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Pharmacological activity and quantitative analysis of flavonoids isolated from the flowers of *Begonia semperflorens* Link et Otto

Jung-Hwa Kwon[1], Hyun-Ji Oh[1], Dong-Sung Lee[2], Seo-Ji In[1], Kyeong-Hwa Seo[3], Jae-Woo Jung[1], Byeong-Ju Cha[1], Dae Young Lee[4*] and Nam-In Baek[1*]

Abstract

Begonia semperflorens Link et Otto has been broadly raised up for ornamental purpose as well comestible blossom. As the reproductive structures of phanerogams, flowers contain various secondary metabolites and have many biological activities. Accordingly, we began the contrivance for isolation and analysis of flavonoids contained in *B. semperflorens* flowers. MeOH extraction of *B. semperflorens* followed solvent fractionation was prosecuted. Column chromatography of non-polar fraction gave four flavonoids using several resins. Identification of the flavonols were established as quercetin (**1**), kaempferol (**2**), astragalin (**3**), and isoquercetin (**4**) by interpreting a variety of spectral information. Quercetin (**1**) and kaempferol (**2**) inhibited NO production and protected against *t*-BHP-induced oxidative stress. Kaempferol (**2**) also protected cell death of glutamate-treated HT22. Quantitative analysis of flavonoid content in *B. semperflorens* flowers was also performed using HPLC experiment.

Keywords: *Begonia semperflorens*, Hepatoprotective, Kaempferol, Neuroprotective, Nitric oxide, Quercetin

Introduction

A flower, as the reproductive organ of a plant, is pollinated by insects, water, and wind and produces various secondary metabolites, including volatiles, pigments, and flavonoids, for alluring pollinating insects as well definite pollination. Pollinators, especially insects, are attracted by floral colors and scents. Volatile compounds have been suggested as the main drivers of visitation decisions by pollinators [1–3]. Many flowers have UV patterns that are specifically visible to insects, and UV-absorbing pigments concentrated in the center of the flower increase its attractiveness [4].

Flowers have been used as ornamental plants for thousands of years because of their flavors, colors, and pleasing shapes. However, many flowers are also used as food ingredients. KFDA acknowledges approximately twenty edible flowers including pansies (*Viola tricolor*), jasmine (*Jasminum polyanthum*), camellia (*Camellia japonica*), peaches (*Prunus persica*), geranium (*Pelargonium inquinans*), and begonias (*Begonia semperflorens*). These flowers include a variety of active components showing anti-inflammatory [5], antioxidant [6], antibacterial [7], and NO-inhibition effects [8]. In addition, the Rural Development Administration (RDA) reported that edible flowers contain a 10-fold higher concentration of antioxidant constituents compared to vegetables and fruits. Among the edible flowers, *B. semperflorens* has a high content of total polyphenols and flavonoids, and NMR and MS analyses have shown it to contain anthocyanins [9, 10]. Therefore, the flowers of *B. semperflorens* were also expected to contain polyphenols and flavonoids.

B. semperflorens (Begoniaceae), native to Brazil, is broadly raised in tropical wetlands areas. This plant is in height by 15–45 cm with broad oval-shaped leaves, and its flowers bloom throughout the growing season until frost. As mentioned above, NMR and MS analyses of *B. semperflorens* flowers have shown the presence of acylated anthocyanins [11]. The anthocyanin cyanidin

*Correspondence: dylee0809@gmail.com; nibaek@khu.ac.kr
[1] Department of Oriental Medicine Biotechnology, Graduate School of Biotechnology, Kyung Hee University, Yongin 17104, Korea
[4] Department Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong 27709, Korea
Full list of author information is available at the end of the article

3-(2^G-xylosylrutinoside) was also reported from the leaves of this plant [12]. Anthocyanins provide photoprotection under stressful conditions [9].

In this study, four flavonoids were isolated from *B. semperflorens* flowers using extraction, fractionation as well repeated chromatography. The flavonoids were identified using spectroscopic methods, NMR, IR, MS. The flavonoids were quantitatively analyzed through HPLC experiment. And antioxidant, hepatoprotective, and neuroprotective effects of the flavonoids were then assessed.

Materials and methods

Plant materials

Begonia semperflorens flowers were acquired in Busan flower plantation, Korea, 2017, and Dr. D.G. Kim of Woosuk University, Jeonju, Korea identified. A voucher specimen (NPCL-20170716) was deposited at the Natural Products Chemistry Laboratory of Kyung Hee University, Yongin, Korea.

Reagents and instrumentation

The reagents and instruments used in this study were same as those used in the previous study [13].

Extraction of *Begonia semperflorens* flowers and isolation of flavonoids

Extraction of the fresh flowers of *B. semperflorens* (3.0 kg) was executed using 100% methanol (MeOH, 18 L) and 80% aqueous MeOH (27 L × 2) at r.t. for 24 h. The filtrates were evaporated under reduced pressure to yield an alcohol extract (Ext, 58 g). Ext was added to water (H_2O, 2 L) and successively fractionated with ethyl acetate (EtOAc, 2 L × 2) and *n*-butanol (*n*-BuOH, 2 L × 2). The evaporated EtOAc Ext (BSE, 11.2 g) was put in application for SiO_2 column chromatography (CC) (7 × 15 cm) and eluated by *n*-hexane:EtOAc (15:1 → 10:1 → 7:1 → 3:1 → 1:1, 7 L of each). Fraction (Fr) BSE-12 (459.0 mg, elution volume/total volume (VET) 0.772–0.797) was subjected to ODS CC (3.5 × 5 cm) and eluated by acetone:H_2O (1:2, 8 L), resulting in 6 Frs (BSE-12-1 to BSE-12-6) with isolation of **1** in BSE-12-3 (4.6 mg, VET 0.070–0.172, TLC using ODS R_f 0.51 in 4:2 acetone:H_2O). Fr BSE-12-5 (126.5 mg, VET 0.175–0.787) was subjected to SiO_2 CC (2.5 × 13 cm) and eluated by $CHCl_3$:MeOH:H_2O (36:3:1 → 25:3:1 → 18:3:1 → 65:35:10, 470 mL of each), resulting in 6 Frs (BSE-12-5-1 to BSE-12-5-6) with isolation of **2** in Fr BSE-12-5-2 (3.5 mg, VET 0.118–0.101, TLC using ODS R_f 0.42 in 4:2 acetone:H_2O). Fr BSE-18 (5.85 g, VET 0.956–1.000) was subjected to SiO_2 CC (5.0 × 13 cm) and eluated by $CHCl_3$:MeOH:H_2O (20:3:1, 15.7 L), resulting in 20 Frs (BSE-18-1 to BSE-18-20). Fr BSE-18-15 (137.3 mg, VET 0.573–0.725) was subjected to SiO_2 CC (3.0 × 14 cm) and eluted by $CHCl_3$:MeOH:H_2O (25:3:1 → 20:3:1, 4.1 L of both), resulting in 7 Frs (BSE-18-15-1 to BSE-18-15-7) with isolation of **3** in Fr BSE-18-15-2 (6.7 mg, VET 0.257–0.324, TLC using ODS R_f 0.43 in 2:2 acetone:H_2O) and **4** in Fr BSE-18-15-4 (22.5 mg, VET 0.545–0.665, TLC using ODS R_f 0.50 in 2:2 acetone:H_2O).

quercetin (**1**) yellow crystals; m.p. 277 °C; IR_v (KBr) 3425, 1660, 1610, and 1505 cm^{-1}; positive FAB/MS (pFABMS) *m/z* 303 $[M+H]^+$.

kaempferol (**2**) light yellow crystals; m.p. 178–180 °C; IR_v (KBr) 3396, 3021, 2867, 1642, and 1609 cm^{-1}; EI/MS *m/z* 286 $[M]^+$, 258, 229, 213, 184, 153, and 121.

astragalin (**3**) yellow crystals; m.p. 230–232 °C; $[\alpha]_D^{25}$ +16.0°; IR_v (KBr) 3420, 1680, and 1628 cm^{-1}; pFABMS *m/z* 449 $[M+H]^+$ and 287.

isoquercetin (**4**) yellow crystals; m.p. 230–232 °C; $[\alpha]_D^{25}$ 230–231°; IR_v (KBr) 3400, 2919, 1656, 1606, and 1508 cm^{-1}; pFABMS *m/z* 465 $[M+H]^+$, 447, 423, 389, 297, and 204.

^{1}H-NMR (400 MHz, CD_3OD, δ_H) and ^{13}C-NMR (100 MHz, CD_3OD, δ_C) see Table 1.

Inhibitory effects on NO production in LPS-induced RAW 264.7

Cell culture of murine macrophage RAW 264.7 cells and measurement of nitrite (NO) production can be referred to literature [14]. Butein was used as a positive control.

Protective effect on cell death of glutamate-treated HT22

Cytoprotective effect was assayed according to the same methods reported in literature [15]. Trolox was used as a positive control.

Protective effect on oxidative stress in treated HepG2 cells by *t*-BHP

Human hepatoma HepG2 cell culture and Hepatoprotective effect assay was accomplished using the same method reported in the previous study [14]. Curcumin was used as a positive control.

Quantitative analysis of the flavonoids isolated from *Begonia semperflorens* flowers

The MeOH Ext of *B. semperflorens* flowers was fractionated using EtOAc and H_2O. The organic phase Fr was utilized to analyze the isolated flavonoids. The flavonoids were diluted to various concentrations to establish calibration curves (**1**: 1.890625, 3.78125, 7.5625, 15.125, and 31.25 μg/mL; **2**: 3.78125, 7.5625, 15.125, 31.25, and 62.5 μg/mL; **3** and **4**: 15.125, 31.25, 62.5, 125, 250 μg/mL).

The equipment and materials for HPLC analysis were as the followings. An Waters 600S (Milford, MA), a

Table 1 ^{1}H- (400 MHz) and ^{13}C-NMR (100 MHz) data of compounds 1-4 from *Begonia semperflorens* flowers (CD_3OD)

Carbon number	δ_C				δ_H, coupling pattern, *J* in Hz			
	1	**2**	**3**	**4**	**1**	**2**	**3**	**4**
2	147.59	148.06	158.54	158.99				
3	137.70	137.27	135.47	135.63				
4	177.12	177.45	179.51	179.42				
5	162.28	162.53	162.80	162.97				
6	99.09	99.28	99.96	99.89	6.73, br. s	6.16, d, 2.0	6.19, d, 2.0	6.17, br. s
7	165.37	165.92	166.13	165.96				
8	94.16	94.45	94.80	94.72	6.68, br, s	6.36, d, 2.0	6.39, d, 2.0	6.36, br. s
9	157.33	158.26	159.08	158.39				
10	104.31	104.54	104.09	105.48				
1′	123.43	123.76	122.80	122.92				
2′	116.48	130.66	132.28	117.58	8.55, br. s	8.07, d, 9.2	8.05, d, 8.8	7.70, d, 2.0
3′	149.66	116.30	116.08	145.84		6.89, d, 9.2	6.88, d, 9.2	
4′	146.91	160.54	161.58	149.82				
5′	116.48	116.30	116.08	115.98	7.35, d, 8.4	6.89, d, 9.2	6.88, d, 9.2	6.85, d, 8.4
6′	120.93	130.66	132.28	123.19	8.08, br. d, 8.4	8.07, d, 9.2	8.05, d, 8.8	7.56, dd, 8.4, 2.0
1″			104.09	104.41			5.24, d, 7.2	5.22, d, 7.2
2″			75.74	75.70				
3″			78.43	78.32				
4″			71.37	71.17				
5″			78.05	78.08				
6″			62.64	62.53			3.68, dd, 12.0, 2.4 3.52, dd, 12.0, 5.2	3.71, dd, 12.0, 2.4 3.58, dd, 12.0, 5.2

reverse phase column (Waters C_{18}, 5 μm, 250 × 4.6 mm). The eluting solvents, aqueous 0.05% trifluoroacetic acid (A) and 100% acetonitrile (B). 0.6 mL/min with gradient of B: 0–5 min, 10–30%; 5–20 min, 30%; 20–23 min, 30–40%; 23–38 min, 40%; 38–43 min, 40–100%. Injection volume, 10 μL. Detection was carried out using a photodiode spectrophotometer at 280 nm. The analysis was repeated three times.

Results and discussion

TLC for alcohol Ext of *B. semperflorens* flowers revealed yellow spots after spraying with a 10% H_2SO_4 solution and heating, indicating the presence of flavonoids in the Ext. The Ext was fractionated into EtOAc, *n*-BuOH, and H_2O Frs through solvent fractionation. And repeated SiO_2 and ODS CC of EtOAc Fr afforded four flavonoid compounds. All compounds were isolated as yellow crystals and exhibited yellow spots on TLC plate after by same treatment, which led to deduction that they were flavonoids. The UV absorption pattern of the compounds confirmed the above-mentioned ratiocination.

The molecular weight (MW) of **1** was determined to be 302 amu based on the molecular ion peak (MIP) $[M+H]^+$ at *m/z* 303 in the pFABMS. IR spectrum showed absorption peaks at 3425 (OH), 1660 (conjugated ketone), and 1610 cm^{-1} (aromatic double bond). The ^{1}H-NMR (PMR) spectrum (400 MHz, CD_3OD) showed two olefin methine proton signals at δ_H 6.68 (br. s, H-8) and 6.73 (br. s, H-6) due to a 1,2,3,5-tetrasubstituted benzene ring and three olefin methine proton signals at 7.35 (d, $J=8.4$ Hz, H-5′, coupling pattern, coupling constant in *J* in Hz), δ_H 8.08 (br. d, 8.4, H-6′) and 8.55 (br. s, H-2′) due to a 1,2,4-trisubstituted benzene ring. The ^{13}C-NMR (CMR) (100 MHz, CD_3OD) spectrum included 15 carbon signals, suggesting **1** was a flavonoid. The five olefin methine carbon signals at δ_C 94.16 (C-8), 99.09 (C-6), 116.48 (C-5′), 116.48 (C-2′), and 120.93 (C-6′); two olefin quaternary carbon signals at 104.31 (C-10) and δ_C 123.43 (C-1′); seven oxygenated olefin quaternary carbon signals at δ_C 137.7 (C-3), 146.91 (C-4′), 147.59 (C-2), 149.66 (C-3′), 157.33 (C-9), 162.28 (C-5), and 165.37 (C-7); one conjugated ketone carbon signal at δ_C 177.12 (C-4) suggested that **1** was a flavonol. **1** was identified to be quercetin through intensive analysis of 2D-NMR (i.e., gHSQC and gHMBC) data as well comparison of the spectroscopic data with reported literature [16].

2 showed very similar NMR signals to those of **1** with the exception of the B-ring structure. The PMR signals

of a *para*-substituted benzene ring at δ_H 6.89 (2H, d, 9.2, H-3′,5′) and 8.07 (2H, d, 9.2, H-2′,6′), as well the CMR signals of four olefin methines at δ_C 116.30 (C-3′,5′) and 130.66 (C-2′,6′), one olefin quaternary at δ_C 123.76 (C-1′), and one oxygenated olefin quaternary at δ_C 160.54 (C-4′) indicated that **2** was 5,7,4′-trihydroxyflavonol, kaempferol. The identification of **2** was confirmed through the molecular weight (MW) of 286 amu, which was 16 amu less than that of **1**.

3 showed similar NMR signals as those of **2** with the exception of additional signals due to a *β*-glucopyranose. The hemiacetal PMR signal at δ_H 5.24 (d, 7.2, H-1″) and the chemical shifts of CMR signals confirmed the presence of a *β*-glucopyranosyl moiety. MW of **3** was determined to be 448 amu from a MIP $[M+H]^+$ at *m/z* 449 in FABMS spectra, which was 162 amu more than that of **2**. The *β*-glucopyranose was revealed to be linked to the 3-OH in the C-ring from the cross-peak between the anomeric PMR signal at δ_H 5.24 (d, 7.2, H-1″) and an oxygenated olefin quaternary CMR signal at δ_C 135.47 (C-3) in the gHMBC spectrum. **3** was identified to be kaempferol 3-*O*-*β*-D-glucopyranoside, astragalin.

4 showed similar NMR signals as those of **1** with the exception of additional *β*-glucopyranose signals. The hemiacetal PMR signal at δ_H 5.22 (d, 7.2, H-1″) and the chemical shifts of CMR signals confirmed the presence of a *β*-glucopyranosyl moiety. MW was determined to be 464 amu from MIP $[M+H]^+$ at *m/z* 465 in pFABMS spectrum, which was 162 amu more than that of **1**. The 3-OH linkage of the *β*-glucopyranose was determined from the cross-peak between the anomeric PMR signal at δ_H 5.22 (d, 7.2, H-1″) and an oxygenated olefin quaternary CMR signal at δ_C 135.63 (C-3) in the gHMBC spectrum. **4** was identified to be quercetin 3-*O*-*β*-D-glucopyranoside, isoquercetin. This study is the first report for isolation of the flavonoids from *B. semperflorens* flowers.

Inhibitory effects on NO production in RAW 264.7 treated by LPS

1 and **2** were estimated for inhibition effect against NO generation in LPS-treated RAW 264.7. LPS-stimulated macrophages were treated with each compound (**1**: 1, 5, 10, or 20 μM; **2**: 5, 10, 20, or 40 μM). As can be seen in Fig. 1, **1** and **2** dose-dependently suppressed NO production in RAW 264.7. **1** and **2** showed slightly lower effect than butein. IC_{50} value of **1** and **2** was respectively estimated as 84.79 and 80.87 μM. Previous studies have also reported the suppressive activity of **1** and **2** on NO generation. Naturally occurring flavonoids are known to modulate various inflammatory and immune processes. Genistein inhibits NO synthase expression and NO generation with IC_{50} value, 26.8 μM [17].

Neuroprotective effects against glutamate-induced cell death in HT22

1-**4** were investigated for their protective effects against glutamate-induced cell death in HT22 cells. Glutamate-stimulated HT-22 cells were treated with the compounds and trolox (100 μM). As shown in Fig. 2, **2** showed considerable protection (99.1%) against glutamate-induced toxicity at the low concentration at 80 μM, which was a

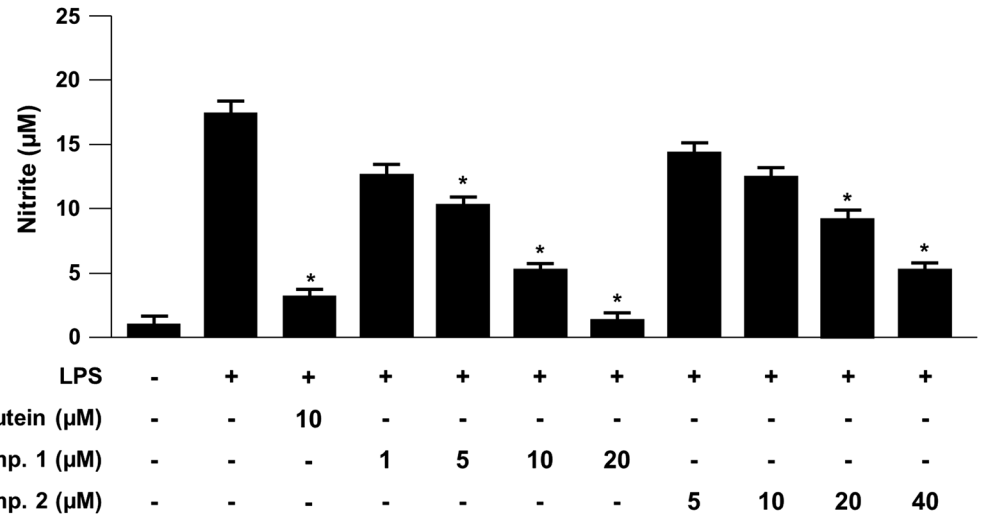

Fig. 1 Inhibitory activity of **1** and **2** on NO generation in RAW 264.7 induced by LPS. The cells were pretreated for 12 h with the indicated concentrations of compounds and stimulated for 18 h with LPS (1 μg/mL). The error bars represent the mean ± SD of three independent experiments. *$p < 0.05$ compared to the LPS-treated control group. Positive control, butein

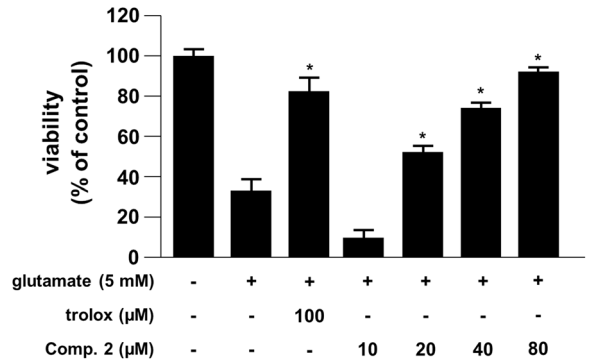

Fig. 2 Neuroprotective activity against cell death in HT22 cells treated by glutamate. Treatment of HT22 with 5 μM glutamate raised the formation of ROS. The error bars represent the mean ± SD of three independent tests. *$p < 0.05$ compared with glutamate-treated group. Positive control, trolox

higher protective effect than trolox (82.0%, 100 μM). The EC_{50} value of **2** was 19.95 μM. A previous study reported that **2** (100 μM) protected HT22 cells against glutamate-induced cytotoxicity by 62.4 ± 2.8% [18].

Hepatoprotective activity against oxidative stress in t-BHP-induced HepG2

To examine the protective effect against *t*-BHP-induced oxidative stress in HepG2, the cells were treated with **1** and **2** (**1**: 5, 10, 20, or 40 μM; **2**: 10, 20, 40, or 80 μM) or curcumin (20 μM). As shown in Fig. 3, **1** and **2** showed high protective effects (86.5 and 78.7%, respectively) against *t*-BHP-induced cytotoxicity at a concentration of 20 μM, which was almost the same as that of the positive control, curcumin. EC_{50} value of **1** and **2** was calculated to be 1.019 and 5.321 μM, respectively. A previous study also reported **1** to show protective effect against *t*-BHP-induced oxidative stress [19].

Many flavonoids have been shown to have various pharmacological activities. Quercetin (**1**) is effective against inflammation, arteriosclerosis, bleeding, allergies, and swelling [10, 20]. Kaempferol (**2**) has antidiabetic [21] and antioxidant as well as anticancer [22] activities. Astragalin (**3**) exhibits antioxidant [23], anti-HIV [24], and anti-allergen [25] activities, and isoquercetin (**4**) shows antioxidant [26], anti-inflammatory [27], and anti-tumor [28] activities. Compounds **1** and **2** were proved to have anti-inflammatory, neuroprotective, and hepatoprotective effects through our experiments and previous studies as well. The compounds are sure to have potential to be developed as new drugs.

Quantitative HPLC analysis of the flavonoids in *Begonia semperflorens* flowers

Using HPLC, each flavonoid peak was clearly separated and identified through comparison of the retention time with those of the standards (Fig. 4). The calibration curves were built using various concentrations of each compound (**1**: 1.890625, 3.78125, 7.5625, 15.125, and 31.25 μg/mL; **2**: 3.78125, 7.5625, 15.125, 31.25, and 62.5 μg/mL; **3** and **4**: 15.125, 31.25, 62.5, 125, and 250 μg/mL). The regression equations and correlation coefficient (r^2 0.9996–1.000) for **1**–**4** are listed in Table 2. The

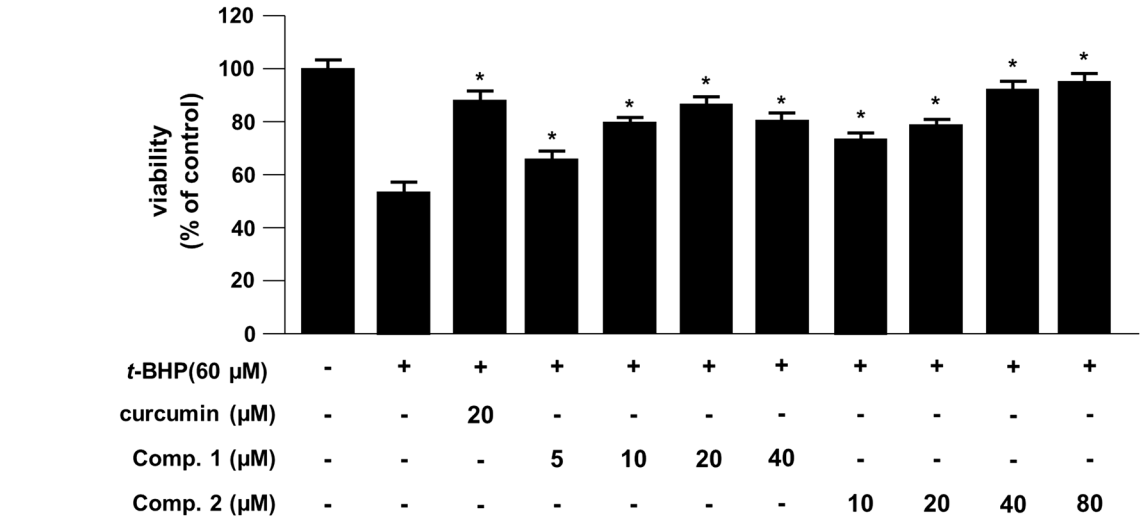

Fig. 3 Hepatoprotective activity against oxidative stress in *t*-BHP-induced HepG2. Cytotoxicity was estimated after incubating the cells with 60 μM *t*-BHP in RPMI. The error bars represent the mean ± SD of three independent tests. *$p < 0.05$ compared with *t*-BHP-treated group. Positive control, curcumin

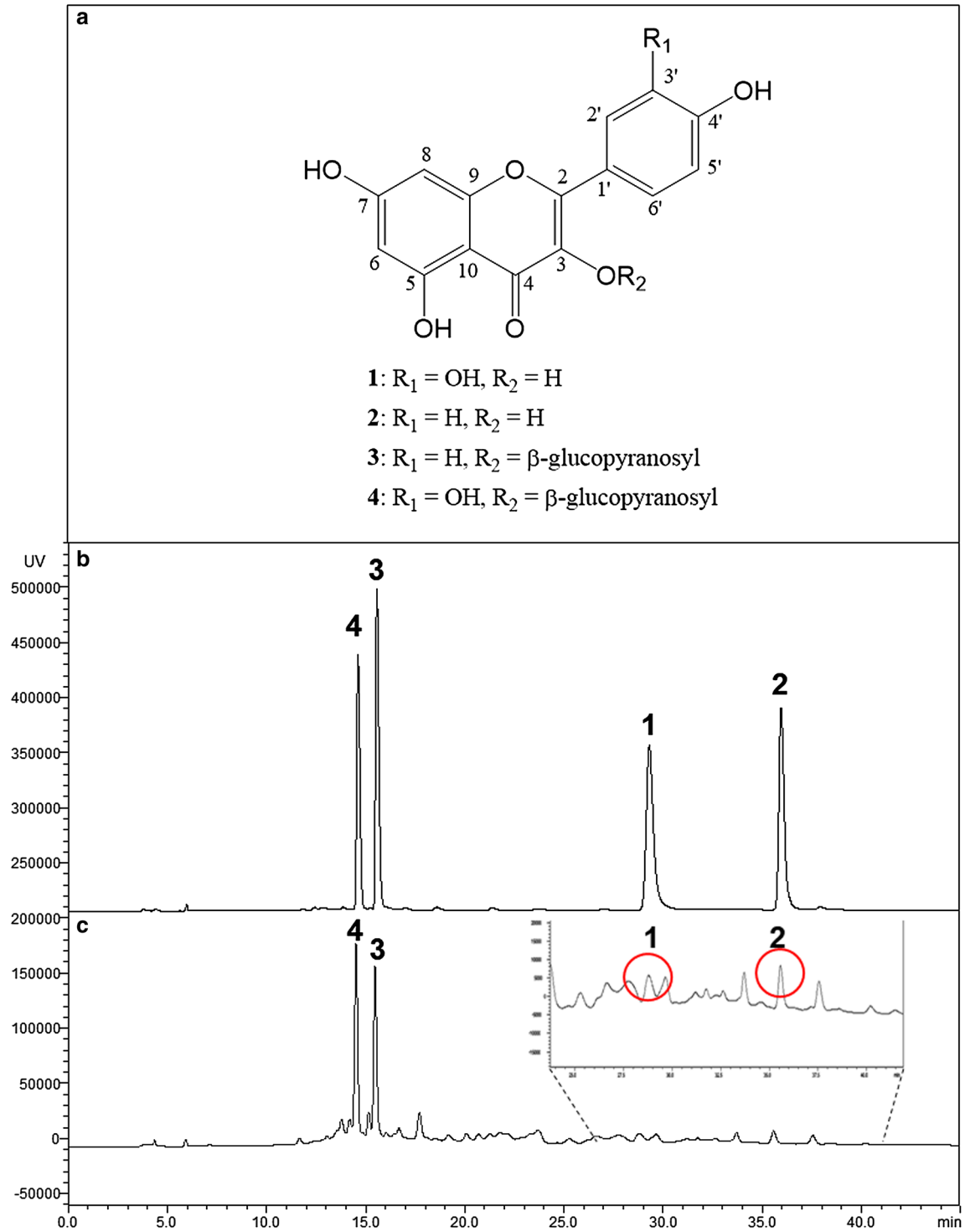

Fig. 4 Molecular structures of flavonoids from *Begonia semperflorens* flowers. **a** HPLC chromatogram of the isolated flavonoids (**b**) and EtOAc fraction (**c**) from the flowers of *Begonia semperflorens*. **1** (quercetin, rt: 29.3′), **2** (kaempferol, rt: 36.0′), **3** (astragalin, rt: 15.6′), **4** (isoquercetin, rt: 14.6′)

Table 2 Contents of flavonoids in EtOAc fraction of *Begonia semperflorens* flowers

Compound	RT (min)	Calibration curve fit	R^2	Content (%)
1	29.3	y = 25,644.2202x− 40,789	0.9997	0.3 ± 0.02
2	36.0	y = 18,150x−10,7941	0.9997	0.8 ± 0.09
3	15.6	y = 49,027x−35,220	1.0000	7.1 ± 0.16
4	14.6	y = 24,033x−32,222	0.9996	11.9 ± 0.03

y = area units, x = concentration in standard solution (ppm)

high value of each r^2 confirmed this analysis to be reliable. The concentrations of **1**–**4** were determined using the peak areas in the chromatogram and the regression equations (Table 2). The contents of **1**–**4** in the EtOAc Fr were calculated to be 0.3 ± 0.02, 0.8 ± 0.09, 7.1 ± 0.16, and 11.9 ± 0.03%, respectively.

Authors' contributions

J-HK and H-JO isolated the compounds and elucidated the structures; J-WJ contributed to the plant materials preparation; D-SL carried out the biological assay and helped with the preparation of the manuscript; S-JI and K-HS performed the NMRs, and HPLC of the samples; DYL assisted the revision of the manuscript; J-WK wrote the paper; N-IB designed and managed the research project. All authors read and approved the final manuscript.

Author details

[1] Department of Oriental Medicine Biotechnology, Graduate School of Biotechnology, Kyung Hee University, Yongin 17104, Korea. [2] Chosun University, Gwangju 61452, Korea. [3] Biological and Genetic Resources Utilization Division, National Institute of Biological Resources, Incheon 22689, Korea. [4] Department Herbal Crop Research, National Institute of Horticultural and Herbal Science, RDA, Eumseong 27709, Korea.

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Competing interests

The authors declare that they have no competing interests.

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