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RESEARCH ARTICLE

A new indole glycoside from the seeds of *Raphanus sativus*

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Abstract A new indole glycoside, β-D-glucopyranosyl 2-(methylthio)-1*H*-indole-3-carboxylate, named raphanuside A (**1**), as well as eight known compounds, β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**2**), (3-*O*-sinapoyl)-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside (**3**), (3-*O*-sinapoyl)-β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**4**), (3,4-*O*-disinapoyl)-β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**5**), isorhamnetin 3,4′-di-*O*-β-D-glucoside (**6**), isorhamnetin 3-*O*-β-D-glucoside-7-*O*-α-L-rhamnoside (**7**), isorhamnetin 3-*O*-β-D-glucoside (**8**) and 3'-*O*-methyl-(−)-epicatechin 7-*O*-β-D-glucoside (**9**) were isolated from the seeds of *Raphanus sativus*. Furthermore, compounds **1**–**3** and **6**–**9**, were isolated from this plant for the first time. The structures of compounds **1**–**9** were identified using 1D and 2D NMR, including ^{1}H–^{1}H COSY, HSQC, HMBC and NOESY spectroscopic analyses. The inhibitory activity of these isolated compounds against interleukin-6 (IL-6) production in TNF-α stimulated MG-63 cells was also examined.

Keywords Indole glycoside · IL-6 inhibitory effect · Brassicaceae · *Raphanus sativus*

Hong-Guang Jin and Hae Ju Ko have equally contributed to this work.

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Introduction

Raphani Semen, the seeds of *Raphanus sativus* L. (Brassicaceae), are used in oriental medicine as a carminative, diuretic, expectorant, and laxative, and is reported to exhibit stomachic, anti-cancer and anti-inflammatory activity (Duke and Ayensu 1985; Yeung 1985; Chopra et al. 1986). Previous phytochemical investigations resulted in the isolation of some glucosinolates responsible for the aforementioned cancer-chemopreventive properties (Daxenbichler et al. 1991; Nastruzzi et al. 2000; Barillari et al. 2005; Duan et al. 2007). Glucosinolates and/or their decomposition products have recently attracted considerable interest due to their anticancer properties (Noyan-Ashraf et al. 2005; Giacoppo et al. 2013). Recently, three new 4-methylthio-butanyl derivatives were isolated from *R. sativus*, and their anti-tumor and anti-neuroinflammatory activities were investigated (Kim et al. 2014). Although many biologically active glucosinolates were isolated from *R. sativus*, only a few studies of its anti-inflammatory components have been performed (Kim et al. 2015). In addition, there has been no investigations into the alkaloid component of *R. sativus*.

In an ongoing investigation into anti-inflammatory compounds from *R. sativus*, the methanol extract of its seeds was investigated. By means of repeated column chromatography using silica gel, MCI gel, Sephadex LH-20, and LiChroprep RP-18, a new indole glycoside, β-D-glucopyranosyl 2-(methylthio)-1*H*-indole-3-carboxylate, named raphanuside A (**1**), was isolated along with eight known compounds. The structures of the known compounds were identified as β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**2**), (3-*O*-sinapoyl)-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside (**3**), (3-*O*-sinapoyl)-β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-

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glucopyranoside (**4**), (3,4-*O*-disinapoyl)-β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**5**), isorhamnetin 3,4′-di-*O*-β-D-glucoside (**6**), isorhamnetin 3-*O*-β-D-glucoside-7-*O*-α-L-rhamnoside (**7**), isorhamnetin 3-*O*-β-D-glucoside (**8**), and 3'-*O*-methyl-(−)-epicatechin 7-*O*-β-D-glucoside (**9**), by comparison of their spectroscopic data with those reported in the literature (Fig. 1). Furthermore, these nine known compounds, with the exception of **4** and **5**, were isolated from this plant for the first time. The inhibitory activity of these isolated compounds against IL-6 production in TNF-α stimulated MG-63 cells was examined.

This paper reports the isolation and structural characterization of these compounds and their inhibitory activities against IL-6 production.

Materials and methods

General experimental procedure

Optical rotations were measured using an Autopol-IV polarimeter. Ultraviolet (UV) spectra were obtained on a Shimadzu UV/Visible Spectrophotometer. Infrared (IR) spectra were recorded on an IMS 85 (Bruker). High-resolution electrospray ionization mass spectrometry (HR-ESI-MS) was performed on a quadrupole time-of-flight (Q-TOF) mass spectrometer (Synapt HDMS system, Waters, USA). Nuclear magnetic resonance (NMR) spectra, including nuclear Overhauser effect spectroscopy (NOESY), correlation spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC), and heteronuclear multiple bond correlation (HMBC) experiments, were recorded on a Varian UNITY INOVA 500 NMR spectrometer (KBSI-Gwangju center) operating at 500 MHz for ^{1}H-NMR, and 125 MHz for ^{13}C-NMR, with chemical shifts given in ppm (δ). TLC was carried out in a precoated Kieselgel 60 F_{254} (art. 5715, Merck) and RP-18 F_{254}s (Art. 15389, Merck) plates. Column chromatography was performed on silica gel 60 (40–63 and 63–200 μm, Merck), MCI gel CHP20P (75–150 μm, Mitsubishi Chemical Co.), and Sephadex LH-20 (25–100 μm, Sigma). Low pressure liquid chromatography was carried out in a Merck Lichroprep Lobar®-A RP-18 (240 × 10 mm^2) column with a FMI QSY-0 pump (ISCO).

Plant materials

The seeds of *R. sativus* were collected in Andong, Gyeongbuk province, Republic of Korea, in August 2013

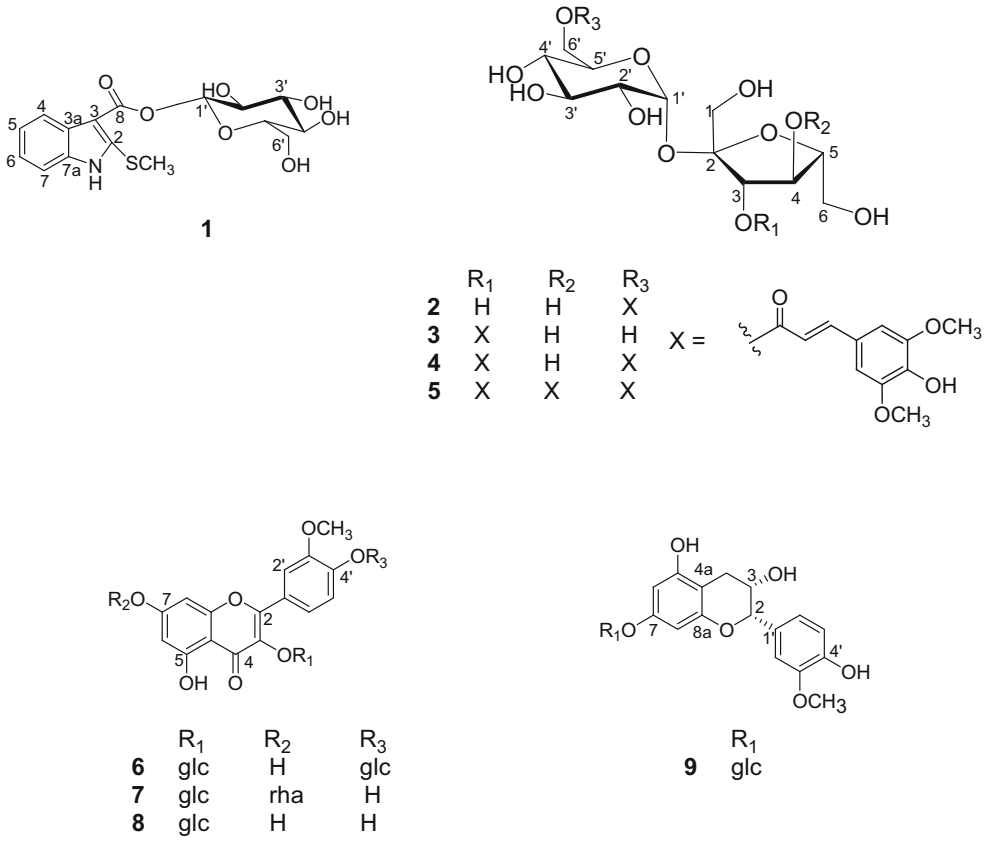

Fig. 1 Structures of compounds **1**–**9**

and identified by Dr. J. H. Lee, Professor of the department of Korean Medicine, Dongguk University. A voucher specimen (CSU-1050-17) was deposited in the Herbarium of the College of Pharmacy, Chosun University.

Extraction and isolation

The air-dried seeds of *R. sativus* (19.1 kg) were pulverized and extracted with MeOH three times for 4 h at 80 °C. The resultant MeOH extract (800.5 g) was suspended in water (3 × 2.0 L) and then partitioned sequentially with equal volumes of *n*-hexane, dichloromethane, ethyl acetate, and *n*-butanol. Each fraction was evaporated in vacuo to yield the residues of *n*-hexane (195.4 g), CH_2Cl_2 (10.3 g), EtOAc (7.3 g), *n*-BuOH (60.6 g) and water (400.3 g) extracts. The *n*-BuOH soluble fraction (22.2 g) was subjected to column chromatography (CC) over a Diaion HP 20 column and eluted with a H_2O/MeOH gradient system (100:0 → 0:100). Fractions were combined based on their TLC pattern to yield subfractions designated B1-B6. Fraction B3 (4.38 g) was further purified by MCI gel CC (MeOH/H_2O, 1:3 → 1:1) to yield five subfractions (B31-B35). Subfraction B31 (0.62 g), containing **1** and **9**, was again purified by silica gel CC ($CHCl_3$/MeOH, 5:1 → 1:1) and finally by Sephadex LH 20 CC (MeOH/H_2O, 1:10) to yield **1** (6.5 mg) and **9** (5.6 mg). In addition, subfraction B33 (1.87 g) was purified by silica gel CC ($CHCl_3$/MeOH/H_2O, 3:1:0.2 → 2:1:0.2) and finally by Lichroprep RP-18 CC (MeOH/H_2O, 1:4 → 1:1) to yield **6** (32.4 mg), **7** (20.8 mg) and **8** (11.2 mg). Fraction B4 (5.4 g) was subjected to MCI gel CC eluting with a MeOH/H_2O (45:55 → 65:35) to yield four subfractions (B41-B44). Subfraction B41 (1.04 g) was then purified by silica gel CC ($CHCl_3$/MeOH/H_2O, 7:1:0.2 → 3:1:0.2) to yield **2** (41.5 mg). Subfraction B42 and B43 (3.39 g) were purified by repeated Lichroprep RP 18 CC (MeOH/H_2O, 1:2 → 1:1) to yield **3** (38.8 mg) and **4** (2.49 g). Fraction B5 (0.83 g) was purified by silica gel CC ($CHCl_3$/MeOH, 10:1 → 3:1) to yield **5** (375.4 mg).

Raphanuside A (**1**): Yellowish amorphous powder; $[\alpha]_D^{20}$ +31.0° (MeOH; *c* 0.26); HR-ESI–MS (negative mode) *m/z*: 368.0797[M–H]$^-$ (calcd for $C_{16}H_{18}NO_7S$, 368.0804); IR ν_{max} (film) cm^{-1}: 3480, 3400, 1620, 1490, 850; UV (MeOH) λ_{max} nm: 320, 253, 272; ^{1}H-NMR (500 MHz, CD_3OD) δ: 7.98 (1H, m, H-4), 7.39 (1H, m, H-7), 7.13 (2H, m, H-5, 6), 5.75 (1H, d, J = 7.5 Hz, H-1′), 3.87 (1H, dd, J = 2.0, 12.0 Hz, H-6′a), 3.72 (1H, dd, J = 5.0, 12.0 Hz, H-6′b), 3.62 (1H, t, J = 8.5 Hz, H-2′), 3.50 (1H, t, J = 8.5 Hz, H-3′), 3.42-3.46 (2H, m, H-4′, 5′), 2.66 (3H, s, H-9-SCH_3); ^{13}C-NMR (125 MHz, CD_3OD) δ: 165.8 (C-8), 148.2 (C-2), 138.5 (C-7a), 128.7 (C-3a), 123.1 (C-6), 122.9 (C-5), 121.6 (C-4), 111.8 (C-7), 103.9 (C-3), 95.5 (C-1′), 79.0 (C-5′), 78.6 (C-3′), 74.4 (C-2′), 71.3 (C-4′), 62.6 (C-6′), 14.6 (C-9–SCH_3).

β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**2**): Amorphous powder; $[\alpha]_D^{20}$ + 10.4° (MeOH; *c* 0.36); IR ν_{max} (film) cm^{-1}: 3408, 1700, 1632, 1608; FAB-MS, *m/z*: 571[M + Na]$^+$; ^{1}H-NMR (500 MHz, CD_3OD) δ: 7.63 (1H, d, J = 16.0 Hz, H-7″), 6.95 (2H, s, H-2″, 6″), 6.46 (1H, d, J = 16.0 Hz, H-8″), 5.42 (1H, d, J = 3.5 Hz, H-1′), 4.51 (1H, dd, J = 1.5, 12.0 Hz, Ha-6′), 4.25 (1H, dd, J = 6.5, 12.0 Hz, Hb-6′), 4.14 (1H, m, H-5′), 4.08 (1H, d, J = 8.0 Hz, H-3), 4.06 (1H, dd, J = 8.0, 8.0 Hz, H-4), 3.87 (6H, s, 3″, 5″-OC$\underline{H}_3$), 3.76-3.81 (3H, m, H-5, 6), 3.74 (1H, dd, J = 9.5, 10.0 Hz, H-3′), 3.61 (1H, d, J = 12.5 Hz, Ha-1), 3.62 (1H, d, J = 12.5 Hz, Hb-1), 3.46 (1H, dd, J = 4.0, 9.5 Hz, H-2′), 3.33 (1H, dd, J = 9.0, 9.5 Hz, H-4′); ^{13}C-NMR (125 MHz, CD_3OD) δ: 169.2 (C-9″), 149.6 (C-3″, 5″), 147.4 (C-7″), 139.9 (C-4″), 126.6 (C-1″), 115.8 (C-8″), 107.1 (C-2″, 6″), 105.3 (C-2), 93.3 (C-1′), 84.0 (C-5), 79.3 (C-3), 76.2 (C-4), 74.8 (C-3′), 73.3 (C-2′), 72.2 (C-5′), 72.0 (C-4′), 65.3 (C-1), 64.4 (C-6′), 64.4 (C-6), 57.0 (C-3″, 5″-OC$\underline{H}_3$).

(3-*O*-sinapoyl)-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside (**3**): Amorphous powder; $[\alpha]_D^{20}$ -28.0° (MeOH; *c* 0.72); IR ν_{max} (film) cm^{-1}: 3408, 1700, 1632, 1608; FAB-MS, *m/z*: 571[M + Na]$^+$;^{1}H-NMR (500 MHz, CD_3OD) δ: 7.71 (1H, d, J = 16.0 Hz, H-7″), 6.97 (2H, s, H-2″, 6″), 6.45 (1H, d, J = 16.0 Hz, H-8″), 5.47 (1H, d, J = 7.5 Hz, H-3), 5.44 (1H, d, J = 3.5 Hz, H-1′), 4.38 (1H, t, J = 8.0 Hz, H-4), 3.92-3.96 (2H, m, H-5, 5′), 3.89 (6H, s, 3″, 5″-OC$\underline{H}_3$), 3.76-3.87 (4H, m, H-6, 6′), 3.67 (1H, dd, J = 9.5, 10.0 Hz, H-3′), 3.66 (1H, d, J = 12.0 Hz, Ha-1), 3.58 (1H, d, J = 12.0 Hz, Hb-1), 3.44 (1H, dd, J = 4.0, 9.5 Hz, H-2′), 3.40 (1H, dd, J = 9.0, 9.5 Hz, H-4′); ^{13}C-NMR (125 MHz, CD_3OD) δ: 168.3 (C-9″), 149.6 (C-3″, 5″), 148.0 (C-7″), 139.8 (C-4″), 126.8 (C-1″), 115.7 (C-8″), 107.2 (C-2″, 6″), 104.9 (C-2), 93.5 (C-1′), 84.3 (C-5), 79.8 (C-3), 75.1 (C-3′), 74.7 (C-4), 74.0 (C-5′), 73.3 (C-2′), 71.3 (C-4′), 65.5 (C-1), 63.1 (C-6), 62.5 (C-6), 57.1 (C-3″-OC$\underline{H}_3$), 57.0 (C-5″-OC$\underline{H}_3$).

(3-*O*-sinapoyl)-β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**4**): Yellowish amorphous powder; $[\alpha]_D^{20}$ -94.0° (MeOH; *c* 0.6); IR ν_{max} (film) cm^{-1}: 3396, 1704, 1632, 1606; FAB-MS, *m/z*: 777[M + Na]$^+$; ^{1}H-NMR (600 MHz, CD_3OD) δ: 7.67 (1H, d, J = 15.6 Hz, H-7″), 7.59 (1H, d, J = 15.6 Hz, H-7‴), 6.93 (2H, s, H-2″, 6″), 6.88 (2H, s, H-2‴, 6‴), 6.47 (1H, d, J = 15.6 Hz, H-8″), 6.46 (1H, d, J = 15.6 Hz, H-8‴), 5.53 (1H, d, J = 3.6 Hz, H-1′), 5.51 (1H, d, J = 7.8 Hz, H-3), 4.68 (1H, dd, J = 1.8, 12.0 Hz, Ha-6′), 4.51 (1H, t, J = 7.8 Hz, H-4), 4.29 (1H, ddd, J = 1.8, 7.2, 9.6 Hz, H-5′), 4.20 (1H, dd, J = 7.2, 12.0 Hz, Hb-6′), 3.97 (1H, ddd, J = 3.0, 6.6, 7.8 Hz, H-5), 3.90 (1H, dd, J = 7.2, 12.0 Hz, Hb-6), 3.87 (6H, s, 3″, 5″-OC$\underline{H}_3$), 3.84 (6H, s, 3‴, 5‴-OC$\underline{H}_3$), 3.82

(1H, dd, $J = 1.8$, 12.0 Hz, Ha-6), 3.67 (1H, dd, $J = 9.0$, 9.6 Hz, H-3′), 3.61 (1H, d, $J = 12.6$ Hz, Ha-1), 3.59 (1H, d, $J = 12.6$ Hz, Hb-1), 3.48 (1H, dd, $J = 3.6$, 9.6 Hz, H-2′), 3.31(1H, dd, $J = 9.0$, 9.6 Hz, H-4′); ^{13}C-NMR (150 MHz, CD_3OD) δ: 169.2 (C-9‴), 168.6 (C-9″), 149.6 (C-3″, 5″), 149.5 (C-3‴, 5‴), 148.0 (C-7″), 147.4 (C-7‴), 139.8 (C-4″), 139.6 (C-4‴), 126.7 (C-1″), 126.6 (C-1‴), 115.9 (C-8″), 115.6 (C-8″), 107.2 (C-2″, 6″), 107.0 (C-2‴, 6‴), 104.9 (C-2), 92.8 (C-1′), 84.5 (C-5), 79.4 (C-3), 75.2 (C-3′), 74.3 (C-4), 73.3 (C-2′), 72.6 (C-5′), 72.1 (C-4′), 65.8 (C-1), 65.8 (C-6′), 64.0 (C-6), 57.0 (C-3″, 5″-O<u>C</u>H$_3$), 56.9 (C-3‴, 5‴-O<u>C</u>H$_3$).

(3,4-*O*-disinapoyl)-β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**5**): Yellow amorphous powder; $[\alpha]_D^{20}$ -28.0° (MeOH; *c* 0.2); IR ν_{max} (film) cm^{-1}: 3400, 1704, 1640, 1600; FAB-MS, *m/z*: 983[M + Na]$^+$; ^{1}H-NMR (500 MHz, CD_3OD) δ: 7.71 (1H, d, $J = 15.5$ Hz, H-7″), 7.56 (1H, d, $J = 15.5$ Hz, H-7⁗), 7.46 (1H, d, $J = 15.5$ Hz, H-7‴), 6.91 (2H, s, H-2″, 6″), 6.81 (2H, s, H-2⁗, 6⁗), 6.76 (2H, s, H-2‴, 6‴), 6.48 (1H, d, $J = 15.5$ Hz, H-8⁗), 6.46 (1H, d, $J = 15.5$ Hz, H-8″), 6.26 (1H, d, $J = 15.5$ Hz, H-8‴), 5.85 (1H, d, $J = 8.0$ Hz, H-3), 5.71 (1H, t, $J = 8.0$ Hz, H-4), 5.57 (1H, d, $J = 3.5$ Hz, H-1′), 4.75 (1H, dd, $J = 1.5$, 12.0 Hz, Ha-6′), 4.38 (1H, ddd, $J = 1.5$, 6.0, 9.5 Hz, H-5′), 4.21 (1H, dd, $J = 6.0$, 12.0 Hz, Hb-6′), 4.20 (1H, m, H-5), 3.99 (1H, dd, $J = 7.0$, 12.5 Hz, Hb-6), 3.94 (1H, dd, $J = 4.5$, 12.5 Hz, Ha-6), 3.84 (12H, s, 3″, 5″, 3‴, 5⁗-O<u>C</u>H$_3$), 3.78 (6H, s, 3‴, 5‴-O<u>C</u>H$_3$), 3.71 (1H, m, H-3′), 3.65 (2H, d, $J = 3.5$ Hz, H-1), 3.51 (1H, dd, $J = 4.0$, 10.0 Hz, H-2′), 3.30 (1H, m, H-4′); ^{13}C-NMR (125 MHz, CD_3OD) δ: 169.4 (C-9⁗), 168.2 (C-9‴), 167.9 (C-9″), 149.5 (C-3⁗, 5‴), 149.5 (C-3″, 5″), 149.4 (C-3⁗, 5⁗), 148.5 (C-7″), 148.4 (C-7‴), 147.2 (C-7⁗), 139.9 (C-4″), 139.8 (C-4‴), 139.6 (C-4⁗), 126.7 (C-1⁗), 126.6 (C-1″), 126.5 (C-1‴), 116.2 (C-8⁗), 115.3 (C-8″), 114.8 (C-8‴), 107.2 (C-2⁗, 6⁗), 107.2 (C-2″, 6″), 107.0 (C-2‴, 6‴), 105.5 (C-2), 93.0 (C-1′), 83.0 (C-5), 77.1 (C-3), 76.6 (C-4), 75.1 (C-3′), 73.2 (C-2′), 73.0 (C-5′), 72.3 (C-4′), 66.1 (C-6′), 65.2 (C-1), 64.2 (C-6), 57.0 (C-3″, 5″, 3⁗, 5⁗-O<u>C</u>H$_3$), 56.9 (C-3‴, 5‴-O<u>C</u>H$_3$).

Isorhamnetin 3,4′-di-*O*-β-D-glucoside (**6**): Yellowish amorphous powder; $[\alpha]_D^{20}$ -85.0° (MeOH; *c* 0.38); IR ν_{max} (film) cm^{-1}: 3480, 3100, 1645, 1610; ESI–MS, *m/z*: 639[M-H]$^-$; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 7.96 (1H, d, $J = 2.0$ Hz, H-2′), 7.53 (1H, dd, $J = 2.0$, 8.5 Hz, H-6′), 7.23 (1H, d, $J = 8.5$ Hz, H-5′), 6.44 (1H, d, $J = 1.5$ Hz, H-8), 6.21 (1H, d, $J = 1.5$ Hz, H-6), 5.58 (1H, d, $J = 7.5$ Hz, H-1″), 5.06 (1H, d, $J = 7.5$ Hz, H-1‴), 3.84 (3H, s, 3′-O<u>C</u>H$_3$), 3.68 (1H, dd, $J = 1.5$, 12.0 Hz, H-6″), 3.58 (1H, dd, $J = 2.0$, 12.0 Hz, H-6‴), 3.46 (1H, dd, $J = 5.0$, 12.0 Hz, H-6″), 3.39 (1H, dd, $J = 5.5$, 12.0 Hz, H-6‴), 3.36 (1H, t, $J = 9.5$ Hz, H-5‴), 3.29-3.30 (2H, m, H-2⁗, 3⁗), 3.18-3.24 (3H, m, H-2″, 3″, 4⁗), 3.10-3.12 (2H, m, H-4″, 5″); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 177.4 (C-4), 164.8 (C-7), 161.2 (C-5), 156.5 (C-8a), 155.6 (C-2), 148.4 (C-4′), 148.0 (C-3′), 133.4 (C-3), 123.7 (C-1′), 121.4 (C-6′), 114.5 (C-5′), 113.5 (C-2′), 103.9 (C-4a), 100.7 (C-1‴), 99.5 (C-1⁗), 98.9 (C-6), 93.8 (C-8), 77.5 (C-5″), 77.1 (C-5⁗), 76.8 (C-3⁗), 76.4 (C-3″), 74.3 (C-2″), 73.1 (C-2⁗), 69.8 (C-4″), 69.5 (C-4⁗), 60.6 (C-6″), 60.5 (C-6⁗), 55.6 (C-3′-O<u>C</u>H$_3$).

Isorhamnetin 3-*O*-β-D-glucoside-7-*O*-α-L-rhamnoside (**7**): Yellowish amorphous powder; $[\alpha]_D^{20}$ -42.0° (MeOH; *c* 0.30); IR ν_{max} (film) cm^{-1}: 3480, 3100, 1645, 1610; ESI–MS, *m/z*: 623[M-H]$^-$; ^{1}H-NMR (500 MHz, CD_3OD) δ: 7.93 (1H, d, $J = 2.0$ Hz, H-2′), 7.62 (1H, dd, $J = 2.0$, 8.5 Hz, H-6′), 6.88 (1H, d, $J = 8.5$ Hz, H-5′), 6.75 (1H, d, $J = 2.0$ Hz, H-8), 6.45 (1H, d, $J = 2.0$ Hz, H-6), 5.57 (1H, s, H-1‴), 5.45 (1H, d, $J = 7.5$ Hz, H-1″), 4.02 (1H, m, H-2‴), 3.94 (3H, s, 3′-O<u>C</u>H$_3$), 3.83 (1H, dd, $J = 3.5$, 10.0 Hz, H-5‴), 3.74 (1H, dd, $J = 2.5$, 12.0 Hz, H-6″), 3.57 (1H, dd, $J = 5.0$, 12.0 Hz, H-6″), 3.56 (1H, m, H-3‴), 3.43-3.49 (4H, m, H-2″, 3″, 4″, 4‴), 3.25 (1H, m, H-5″), 1.25 (3H, d, $J = 6.0$ Hz, H-6‴); ^{13}C-NMR (125 MHz, CD_3OD) δ: 179.6 (C-4), 163.6 (C-7), 163.0 (C-5), 159.5 (C-2), 158.1 (C-8a), 152.6 (C-4′), 148.9 (C-3′), 135.5 (C-3), 124.3 (C-5′), 122.2 (C-1′), 116.5 (C-6′), 114.4 (C-2′), 107.6 (C-4a), 103.5 (C-1″), 100.7 (C-6), 99.9 (C-1‴), 95.6 (C-8), 78.7 (C-5″), 78.2 (C-3″), 76.1 (C-2″), 73.7 (C-4‴), 72.2 (C-5‴), 71.8 (C-2‴), 71.6 (C-4″), 71.4 (C-3‴), 62.6 (C-6″), 56.8 (C-3′-O<u>C</u>H$_3$), 18.2 (C-6‴).

Isorhamnetin 3-*O*-β-D-glucoside (**8**): Yellowish amorphous powder; $[\alpha]_D^{20}$ -21.7° (MeOH; *c* 0.06); IR ν_{max} (film) cm^{-1}: 3480, 3100, 1645, 1610; ESI–MS, *m/z*: 477[M-H]$^-$; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 7.93 (1H, d, $J = 2.0$ Hz, H-2′), 7.45 (1H, dd, $J = 2.0$, 8.5 Hz, H-6′), 6.89 (1H, d, $J = 8.5$ Hz, H-5′), 6.30 (1H, d, $J = 1.5$ Hz, H-8), 6.09 (1H, d, $J = 1.5$ Hz, H-6), 5.54 (1H, d, $J = 7.5$ Hz, H-1″), 3.83 (3H, s, 3′-O<u>C</u>H$_3$), 3.58 (1H, dd, $J = 2.5$, 12.0 Hz, H-6″), 3.38 (1H, dd, $J = 5.0$, 12.0 Hz, H-6″), 3.21-3.23 (2H, m, H-2″, 3″), 3.10-3.12 (2H, m, H-4″, 5″); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 176.7 (C-4), 161.1 (C-5, 7), 156.7 (C-8a), 155.6 (C-2), 149.4 (C-4′), 146.8 (C-3′), 132.7 (C-3), 121.8 (C-6′), 121.1 (C-1′), 115.2 (C-5′), 113.4 (C-2′), 102.8 (C-4a), 100.9 (C-1″), 99.5 (C-6), 94.0 (C-8), 77.4 (C-5″), 76.4 (C-3″), 74.3 (C-2″), 69.8 (C-4″), 60.5 (C-6″), 55.6 (C-3′-O<u>C</u>H$_3$).

3′-*O*-methyl-(−)-epicatechin 7-*O*-β-D-glucoside (**9**): Colorless powder; $[\alpha]_D^{20}$ -77.3° (EtOH; *c* 0.42); IR ν_{max} (film) cm^{-1}: 3400, 1704, 1632, 1600; ESI–MS, *m/z*: 465[M-H]$^-$; ^{1}H-NMR (500 MHz, DMSO-d_6) δ: 7.04 (1H, d, $J = 2.0$ Hz, H-2′), 6.83 (1H, dd, $J = 2.0$, 8.0 Hz, H-6′), 6.72 (1H, d, $J = 8.0$ Hz, H-5′), 6.10 (1H, d, $J = 2.0$ Hz, H-8), 6.02 (1H, d, $J = 2.0$ Hz, H-6), 4.82 (1H, s, H-2), 4.70 (1H, d, $J = 7.5$ Hz, H-1″), 4.05 (1H, m, H-3), 3.76

(3H, s, 3′-OC<u>H</u>$_3$), 3. 08 (1H, dd, $J = 2.0$, 12.0 Hz, H-6″), 3.65 (1H, dd, $J = 5.0$, 12.0 Hz, H-6″), 3.12-3.43 (4H, m, H-2″, 3″, 4″, 5″), 2.73 (1H, dd, $J = 4.5$, 17.0 Hz, H-4), 2.54 (1H, dd, $J = 2.5$, 17.0 Hz, H-4); ^{13}C-NMR (125 MHz, DMSO-d_6) δ: 156.6 (C-7), 156.5 (C-5), 155.7 (C-8a), 146.9 (C-3′), 145.8 (C-4′), 130.4 (C-1′), 119.7 (C-6′), 114.7 (C-5′), 111.6 (C-2′), 101.5 (C-4a), 100.7 (C-1″), 96.4 (C-8), 95.1 (C-6), 78.3 (C-2), 76.9 (C-3″), 76.6 (C-5″), 73.2 (C-2″), 69.6 (C-4″), 64.6 (C-3), 60.3 (C-6″), 55.6 (C-3′-OC<u>H</u>$_3$), 30.7 (C-4).

Bioassay of IL-6

An IL-6 bioassay was carried out using a slight modification of an established method (Kim et al. 2003; Liu et al. 2006). Briefly, 500 μL of the MG-63 cells (3×10^4 cells/mL) in Dulbecco′s modified Eagle′s medium (DMEM) containing 10 % fatal bovine serum (FBS) were dispensed into a 24-well plate; the culture was incubated for 24 h at 37 °C. Then, 5 μL of TNF-α (10 ng/mL), 5 μL of BAY 11-7085 (10 ng/mL) and 5 μL of DMSO with or without the compounds (100 μg/mL) were added. After incubation at 37 °C with 5 % CO_2 for 24 h, the medium was stored at −20 °C until measurement. The IL-6 content of the medium was measured in an enzyme-linked immunosorbent assay (ELISA) procedure. The 96-well plates were coated with 100 μL of purified rat anti-human IL-6 monoclonal antibody in 0.1 M $NaHCO_3$ (pH 9.6) by overnight incubation at 4 °C. The wells were blocked with 200 μL of 3 % BSA in PBS for 2 h at room temperature and then incubated with 100 μL of specific antibody for 2 h at rt. Horseradish peroxidase (HRP) conjugated rabbit anti-goat IgG (100 μL, 1:1000 dilution) was added to each well and incubated for 2 h at rt. TMB (3,3′,5,5′-tetramethyl-benzidine) substrate solution (100 μL) was added and incubated for 10 min at rt. The color reaction was stopped by addition of 50 μL of 0.4 N HCl and the optical density was measured at 450 nm using a Microplate Reader (Molecular Devices Co., Ltd., U.S.A.).

Results and discussion

Repeated column chromatography of the *n*-BuOH soluble fraction of the seeds of *R. sativus* yielded a new indole glycoside, named raphanuside A (**1**), as well as eight known phenolic compounds **2**–**9** (Fig. 1).

Compound **1** was obtained as a yellowish amorphous powder, $[\alpha]_D^{20}$ +31.0° (MeOH; *c* 0.26). Its molecular formula was determined to be $C_{16}H_{18}O_7NS$ by HR-ESI–MS (negative mode) *m/z*: 368.0797 $[M-H]^-$ (calcd for $C_{16}H_{18}O_7NS$, 368.0804). In the IR spectrum, absorption bands for the imino (3480, 3420, 3300 cm^{-1}) and carbonyl (1620 cm^{-1}) groups were observed. The ^{1}H-NMR spectrum of **1** showed four aromatic protons at δ_H 7.98 (1H, m, H-4), 7.39 (1H, m, H-7) and 7.13 (2H, m, H-5,6), a glucosyl anomeric proton at δ_H 5.75 (1H, d, $J = 7.5$ Hz, H-1′), thiomethyl protons at δ_H 2.66 (3H, s, –SCH_3) and characteristic signals attributable to the glucose moiety. Furthermore, in the ^{1}H-NMR spectrum measured in pyridine-d_5 instead of CD_3OD, the -NH group was identified at δ_H 12.85 (br. s) and confirmed by its disappearance in a D_2O exchange experiment. In the ^{13}C-NMR spectrum, 13 carbon signals appeared in addition to those of the sugar unit, including one carbonyl carbon at δ_C 165.8 (C-8), four aromatic methine carbons at δ_C 123.1 (C-6), 122.9 (C-5), 121.6 (C-4) and 111.8 (C-7), four quaternary carbons at δ_C 148.2 (C-2), 138.5 (C-7a), 128.7 (C-3a) and 103.9 (C-3), one thiomethyl carbon at δ_C 14.6 (–SCH_3). These spectral data indicated that **1** was to an indole 3-carboxylate ester. The signals from the sugar unit appeared at δ_H 5.75 (1H, d, $J = 7.5$ Hz, H-1′), 3.62 (1H, t, $J = 8.5$ Hz, H-2′), 3.50 (1H, t, $J = 8.5$ Hz, H-3′), 3.42-3.46 (2H, m, H-4′, H-5′), 3.87 (1H, dd, $J = 2.0$, 12.0 Hz, H-6′a), 3.72 (1H, dd, $J = 5.0$, 12.0 Hz, H-6′b) [δ_C 95.5 (C-1′), 74.4 (C-2′), 78.6 (C-3′), 71.3 (C-4′), 79.0 (C-5′), 62.6 (C-6′)] which strongly supported the presence of D-glucopyranose (Ishimaru et al. 1987; Jin et al. 2014). In addition, acid hydrolysis of **1** in refluxing 1 N-HCl/MeOH afforded (+)-D-glucose which was detected by direct comparison with an authentic sample using co-TLC (Kim et al. 2004). The coupling constant ($J = 7.5$ Hz) of the anomeric proton of D-glucose indicated it to be in the β-form (Ishimaru et al. 1987). The glycosidic linkage was established by an HMBC experiment and comparison of the reported NMR data (Jin et al. 2011), with the correlation between H-1′ and C-8 suggesting that glucose was attached to C-8 of indole 3-carboxylate moiety. Furthermore, long-range correlations were observed between the following protons and carbons: H-4 and C-7a; H-5 and C-3a; H-6 and C-7a; H′-1 and C-8; –SCH_3 and C-3 (Fig. 2). In the ^{1}H-^{1}H COSY spectrum, the aromatic proton of H-4 showed couplings with H-5, H-6 and H-7. Accordingly, the structure of **1** was proposed to be β-D-glucopyranosyl 2-(methylthio)-1*H*-indole-3-carboxylate, and named, raphanuside A.

Compound **2** was obtained as an amorphous powder, $[\alpha]_D^{20}$ + 10.4° (MeOH; *c* 0.36). The positive mode FAB-MS spectrum showed a quasi-molecular ion peaks at *m/z* 571$[M + Na]^+$, consistent with a molecular formula of $C_{23}H_{32}O_{15}$. The ^{1}H-NMR spectrum of **2** indicated a *trans* olefinic protons at [δ_H 7.63 (1H, d, $J = 16.0$ Hz, H-7″), 6.46 (1H, d, $J = 16.0$ Hz, H-8″)], a 1,3,4,5-tetrasubstituted aromatic ring protons at [δ_H 6.95 (2H, s, H-2″, 6″)] and two methoxy groups at δ_H 3.87 (6H, s). Furthermore, the ^{1}H and ^{13}C NMR spectrum showed a characteristic α-anomeric resonance at δ_H 5.42 with a small coupling

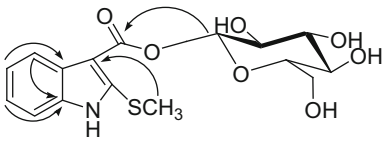

Fig. 2 Key HMBC (H → C) correlations of compound **1**

constant (d, $J = 3.5$ Hz, H-1′), together with 12 oxygenated carbon signals in the range δ_C 105.3–64.4. These data implied that compound **2** had a disaccharide moiety composed of a pentose and hexose unit. On the basis of chemical evidence, detailed analysis by ^{1}H–^{1}H COSY, HMQC, and HMBC experiments, and comparison of spectroscopic data with literature reports, compound **2** was identified as β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-glucopyranoside, sibiricose A1, which was previously isolated from the roots of *Polygala sibirica* (Ikeya et al. 1991; Miyase et al. 1999).

The structures of seven other compounds were identified as (3-*O*-sinapoyl)-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside (**3**) (Bashir et al. 1993; Miyase et al. 1999), (3-*O*-sinapoyl)-β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**4**) (Bashir et al. 1993; Kim et al. 2014), (3,4-*O*-disinapoyl)-β-D-fructofuranosyl-(2 → 1)-(6-*O*-sinapoyl)-α-D-glucopyranoside (**5**) (De Tommasi et al. 1993; Kim et al. 2014), isorhamnetin 3,4′-di-*O*-β-D-glucoside (**6**) (Nørbæk et al. 1999; Park et al. 2007), isorhamnetin 3-*O*-β-D-glucoside-7-*O*-α-L-rhamnoside (**7**) (Park et al. 2007), isorhamnetin 3-*O*-β-D-glucoside (**8**) (Woo et al. 1983) and 3'-*O*-methyl-(−)-epicatechin 7-*O*-β-D-glucoside (**9**) (Korver and Wilkins 1971; Tschesche et al. 1980; Morimoto et al. 1985), by comparing their spectroscopic data with literature reports.

Interleukin-6 (IL-6) is a cytokine that can facilitate autoimmune phenomena, amplify acute inflammation and promote evolution into a chronic inflammatory state (Fonseca et al. 2009). Dysregulation of IL-6 production has been implicated in a variety of inflammatory and autoimmune disease states, including rheumatoid arthritis, cardiac myxoma, Castleman′s disease and mesangial proliferative glomerulonephritis (Hirano et al. 1990). The proinflammatory cytokines IL-1 and TNF-α markedly stimulate the production of IL-6 (Van Damme et al. 1987). Identification of plant-derived compounds, such as phenolic compounds, able to selectively interfere with the production and/or function of cytokines could offer an important alternative for the treatment of many inflammatory diseases (Calixto et al. 2004; Comalada et al. 2006). It has been observed that several flavonoids are able to decrease the expression of different proinflammatory cytokines/chemokines, including TNF-α, IL-1β, IL-6, IL-8, and MCP-1, in many cell types such as lipopolysaccharide (LPS)-activated mouse primary macrophages, phorbol 12-myristate 13-acetate (PMA) or phytohemagglutinin (PHA) stimulated human peripheral blood mononuclear cell (Comalada et al. 2006), activated human astrocytes (Sharma et al. 2007), activated human mast cell line HMC-1 (Min et al. 2007), nasal mucosal fibroblasts and A549 bronchial epithelial cells (Kim et al. 2006).

Table 1 Inhibitory effect of compounds **1–9** against IL-6 production in TNF-α stimulated MG 63 cells

Treatment	IL-6 (pg/mL)	Inhibition (%)
None	19.7 ± 3.7	–
TNF-α	250.6 ± 3.4	–
BAY 11-7085	30.2 ± 2.1**	87.9**
Compound 1	215.0 ± 6.8*	14.0*
Compound 2	142.8 ± 3.8**	42.9**
Compound 3	138.3 ± 3.9**	44.7**
Compound 4	133.8 ± 5.8**	46.6**
Compound 5	107.6 ± 10.5**	57.1**
Compound 6	177.5 ± 5.0**	29.0**
Compound 7	172.0 ± 4.4**	31.2**
Compound 8	182.8 ± 5.4**	26.9**
Compound 9	193.5 ± 4.3**	22.6**

MG-63 cells (3×10^4) were incubated for 24 h. Cultures were incubated with or without compounds (100 μg/mL) for 30 min and then stimulated with TNF-α (10 ng/mL) for 24 h. IL-6 in the supernatant was measured by ELISA as described in Materials and Methods. Results are expressed as the mean ± SE from three different experiments. BAY 11-7085 was used as a positive control. * $P < 0.05$ or ** $P < 0.01$ compared with TNF-α treated value

In our study, the inhibitory activity of the isolated compounds (**1–9**) against IL-6 production in TNF-α stimulated MG-63 cells was examined. Among them, compounds **2–5** showed moderate inhibitory activity against IL-6 production in TNF-α stimulated MG-63 cells, while raphanuside A (**1**), and compounds **6–9** showed negligible activity (Table 1). Recently, Kim et al. reported the inhibitory effects of phenylpropanoid sucrosides from the seeds of *R. sativus* on NO production in LPS-activated murine microglia BV-2 cells (Kim et al. 2015).

In conclusion, this paper reports the isolation, characterization and inhibitory activity of nine isolates, including one new compound and eight known compounds, from the seeds of *R. sativus*.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest

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