Article

Chemical Constituents of the Seeds of *Raphanus sativus* and their Biological Activity

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As part of our ongoing search for bioactive constituents of natural Korean medicinal resources, a bioassay-guided fractionation and chemical investigation of the MeOH extract of *Raphanus sativus* (Brassicaceae) seeds resulted in the isolation and identification of fifteen compounds, including a new phenolic compound. The structure of the new compound was determined by extensive spectroscopic analysis and the Mosher's method. One of the compounds has been recently reported as a synthetic product. Some compounds showed moderate antiproliferative activities against the tumor cell lines A549, SK-OV-3, SK-MEL-2, and HCT-15 with IC_{50} values in the range of 5.62 to 28.88 μM. Moreover, the anti-neuroinflammatory activities of the isolates were determined by measuring the nitric oxide (NO) levels in the medium using murine microglia BV-2 cells. With exception of one specific compound, all the others inhibited the lipopolysaccharide (LPS)-stimulated NO production (IC_{50} values < 200 μM).

Keywords: *Raphanus sativus*, Brassicaceae, cytotoxicity, anti-inflammation, Mosher's method

Introduction

The edible root vegetable *Raphanus sativus* L. (Brassicaceae), commonly known as radish, is one of the most widely grown and consumed vegetables throughout the world. In China, it has been used as a traditional herbal medicine for over 1400 years, recorded in 'Tang Materia Medica', the first Chinese pharmacopoeia.[1] Different parts of *R. sativus*, including roots, seeds, and leaves, are known to possess a variety of medicinal properties.[2] In particular, the seeds of *R. sativus*, also known as Raphani Semen, have been used as a traditional Korean medicine since ancient times, as carminative, diuretic, expectorant, laxative, and stomachic agents, and have also been used as anti-cancer and/or anti-inflammatory agents.[3-5] These effects have been supported by previous phytochemical investigations reporting pharmacologically active glucosinolates responsible for cancer-chemoprotective properties.[1,6-8]

In a previous work,[9,10] we found that the MeOH extract of Raphani Semen exhibits significant cytotoxic activity against the human tumor cell lines, A549, SK-OV-3,

SK-MEL-2, and HCT-15, in addition to inhibitory effects on nitric oxide (NO) production in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. This observation led to the isolation and identification of 4-methylthio-butanyl derivatives and phenylpropanoid sucrosides that correlated with the cytotoxic and anti-inflammatory activities.[9,10] Using a bioassay-guided methodology, we are now reporting the isolation of fifteen compounds (**1-15**), including a new phenolic (**1**), from the most active CHCl₃-soluble fraction of the MeOH extract (Figure 1). In addition, their antiproliferative activities against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines, and their inhibitory effects on NO production in a LPS-activated BV2 cell line, have been evaluated.

Experimental

General experimental procedures

Melting point was determined on a Gallenkamp melting point apparatus and is uncorrected. Infrared (IR) spectra were recorded on a Bruker IFS-66/S FTIR spectrometer (Bruker, Karlsruhe, Germany). UV
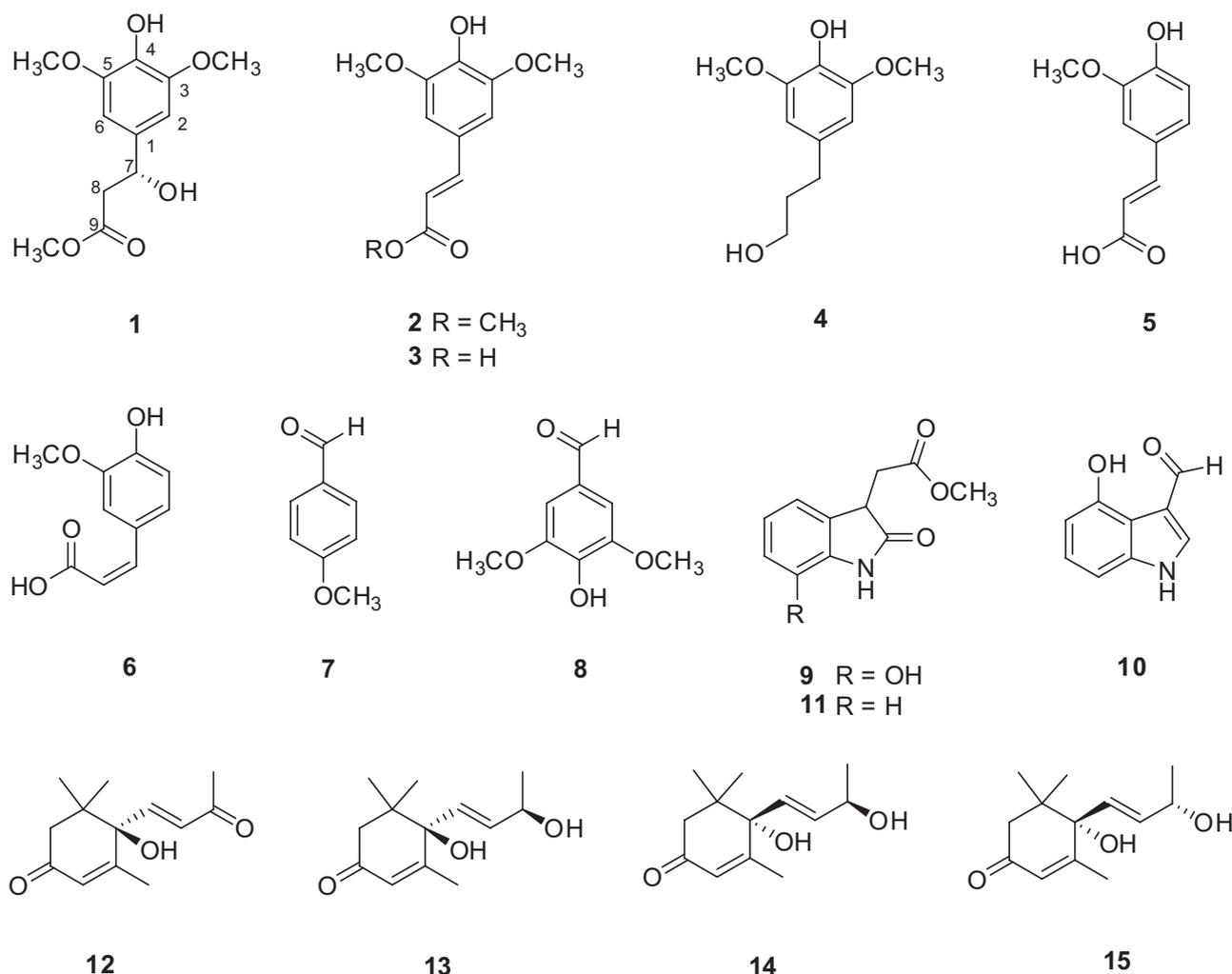
Figure 1. Structures of compounds **1**-**15** isolated from *R. sativus*.

spectra were recorded with a Shimadzu UV-1601 UV-Visible spectrophotometer (Shimadzu, Tokyo, Japan). High-resolution electrospray ionisation mass spectrometry (HR-ESIMS) and ESIMS spectra were recorded on a Micromass QTOF2-MS (MicroMass, Waters, Milford, MA, USA). Nuclear magnetic resonance (NMR) spectra, including ^{1}H-^{1}H COSY, HMQC, and HMBC experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer (Varian, Palo Alto, CA, USA) operating at 500 MHz (^{1}H) and 125 MHz (^{13}C), with chemical shifts given in ppm (δ). Semi-preparative high performance liquid chromatography (HPLC) was conducted using a Gilson 306 pump (Gilson, Middleton, WI) with Shodex refractive index detector (Shodex, New York, NY). Silica gel 60 and RP-C$_{18}$ silica gel (230-400 mesh, Merck, Darmstadt, Germany) were used for column chromatography. Low-pressure liquid chromatography (LPLC) was carried out over a LiChroprep Lobar-A Si 60 column (240 mm × 10 mm i.d.; Merck) with a FMI QSY-0 pump. The packing material for molecular sieve column

chromatography was Sephadex LH-20 (Pharmacia, Uppsala, Sweden). Merck precoated silica gel F$_{254}$ plates and RP-18 F$_{254s}$ plates (Merck, Darmstadt, Germany) were used for thin layer chromatography (TLC). Spots were detected on TLC under UV light or by heating after spraying with 10% H$_2$SO$_4$ in C$_2$H$_5$OH (v/v).

Plant material

The seeds of *R. sativus* were purchased at the Kyungdong herbal market, Seoul, Korea, in January 2010, and identified by one of the authors (K. R. Lee). A voucher specimen (SKKU-2010-01) has been deposited in the herbarium of the School of Pharmacy, Sungkyunkwan University, Suwon, Korea.

Extraction and isolation

The dried seeds of *R. sativus* (3.6 kg) were ground and extracted twice with 80% aqueous MeOH at room

temperature, and subsequently filtered. The filtrate was evaporated *in vacuo* to obtain a crude extract (325 g), which was fractionated by sequential liquid-liquid partitioning of H_2O with *n*-hexane, $CHCl_3$, and *n*-BuOH to yield 12, 8, and 30 g of residues, respectively. Each fraction was evaluated for cytotoxicity against A549, SK-OV-3, SK-MEL-2, and HCT-15 cell lines using a sulforhodamine B (SRB) assay. The $CHCl_3$-soluble fraction exhibited the highest cytotoxic activity against the tested tumor cell lines. It also showed potent inhibition of NO production in LPS-stimulated BV-2 cells. The $CHCl_3$-soluble fraction (8 g) was separated by column chromatography on a reversed-phase C18 column using a gradient of increasing MeOH in H_2O [40% MeOH (1.5 L), 60% MeOH (1.5 L), and 100% MeOH (2.0 L)] to give 13 fractions [C1-C13; C1-C3 (each 0.5 L of 40% MeOH), C4-C8 (each 0.3 L of 60% MeOH), and C9-C13 (each 0.4 L of 100% MeOH)], whose compositions were monitored by TLC. Fraction C4 (180 mg) was subjected to semi-preparative reversed-phase HPLC using a 250 mm × 10 mm i.d., 10 μm, Econosil RP-18 column (Alltech, Nicholasville, KY, USA), with MeOH-H_2O (2:3) as eluent, to yield fraction C41. Fraction C41 (55 mg) was reapplied to semi-preparative normal-phase HPLC using a 250 mm × 10 mm i.d., 5 μm, Apollo Silica column (Alltech), with $CHCl_3$-MeOH (30:1) as eluent, to yield compounds **1** (8 mg) and **4** (6 mg). Fraction C5 (530 mg) was applied to LPLC on a 240 mm × 10 mm i.d., 40-63 μm, LiChroprep Lobar-A Si 60 column (Merck), with *n*-hexane-EtOAc (1:1, 900 mL) as eluent, to give three fractions [C51-C53; C51 (200 mL), C52 (200 mL), and C53 (500 mL)]. Fraction C51 (182 mg) was purified using semi-preparative normal-phase HPLC with *n*-hexane-$CHCl_3$-EtOAc (1:1:2) as eluent, to yield compounds **7** (5 mg), **8** (15 mg), **12** (7 mg), **13** (6 mg), and **14** (8 mg). Fraction C52 (162 mg) was also purified using semi-preparative normal-phase HPLC with $CHCl_3$-MeOH (30:1) as eluent, to yield compound **15** (9 mg). Fraction C7 (400 mg) was subjected to a Sephadex LH-20 column using CH_2Cl_2-MeOH (1:1) as eluent, to yield two fractions (C71 and C72). Fraction C72 (150 mg) was then applied to semi-preparative normal-phase HPLC with *n*-hexane-$CHCl_3$-EtOAc (1:0.1:1) as eluent, to afford compounds **5** (5 mg), **6** (5 mg), **9** (6 mg), and **11** (6 mg). Fraction C8 (350 mg) was separated over a Sephadex LH-20 column using CH_2Cl_2-MeOH (1:1) as eluent, to yield compound **10** (7 mg). Fraction C10 (280 mg) was also applied to a Sephadex LH-20 column using the same eluent, to give two fractions (C101 and C102). Fraction C101 (75 mg) was further purified using semi-preparative normal-phase HPLC with *n*-hexane-$CHCl_3$-EtOAc (1:0.1:1) as eluent, to yield compounds **2** (33 mg) and **3** (9 mg).

Methyl (3*R*)-hydroxy-3-(4-hydroxy-3,5-dimethoxyphenyl) propanoate (**1**)

Colorless gum; $[\alpha]_D^{25}$ +17.3 (*c* 0.40, MeOH); UV (MeOH) λ_{max} / nm (log ε) 270 (1.2), 238 (2.8), 216 (3.9); IR (KBr) ν_{max} / cm^{-1} 3357, 2946, 2832, 1720, 1451, 1116, 1032, 674; ^1H (500 MHz) and ^{13}C (125 MHz) NMR, see Table 1; ESIMS (positive-ion mode) *m/z* 279 [M + Na]$^+$; HRESIMS (negative-ion mode) *m/z* 255.0877 [M − H]$^+$ (calcd. for $C_{12}H_{15}O_6$, 255.0869).

Preparation of (*R*)- and (*S*)-MTPA ester derivatives of **1**

4-(Dimethylamino)pyridine (DMAP, 1 mg) and (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl, 10 μL) were added to a stirred solution of **1** (2.0 mg) in pyridine (400 μL), and the mixture was further stirred at room temperature for 16 h. The reaction mixture was then passed through a silica gel Waters Sep-Pak Vac 6cc and eluted with *n*-hexane-EtOAc (15:1) to give the (*R*)-Mosher ester **1r**. Treatment of **1** (2.0 mg) with (*R*)-MTPA-Cl (10 μL) as described above, yielded the corresponding (*S*)-MTPA ester **1s**.

Cytotoxicity assays

All tumor cell cultures were maintained using RPMI1640 cell growth medium (Gibco, Carlsbad, CA), supplemented with 5% fetal bovine serum (FBS) (Gibco), 100 units mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. The human tumor cell lines, A549 (non-small cell lung carcinoma), SK-OV-3 (ovarian malignant ascites), SK-MEL-2 (skin melanoma), and HCT-15 (colon adenocarcinoma) were provided by the National Cancer Institute (NCI). The cytotoxicity of the isolates against cultured human tumor cell lines was evaluated by the sulforhodamine B (SRB) method. Each tumor cell line was inoculated into standard 96-well flat-bottom microplates and incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2. The attached cells were then incubated with the serially diluted compounds with the control cultures receiving the carrier solvent (0.1% dimethyl sulfoxide). After continuous exposure to the compounds for 48 h, the culture medium was removed from each well and the cells were fixed with 10% cold trichloroacetic acid at 4 °C for 1 h. After washing with tap water, the cells were stained with 0.4% SRB dye and incubated for 30 min at room temperature. The cells were washed again and solubilized with 10 mM unbuffered Tris base solution (pH 10.5). The absorbance was measured spectrophotometrically at 520 nm using a microtiter plate reader. Doxorubicin

(purity ≥ 98%; Sigma) was used as a positive control. Tested compounds were demonstrated to be pure as evidenced by NMR and HPLC analysis (purity ≥ 95%).

Measurement of NO production

The murine microglial BV2 cell line was generously provided by PhD E. Choi from Korea University (Seoul, Korea), and maintained in Dulbecco's modified Eagle (DMEM) medium supplemented with 5% FBS, 100 units mL^{-1} penicillin, and 100 µg mL^{-1} streptomycin. All cells were incubated at 37 °C in a humidified incubator with 5% CO_2. BV-2 cells were plated into a 96-well plate (3×10^4 cells well^{-1}). Following culture for 24 h, cells were pretreated with the compounds for 30 min, and subsequently stimulated with 100 ng mL^{-1} of LPS for a further 24 h. Control cultures received the carrier solvent (0.1% dimethyl sulfoxide). Nitrite, a soluble oxidation product of NO, was measured in the culture media using the Griess reaction. The supernatant (50 µL) was harvested and mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-napthylethylenediamine dihydrochloride in 5% phosphoric acid). After 10 min, the absorbance at 570 nm was measured using a microplate reader. Sodium nitrite was used as a standard to calculate the NO_2^- concentration. N^G-monomethyl-L-arginine (NMMA, Sigma, St. Louis, MO, USA), a well-known NO synthase inhibitor, was tested as a positive control.

Results and Discussion

Compound **1** was isolated as a colorless gum. The molecular formula of **1** was determined to be $C_{12}H_{16}O_6$ by the negative mode HRESIMS at *m/z* 255.0877 [M − H]$^+$ (calcd. for $C_{12}H_{15}O_6$, 255.0869). The IR spectrum displayed absorption bands associated with hydroxyl (3357 cm^{-1}) and carbonyl ester (1720 cm^{-1}) groups. The ^{1}H NMR spectrum of **1** (Table 1) showed characteristic signals attributable to a 1,3,4,5-tetrasubstituted aromatic ring at δ_H 6.54 (2H, s, H-2 and H-6), one downfield shifted methine at δ_H 5.00 (1H, ddd, *J* 9.0, 4.0, 3.0 Hz, H-7), one methylene adjacent to a carbonyl group at δ_H 2.70 (1H, dd, *J* 16.0, 9.0 Hz, H-8a) and 2.62 (1H, dd, *J* 16.0, 4.0 Hz, H-8b), and three methoxy groups, two that were overlapping at δ_H 3.83 (6H, s) and another at δ_H 3.66 (3H, s). The ^{13}C NMR spectrum of **1** (Table 1) exhibited a total of 12 carbons including six aromatic carbons in the range δ_C 147.1-102.3, one oxygenated carbon at δ_C 70.5, one methylene carbon at δ_C 43.4, one carbonyl carbon at δ_C 172.8, and three methoxy carbons at δ_C 56.3 (× 2) and 51.9. Interpretation of the 2D NMR spectra correlations,

including ^{1}H-^{1}H correlated spectroscopy (COSY), heteronuclear multiple quantum correlation (HMQC), and heteronuclear multiple bond correlation (HMBC), revealed compound **1** to be the sinapic acid derivative, methyl 3-hydroxy-3-(4-hydroxy-3,5-dimethoxyphenyl) propanoate (Figure 2). The absolute configuration of C-7 of **1** was established on the basis of the modified Mosher's method.[11] Treatment of **1** with (*S*)-(+)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*S*)-MTPA-Cl] and DMAP in pyridine gave the (*R*)-MTPA ester **1r**. Similar treatment of **1** with (*R*)-(−)-MTPA-Cl afforded the (*S*)-MTPA ester **1s**. Analysis of the ^{1}H NMR chemical shift differences ($\Delta\delta_{S-R}$) [see Supporting Information (SI) section] of the two MTPA esters allowed the assignment of the absolute configuration of C-7 as *R* (Figure 3). Thus, the structure of **1** was elucidated as methyl (3*R*)-hydroxy-3-(4-hydroxy-3,5-dimethoxyphenyl) propanoate, which appears to be the epimer of methyl (3*S*)-hydroxy-3-(4-hydroxy-3,5-dimethoxyphenyl) propanoate, previously reported in *Ruta graveolens* L.[12] The spectroscopic data of the known epimer is similar to those of **1** (Table 1), except for the splitting pattern of H-7,[12] however, its optical rotation value ($[\alpha]_D^{25}$ −1.03 in MeOH) was easily distinguishable from that of **1** ($[\alpha]_D^{25}$ +17.3 in MeOH).[12]

Table 1. ^{1}H and ^{13}C NMR data of compound **1** in CDCl$_3$^{a}

Position	1	
	δ_H / ppm	δ_C / ppm
1	−	133.7
2	6.54 (s)	102.3
3	−	147.1
4	−	134.2
5	−	147.1
6	6.54 (s)	102.3
7	5.00 (ddd, 9.0, 4.0, 3.0 Hz)	70.5
8	2.70 (dd, 16.0, 9.0 Hz) 2.62 (dd, 16.0, 4.0 Hz)	43.4
9	−	172.8
4-OH	5.41 (s)	−
7-OH	3.06 (d, 3.0 Hz)	−
3,5-OCH₃	3.83 (s)	56.3
9-OCH₃	3.66 (s)	51.9

^{1}H and ^{13}C NMR data were recorded at 500 and 125 MHz, respectively. Coupling constants (in Hz) are given in parentheses.

The known compounds were identified as sinapic acid methyl ester (**2**),[13] sinapic acid (**3**),[14] dihydrosinapic alcohol (**4**),[15] (*E*)-ferulic acid (**5**),[16] (*Z*)-ferulic acid (**6**),[16] *p*-anisic aldehyde (**7**),[17] and syringic aldehyde (**8**),[18] by comparison of their spectroscopic data with previously reported literature
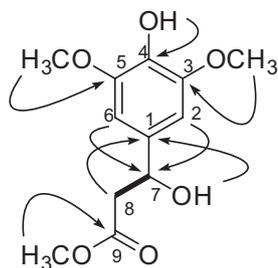
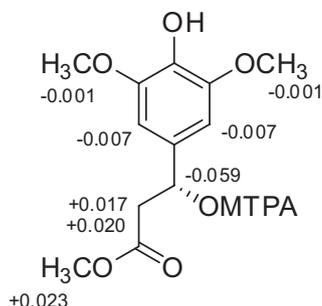**Figure 2.** The ^{1}H-^{1}H COSY correlations (bond) and key HMBC correlations (H→C) of **1**.

Figure 3. $\Delta\delta$ Values ($\delta_S - \delta_R$) in ppm of the two MTPA esters of 1.

values. Furthermore, three indole alkaloids were identified as methyl 7-hydroxyoxindole-3-acetate (**9**),[19] 4-hydroxy-3-indolecarbaldehyde (**10**),[20] and methyl oxindole-3-acetate (**11**),[21] by comparison with previously published data. On the other hand, methyl 7-hydroxyoxindole-3-acetate (**9**) has been recently reported as a synthetic product.[19] Compound **9** appears to be an artifact of extraction with MeOH, since its free acid precursor has been reported to be present in *Zea mays*.[19,22] Other known megastigmane derivatives were also identified as (−)-dihydrovomifoliol (**12**),[23] (6*R*,9*R*)-vomifoliol (**13**),[24] (6*S*,9*R*)-vomifoliol (**14**),[24] and (6*S*,9*S*)-vomifoliol (**15**),[24] by comparison of their spectroscopic data with previously reported values. The absolute configurations of compounds **12**-**15** were established on the basis of their NMR data, optical rotation values, and circular dichroism (CD) data.[23,24]

The antiproliferative activities of compounds **1**-**15** were evaluated by determining their inhibitory effects on four human tumor cell lines, namely A549, SK-OV-3, SK-MEL-2, and HCT-15, using the SRB bioassay.[25] The results (Table 2) showed that compounds **2**, **3**, and **9**-**11** displayed moderate antiproliferative activities against all the tumor cell lines. However, the other compounds were inactive (IC$_{50}$ > 30 μM) in all four cell lines. In particular, among the isolates, indole alkaloids **9**-**11** showed good antiproliferative effects against all the tumor cells tested, with IC$_{50}$ values ranging from 5.62 to 28.88 μM.

Compounds **1**-**15** were also examined for their anti-neuroinflammatory activities by measuring the NO levels produced in LPS-activated BV-2 cells, a microglial cell

Table 2. Antiproliferative activities of compounds **2**, **3**, and **9**-**11** isolated from *R. sativus*

Compound	IC$_{50}$ / μM [a]			
	A549	SK-OV-3	SK-MEL-2	HCT-15
2	28.47	12.51	14.41	7.14
3	25.89	18.15	7.98	9.45
9	18.90	23.31	10.86	5.62
10	22.19	27.53	16.29	8.45
11	28.88	25.18	15.20	14.53
Doxorubicin[b]	0.007	0.011	0.001	0.036

[a]IC$_{50}$ value of compounds against each cancer cell line. The IC$_{50}$ value was defined as the concentration (μM) causing 50% inhibition of cell growth *in vitro*; [b]Doxorubicin as positive control.

line. The results (Table 3) showed that all the compounds, with the exception of compound **6**, inhibited NO production (IC$_{50}$ values < 200 μM). These compounds had no effect on cell viability in LPS-treated BV-2 cells at their respective IC$_{50}$ values (data not shown). Among these, compounds **2**, **4**, and **12** significantly inhibited NO levels with IC$_{50}$ values of 18.99, 18.69, and 15.60 μM, respectively. In particular, compound **12** showed potent inhibition of NO in LPS-stimulated NO production with an IC$_{50}$ of 15.60 μM, which is a lower concentration than that displayed by the positive control N^G-nonomethyl-L-arginine (IC$_{50}$ 19.15 μM). Excess production of NO by activated microglia induces neuronal cell death,[26,27] which consequently leads to various neurodegeneration disorders of the CNS. Therefore, the active compounds **2**, **4**, and **12** could be considered potential candidates in the prevention of the progressive damage resulting from neurodegenerative diseases.

Table 3. Inhibitory effects of compounds **1**-**15** on NO production in LPS-activated BV-2 cells

Compound	IC$_{50}$ / μM[a]	Compound	IC$_{50}$ / μM[a]
1	58.74	**9**	166.71
2	18.99	**10**	36.91
3	24.05	**11**	27.15
4	18.69	**12**	15.60
5	41.25	**13**	76.50
6	> 200	**14**	39.24
7	44.76	**15**	53.21
8	31.41	NMMA[b]	19.15

[a]IC$_{50}$ value of each compound was defined as the concentration (μM) that caused 50% inhibition of NO production in LPS-activated BV-2 cells; [b]NMMA as positive control.

Conclusions

The bioassay-guided fractionation and chemical investigation of the MeOH extract of *R. sativus* seeds resulted in the isolation and identification of fifteen

compounds (**1**-**15**), including a new phenolic compound, methyl (3*R*)-hydroxy-3-(4-hydroxy-3,5-dimethoxyphenyl) propanoate (**1**). Among the isolates, three indole alkaloids (**9**-**11**) showed good antiproliferative effects against all the tumor cells tested, with IC_{50} values ranging from 5.62 to 28.88 μM. In addition, compounds **2**, **4**, and **12** significantly inhibited LPS-stimulated NO production in murine microglia BV-2 cells with IC_{50} values of 18.99, 18.69, and 15.60 μM, respectively. These results suggest that the above mentioned active compounds with cytotoxic or anti-neuroinflammatory activities, could be considered as lead molecules for drug development related to various cancers or neurodegenerative diseases.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as a PDF file.

Acknowledgments

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