as Trolox equivalents. BA (20 µg/mL) and quercetin (5 µg/mL) were equivalent to a concentration of 11.91 ± 0.23 and 160.32 ± 2.75 µM Trolox, respectively.

BA has one free aromatic hydroxyl group in its structure (Figure 1). Obviously, the aromatic hydroxyl group is very important for the antioxidative effects of the compounds (20). Interestingly, chalcones with only two adjacent hydroxyl groups were almost fully effective.

It is interesting to note that in the present study BA, like other chalcones which have been reported to be anti-inflammatory agents, was found to have a significant anti-inflammatory effect which was much more evident 50 and 25 µM concentrations of the compound. The same BA showed significant antioxidant property. It has been previously documented that compounds with antioxidant proper-

**Table 1.** Toxicity of boesenbergin A (BA) on different cell types.

Cell line	Cancer of origin	IC$_{50}$ ± SD (µg/mL)	
		BA	Paclitaxel
A549	Non-small cell lung cancer	20.22 ± 3.15[b]	5.81 ± 1.03
PC3	Prostate adenocarcinoma	10.69 ± 2.64[a]	0.08 ± 0.03
HepG2	Human hepatocellular carcinoma	20.31 ± 1.34[b]	1.18 ± 0.24
HT-29	Human colon adenocarcinoma	94.10 ± 1.19[c]	0.06 ± 0.02
WRL-68	Normal hepatic cells	9.32 ± 0.24[a]	0.10 ± 0.05

IC_{50} values were obtained with the MTT assay. Data are reported as means ± SD for measurements in triplicate. Means followed by different superscript letters differed significantly. Data were analyzed statistically by one-way ANOVA and the Tukey *post hoc* test (P < 0.05).

Table 2. Oxygen radical absorbance capacity (ORAC) assay.

Sample	Net AUC	Equivalent concentration of Trolox at 20 µg/mL (µM)
Boesenbergin A	2.17	11.91 ± 0.23
Quercetin	21.98	160.32 ± 2.75*

The net AUC was calculated by subtracting the blank AUC from the AUC of each sample, the standards, and the positive control. Final ORAC values are reported as the equivalent concentration of Trolox that produces the same level of antioxidant activity as the samples at 20 µg/mL. AUC = area under curve. *P < 0.05, quercetin compared to boesenbergin A (*t*-test).
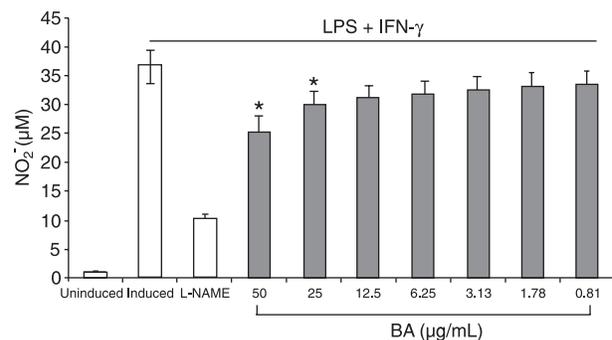
Figure 4. Effect of boesenbergin A (BA) on nitric oxide (NO) inhibition in RAW cells. Cells were pretreated for 24 h with the indicated doses of BA and stimulated with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) to activate NO production. L-NAME (Sigma Aldrich, USA) was used as a positive control at a concentration of 250 µM. Data are reported as the average of three experiments. Differences between induced and treatment were analyzed by one-way ANOVA and the Tukey *post hoc* test (*P < 0.05).

ties could be expected to have anti-inflammatory activity (21,22). In addition, the development of cyclooxygenase- and lipoxygenase-mediated proinflammatory intermediates from arachidonic acid is closely related to the participation of ROS (23), and has led to many investigations of the anti-inflammatory activity of antioxidant compounds (24). In agreement with this theory, BA could be considered as a chalcone with multiple pharmacological properties.

The significant anticancer and anti-inflammatory effect of BA was clearly shown in the present investigation. This finding confirms that there is a close relationship between inflammation and cancer (25). Numerous experimental, epidemiologic, and clinical studies have suggested that nonsteroidal anti-inflammatory agents, particularly the highly selective cyclooxygenase (COX)-2 inhibitors, are promising anticancer agents (26). A closely related compound,

panduratin A, which is also a chalcone isolated from Boesenbergia species has shown inhibition of COX-2 enzymes (17). Therefore, the anti-inflammatory and anti-cancer effect of BA observed in the present study can be considered to parallel the properties of panduratin A. Additionally, inflammatory cells are quite capable of producing genotoxic effect in the neighboring cells, and therefore may lead to tumor progression (27). A study by Lundholm et al. (28) clearly reported that anti-inflammatory drugs administered to cancer patients not only can increase the pain threshold, but can also prolong survival time.

Acknowledgments

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