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Anti-inflammatory and Cytotoxic Activities of Phenolic Compounds from *Broussonetia kazinoki*

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Abstract – The phytochemical investigation of *Broussonetia kazinoki* roots led to the isolation of ten compounds, including six flavonoids (**1**–**6**), two lignans (**7** and **8**), and two coumarins (**9** and **10**) by comparing their ^{1}H and ^{13}C NMR spectra with reference values. To the best of our knowledge, compounds **9** and **10** were isolated from this plant for the first time. Among the ten isolates, compounds **2**, **4**, and **6** exhibited inhibitory effects against lipopolysaccharide (LPS)-induced nitric oxide (NO) production in macrophage RAW264.7 cells with IC_{50} values of 11.98, 10.16, and 24.06 μM, respectively. Furthermore, compounds **2**, **4**, and **6** reduced LPS-induced inducible nitric oxide synthase (iNOS) expression in a dose-dependent manner. Pre-incubation of cells with these compounds also significantly suppressed LPS-induced COX-2 protein expression. Compounds **2**, **4**, and **6** also showed cytotoxic activity against HL-60 cells with IC_{50} values ranging between 46.43 and 94.06 μM.
Keywords – *Broussonetia kazinoki*, Moraceae, flavonoid, lignan, anti-inflammatory activity, cytotoxic activity

Introduction

Inflammation, a critical host immune response to irritation or infection, contributes to the initiation and progression of many diseases.[1] In the process of inflammation, mediators and pro-inflammatory cytokine play important roles with the presence of interleukin 1β (IL-1β), interleukin 6 (IL-6), nitric oxide (NO), nitric oxide synthase (iNOS), and cyclooxygenase (COX).[2] However, the overproduction of NO, which is generated by activated inflammatory cells like mononuclear phagocytes, neutrophils, macrophages, and eosinophils in the inflammation process, is the cause of inflammatory diseases such as autoimmune diseases and rheumatoid arthritis.[3,4] NO is also involved in many pathological and physiological processes in both chronic and acute inflammatory disorders as a critical cellular signaling molecule.[5] Thus, the inhibition of NO production by natural products has been investigated to discover new anti-inflammatory drugs.

Cancer is a generic term for a set of diseases characterized by the presence of abnormal cells that multiply rapidly beyond their normal limits, causing them to invade other tissues and organs.[6] The disease states bearing relevance to cancer or a cancer symptom can be reflected by ethnopharmacological usages such as inflammatory, skin disorders, parasitic, and infectious.[7] Treatment of metastatic cancer mainly relies on chemotherapeutic agents which kill tumor cells in several ways such as interfering with cell division to stop cell growing and dividing or weakening cell membrane that makes cell explodes.[8] Plant is a potential source for discovering new anticancer agents since it has provided many effective anticancer drugs in current use such as irinotecan, vinblastine, taxanes, vincristine, etc.[7]

Broussonetia kazinoki, a member of the Moraceae family, which grows naturally in Korea, China, and Japan, has long been employed in folk medicine as a diuretic, a suppressant of edema, and as a tonic to treat various conditions.[9] According to the *Flora of China*, the inner bark fiber is used as a raw material for producing paper, and the wood is used for furniture.[10] Previous phytochemical investigations of *B. kazinoki* reported the isolation of several active compounds including flavonoids, alkaloids, and 1,3-diphenylpropanes, which have been demonstrated to exert anti-cancer, anti-inflammatory, and depigmenting effects.[10,11] In our search for biologically active compounds from *B. kazinoki*, ten compounds were isolated from the methanol extract of the roots. All

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isolates were evaluated for anti-inflammatory and cytotoxic activities against three human cancer cell lines (Hela, HL-60, and MCF-7).

Experimental

General experiment procedures – IR and UV spectra were measured by a JASCO FT/IR-4100 spectrometer and a J-1500 circular dichroism spectrophotometer, respectively. NMR spectra were measured by Varian Unity Inova 400 MHz spectrometers. Preparative high-performance liquid chromatography (HPLC) was carried out on a YMC Pak ODS-A column (20 × 250 mm, 5 μm particle size; YMC Co., Ltd., Japan) and a Waters 2487 controller system with a UV detector (UV/VIS - 156). Merck precoated silica gel F_{254} and RP-C18 F_{254s} plates were used to perform thin-layer chromatography (TLC). Column chromatography (CC) was performed using silica gel 60 (Merck, 230–400 mesh) and RP-C18 (Merck, 75 mesh).

Plant materials – The roots of *B. kazinoki* were collected in May 2018, at Noeun-dong of Daejeon, Korea, and identified by Professor Byung Sun Min (one of the authors). A voucher specimen (CUD-1142-1) was deposited at the Herbarium of the College of Pharmacy, Daegu Catholic University, Korea.

Extraction and isolation – The air-dried roots of *B. kazinoki* (7 kg) were extracted using MeOH (15 L × 3). The combined extracts were concentrated to obtain a crude extract (330 g), which was suspended in water (0.5 L) and successively partitioned with hexane, CH_2Cl_2 (MC), EtOAc, and BuOH. The MC fraction (45 g) was chromatographed on a silica gel column, eluting with a gradient mixture of hexane:acetone (10:1–0:1, v/v) to obtain four fractions (M1–M4). Fraction M4 (6 g) was eluted on a silica gel column (CH_2Cl_2:MeOH, 5:1, v/v) to yield six subfractions (M4.1–M4.6). Subfraction M4.2 (750 mg) was separated by Sephadex LH-20 to yield **1** (8 mg), **2** (4 mg), and **3** (5 mg), respectively. Subfraction M4.5 (200 mg) was fractionated using a RP-C18 silica gel column (MeOH:H_2O, 1:1, v/v) to obtain **7** (5 mg).

The EtOAc-soluble fraction (32 g) was subjected to silica gel chromatography (CH_2Cl_2:MeOH, 20:1–1:5, v/v) to generate five fractions (E1–E5). Fraction E2 (1 g) was purified using RP-C18 silica gel CC (MeOH:H_2O, 2:1, v/v) to afford **4** (4 mg), **5** (25 mg), and **6** (4 mg), respectively. Fraction E4 (8 g) was fractionated by silica gel column (CH_2Cl_2:MeOH, 2:1, v/v) to produce 8 subfractions (E4.1–E4.8). Subfraction E4.3 (350 mg) was separated using a RP-C18 silica gel column (MeOH:H_2O, 2:1, v/v) to obtain **8** (4 mg). Subfraction E4.5 (230 mg) was purified by HPLC (MeOH:H_2O, 60:40, v/v, flow rate: 5 mL/min) to obtain **9** (11 mg, t_R 17.5 min) and **10** (6 mg, t_R 25.5 min), respectively.

Determination of nitric oxide (NO) production and cell viability – The RAW264.7 cells were stimulated with or without 1 μg/mL of LPS (Sigma Chemical Co.) for 24 h in the presence or absence of the test compounds (10 μM). The cell culture supernatant (100 μL) was then reacted with 100 μL of Griess reagent (0.1% naphthyl ethylenediamine dihydrochloride in distilled water and 1% sulfanilamide in 5% phosphoric acid). Cell viability was measured with an MTT-based colorimetric assay.[9]

Western blot analysis – 50 μg of protein extracted from cells in ice-cold lysis buffer (1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 1 μg/mL leupeptin, 1 mM sodium vanadate, 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl) was separated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and followed by transferring to a polyvinylidene difluoride membrane. The membrane was incubated with the corresponding antibody after blocking with 5% skim milk. The antibodies against COX-2 and iNOS were obtained from Santa Cruz Biotechnology and the antibody for tubulin was purchased from Sigma-Aldrich. Proteins were visualized by enhanced chemiluminescence after binding of an appropriate secondary antibody coupled to horseradish peroxidase according to the instructions of the manufacturer.[9]

Cytotoxic activity – HL-60, MCF-7, and Hela cancer cells were cultured in Dulbecco's modified Eagle's medium/F-12 with L-glutamine, 15mM HEPES buffer, and pyridoxine hydrochloride with 1% penicillin-streptomycin and 10% fetal bovine serum in a 96-well plate at a density of 6×10^4 cells/mL. The cells were treated with the isolated compounds after reaching confluence (2×10^5 cells/mL). The compounds were dissolved in DMSO and made at various concentrations. The experiment proceeded at 37 °C in a humidified 5% CO_2 atmosphere for 48 h. Supernatants were discarded at the end of this period. Dulbecco's phosphate buffered saline was used to wash the adherent cells, and then each well was added with 20 μL of MTT stock solution (5 mg/mL), and the plates were incubated at 37 °C for 3 h. Each well was added with 100 μL DMSO to solubilize the water-insoluble purple formazan crystals. After 1 h, the absorbance was measured by a microplate reader at 570 nm. Doxorubicin was used as a positive control. The 50% reduction in cell number relative to the control (IC_{50}) was estimated visually. The results are presented as mean ± standard error of the mean (SEM).[10]

Statistical analysis – The data were described as the mean ± standard error of mean (SEM). Two-tailed unpaired student's t-test performed the statistical analyses and $p < 0.01$ was regarded statistically significant.

Results and Discussion

In an additional search for interesting chemical substances and bioactive compounds from nature, we carried on a phytochemical investigation on the roots of *B. kazinoki*. The crude hexane, CH_2Cl_2, EtOAc, and BuOH extracts from the roots of *B. kazinoki* were evaluated for their cytotoxic and anti-inflammatory activities to choose the fractions for further isolations. The CH_2Cl_2 and EtOAc extracts significantly inhibited NO production with IC_{50} values of 23.34 and 24.73 μg/mL, respectively (Fig. 1). The hexane and BuOH extracts exhibited weak and no activity against NO production, respectively (Fig. 1). In the cytotoxic activity assay, the CH_2Cl_2 and EtOAc extracts induced the cell death of HeLa cells (IC_{50} = 82.79 and 97.25 μg/mL, respectively) and HL-60 cells (IC_{50} = 69.77 and 73.56 μg/mL, respectively), whereas the hexane extract showed weak activity (IC_{50} = 91.54 μg/mL) against only HeLa cells (Table 1). The BuOH extract had no

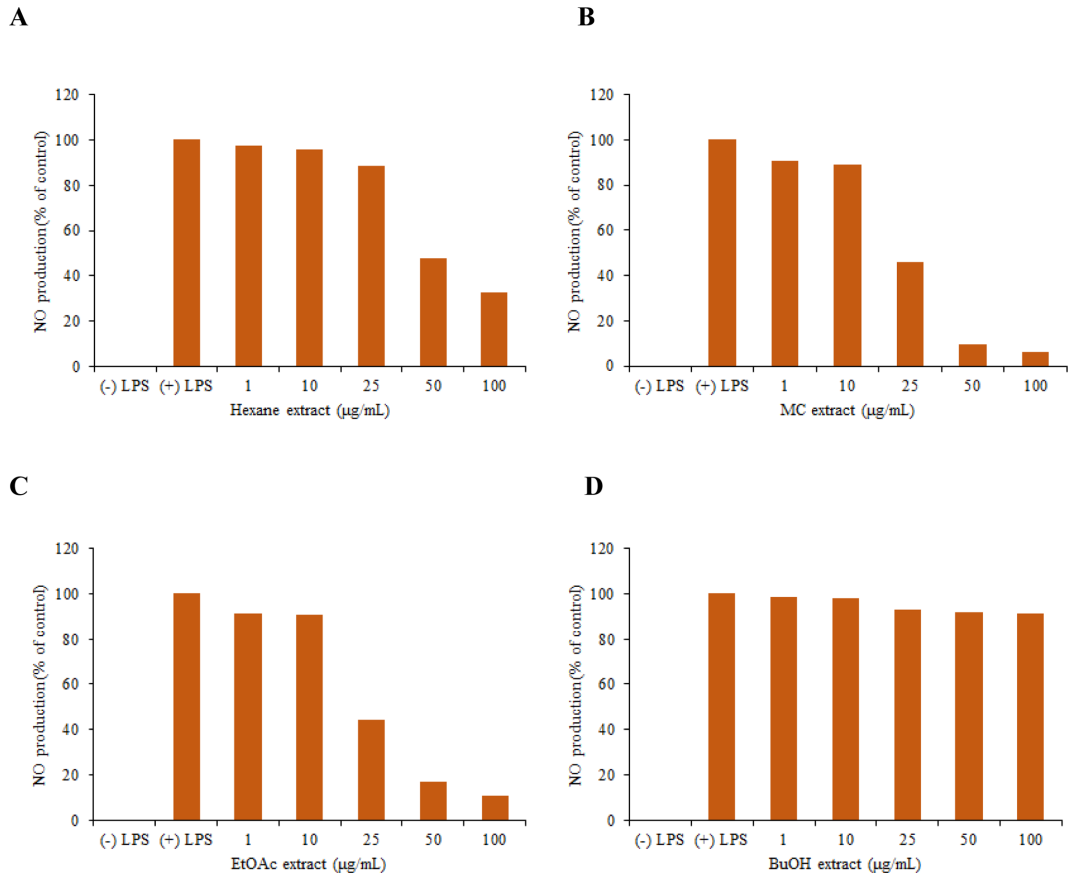

Fig. 1. The anti-inflammatory effects of hexane (**A**), MC (**B**), EtOAc (**C**), and BuOH (**D**) extracts from *B. kazinoki* on LPS-stimulated inflammation in RAW264.7 cells through the measurement of accumulated nitrite.

Table 1. Cytotoxic activity of four extracts against Hela, HL-60, and MCF-7 cell lines

Extracts	IC_{50} (μg/mL)[a]		
	Hela	HL-60	MCF-7
hexane	91.54 ± 0.14	>100	>100
MC	82.79 ± 0.86	69.77 ± 2.48	>100
EtOAc	97.25 ± 0.27	73.56 ± 0.25	>100
BuOH	>100	>100	>100

[a]These data are expressed as the mean ± SEM of triplicate experiments.

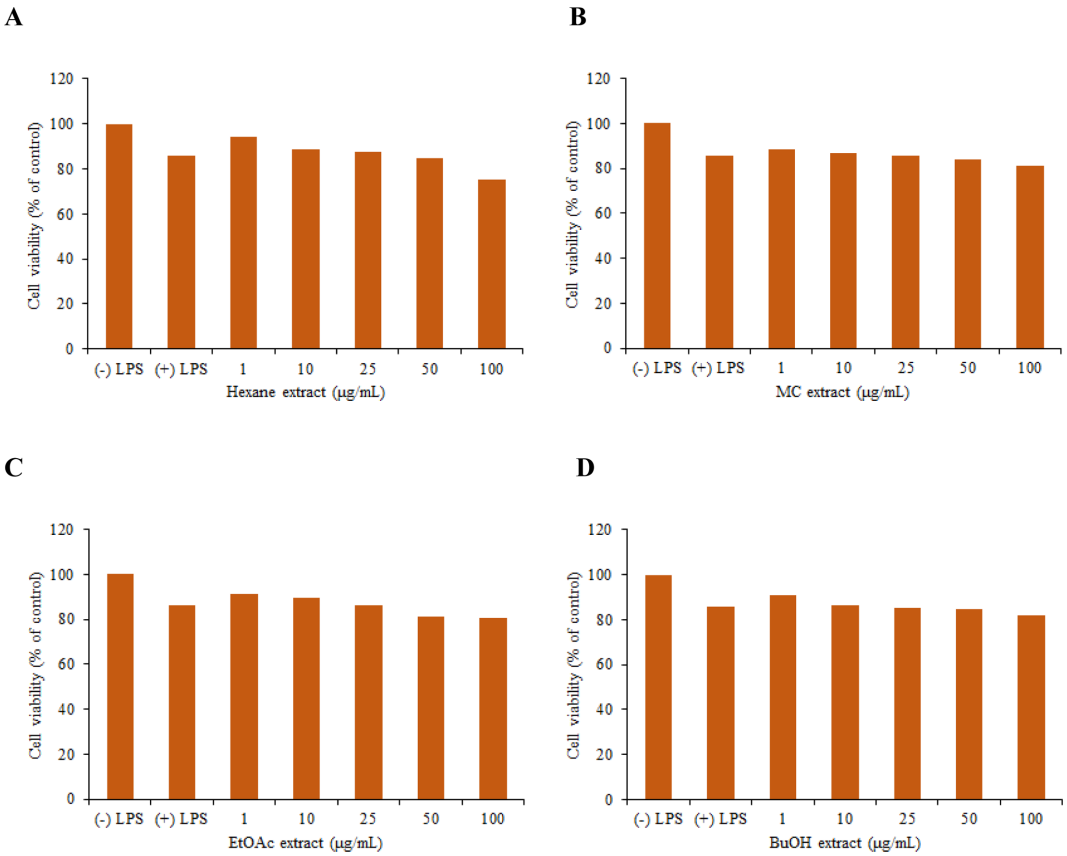

Fig. 2. The cytotoxicity of hexane (**A**), MC (**B**), EtOAc (**C**), and BuOH (**D**) extracts from *B. kazinoki* on LPS-stimulated inflammation in RAW264.7 cells.

activity in any of the three cancer cell lines (Table 1). After 24 hours, cell viability was examined by the MTT assay, which indicated that all extracts did not induce RAW264.7 cell death (Fig. 2). The NO inhibition, cytotoxic activity, and cell viability assay results made available important preliminary data for selecting the MC and EtOAc extracts for further phytochemical studies.

In our efforts to characterize the compounds responsible for the cytotoxic and anti-inflammatory effects, the chemical investigation of *B. kazinoki* roots using column chromatography led to the isolation of ten compounds, which were determined to be hesperetin (**1**),[12] eriodictyol (**2**),[13] dihydrokaempferol (**3**),[14] apigenin (**4**),[15] chrysoeriol (**5**),[16] kaempferol (**6**),[17] pinoresinol (**7**),[18] syringaresinol-4-O-β-D-glucopyranoside (**8**),[19] 3'-hydroxymarmesin-1'-O-β-glucopyranosyl (**9**),[20] and marmesinin (**10**)[20] based on spectroscopic and physical data compared to the previously reported data (Fig. 3). Among them, compounds **9** and **10** were isolated from this plant for the first time.

All the isolated compounds (**1**–**10**) were assessed for anti-inflammatory effects on the LPS-induced production of NO in RAW264.7 cells. Quercetin was used as a positive control with an IC_{50} of 35.12 ± 0.89 μM. The rates of NO production inhibition are shown in Table 2. All isolates were also tested for their cytotoxicity against RAW 264.7 cells by the MTT assay to determine whether the inhibitory effect of these compounds was related to toxicity. Based on the bioassay results, it was concluded that compounds **2**, **4**, **5**, and **6** possessed stronger anti-inflammatory activity compared to that of the positive control, which was concentration-dependent, with IC_{50} values ranging from 10.16 to 24.06 μM, whereas the other compounds show no activity (Table 2). Compound **5** also showed mild cytotoxicity (IC_{50} = 82.53 μM), suggesting that the anti-inflammatory effect of **5** might be due to its toxicity. Compounds **2**, **4**, and **6** did not decrease cell viability, indicating that the inhibitory effect on NO production was not mediated by cytotoxicity. Compound **6**, with one more hydroxy group than **4**, exhibited less potent activity, implying that the attachment of a hydroxy group at C-3 could influence NO production. Although both **1** and **2** possessed a flavanone skeleton, **1** with C-4' methoxylation was inactive, whereas **2** showed strong activity, indicating that the methoxy group at C-4' played

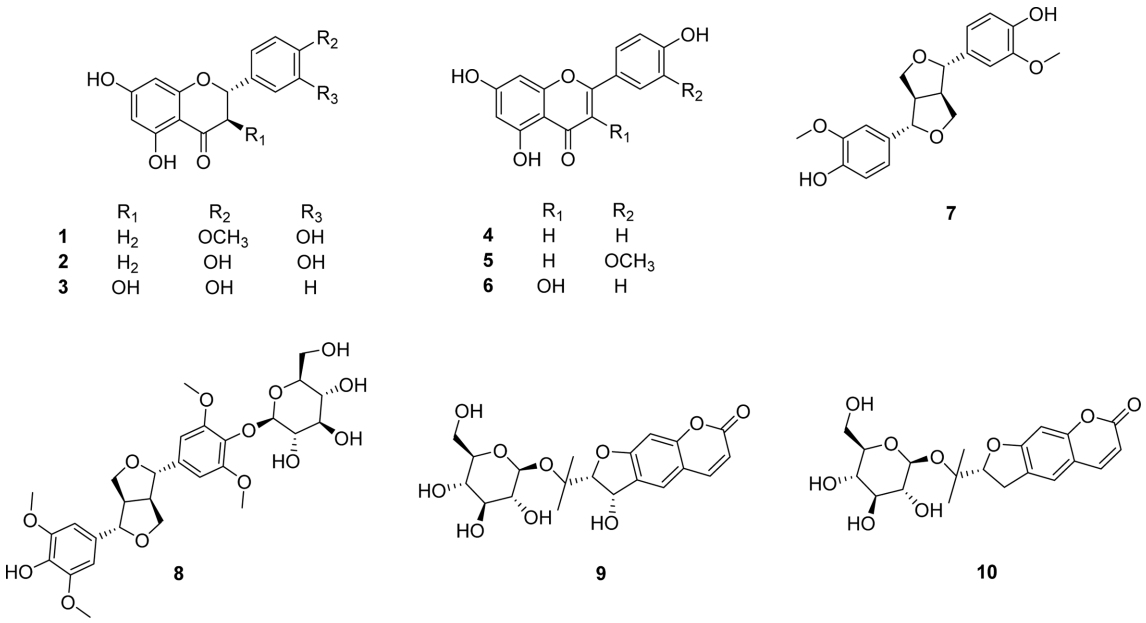

Fig. 3. Chemical structures of compounds **1**–**10**.

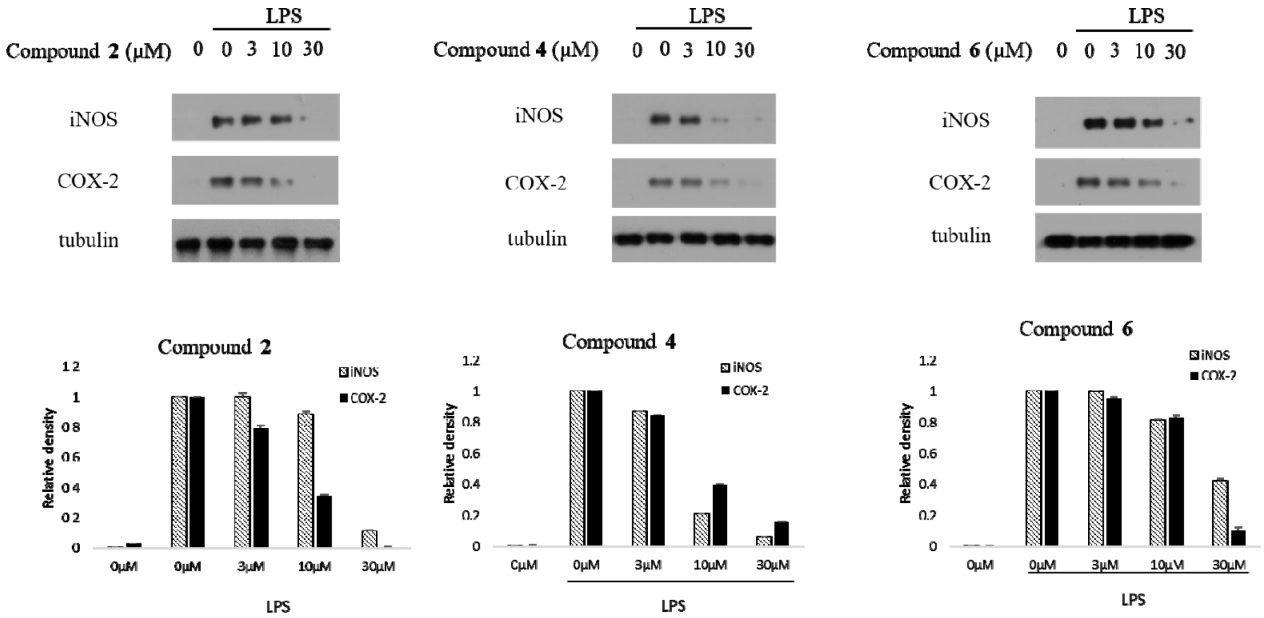

Fig. 4. Effects of compounds **2**, **4**, and **6** on the LPS-induced iNOS and COX-2 protein levels in RAW264.7 cells. (A) The cells were sampled and lysed following treatment for 18 h, and the protein levels of iNOS and COX-2 were determined by western blot analysis. Tubulin was used as the internal control. (B) Data analysis was performed using ImageJ software (ImageJ) by measuring the integrated band densities following background subtraction.

an important role in anti-inflammatory activity.

Because compounds **2**, **4**, and **6** exhibited strong inhibitory effects on the LPS-induced production of NO in macrophage RAW264.7 cells, we then investigated the inhibitory effects of these compounds on the expression levels of iNOS and COX-2. RAW264.7 cells were stimulated with 1 μg/mL of LPS for 18 h in the presence of increasing concentrations of these compounds, and the expression levels of iNOS and COX-2 were determined by Western blot analyses. In unstimulated RAW264.7 cells, the iNOS and COX-2 protein levels were undetectable but were significantly elevated after treatment with LPS. As shown in Figure 4, all three compounds dose-dependently reduced the LPS-induced iNOS expression

Table 2. Cytotoxic activity against Hela, HL-60, and MCF-7 and inhibitory effects on NO production of compounds **1**–**10**

Compounds	IC_{50} (μM)[a]				
	Anticancer			Anti-inflammatory	
	Hela	HL-60	MCF-7	NO inhibition	Cell viability
1	>100	>100	>100	>100	>100
2	>100	93.24 ± 2.58	>100	11.98 ± 0.23	>100
3	>100	>100	>100	>100	>100
4	49.26 ± 0.02	72.49 ± 0.24	>100	10.16 ± 0.65	>100
5	>100	46.43 ± 1.02	>100	17.09 ± 0.80	82.53 ± 0.95
6	>100	94.06 ± 2.75	>100	24.06 ± 0.29	>100
7	>100	>100	>100	>100	>100
8	>100	>100	>100	>100	>100
9	>100	>100	>100	>100	>100
10	>100	>100	>100	>100	>100
Doxorubicin[b]	2.25 ± 0.41	0.23 ± 0.01	17.03 ± 0.92	–	–
Quercetin[c]	–	–	–	35.12 ± 0.89	>100

[a]These data are expressed as the mean ± SEM of triplicate experiments.
[b]Positive controls for cytotoxic assay.
[c]Positive controls for NO inhibition assay.

but had no effect on tubulin expression. The results demonstrated that compounds **2**, **4**, and **6** inhibited iNOS activity in LPS stimulated RAW264.7 cells, implying that these compounds could suppress LPS-induced iNOS expressions at the transcriptional level. Moreover, pre-incubation of cells with these compounds greatly suppressed LPS-induced COX-2 protein production. At the same concentration, compounds **4** and **6** displayed equivalent suppressions of iNOS and COX-2 protein expression. However, at a concentration of 30 μM, compound **6** strongly suppressed COX-2 protein expression compared to that of iNOS. Compound **2** significantly inhibited COX-2 protein expression compared to that of iNOS at all three treatment concentrations. Especially, at a concentration of 30 μM, it showed approximately 100% relative intensity inhibition of COX-2 protein expression.

The family of nitric oxide synthases (NOS) includes neuronal NOS (nNOS), endothelial NOS (eNOS), and iNOS. Various studies have shown that NOS activity was related to proliferation rates, tumor grade, and the expression of critical signaling components associated with cancer progression in tumor cells of varied histogenetic origins.[21] High levels of NOS expression appear to be cytotoxic or cytostatic for tumor cells, whereas low NOS activity can have the reverse effect and stimulate tumor growth.[21] Thus, compounds that can inhibit NOS expression and possess cytotoxic effects against cancer cell lines are potential drugs for treating inflammation in cancer patients. In our study, ten isolated compounds were further examined for their cytotoxic effects against three cancer cell lines MCF-7, Hela, and HL-60. Doxorubicin was used as a positive control. As shown in Table 2, in the presence of the tested compounds, HL-60 cells were more sensitive than the two other cell lines. All compounds were inactive against the three cancer cell lines except for four compounds **2**, **4**, **5**, and **6**, which showed weak activity against HL-60 cells with IC_{50} values ranging from 46.43 to 94.06 μM and compound **4**, which was cytotoxic against Hela cells (IC_{50} = 49.26 μM). The flavone skeleton (compounds **4** and **5)** exhibited stronger activity than **1** and **2** (flavanone skeleton), **3** (flavanonol skeleton), and **6** (flavonol skeleton), suggesting that the flavone skeleton might be important for cytotoxic activity. The methoxy group in **5** increased its cytotoxic effects, with IC_{50} values lower than those of **4**, indicating that the addition of a methoxy group positively influenced this activity.

In conclusion, ten compounds were isolated from the roots of *B. kazinoki*. Two coumarin derivatives **9** and **10** were reported from this plant for the first time. In this study, we provided evidence showing that *B. kazinoki* could be a potential source for discovering new agents for anti-cancer and anti-inflammatory drugs. However, further studies are required to explore their molecular mechanisms in more detail.

Declaration of Competing Interest

The authors declare no competing financial interest.

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