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RESEARCH ARTICLE

Simultaneous analysis and peroxynitrite-scavenging activity of galloylated flavonoid glycosides and ellagic acid in *Euphorbia supina*

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Abstract The herbs of *Euphorbia supina* (Euphorbiaceae) have been used to treat hemorrhage, chronic bronchitis, hepatitis, jaundice, diarrhea, gastritis, and hemorrhoids as a medicinal herb. This work is aimed to qualitatively and quantitatively analyze the polyphenols with peroxynitrite-scavenging activities. The eight compounds: gallic acid, methyl gallate, avicularin, astragalin, juglanin, isoquercitrin 6″-gallate, astragalin 6″-gallate, and ellagic acid, were isolated from *E. supina* and used for HPLC analysis and peroxynitrite ($ONOO^-$)-scavenging assay. Simultaneous analysis of the eight compounds was performed on MeOH extract and its fractions. The contents in MeOH extract and peroxynitrite-scavenging activities of the dimer of gallic acid, ellagic acid (15.64 mg/g; IC_{50} 0.89 μM), and two galloylated flavonoid glycosides, astragalin 6″-gallate (13.72 mg/g; IC_{50} 1.43 μM) and isoquercitrin 6″-gallate (16.99 mg/g; IC_{50} 1.75 μM), were high, compared to other compounds. The legendary uses of *E. supina* could be attributed to the high content of polyphenols, particularly ellagic acid, isoquercitrin 6″-gallate, and astragalin 6″-gallate as active principles.

Keywords Euphorbiaceae · *Euphorbia supina* · Galloylated flavonoid glycosides · Peroxynitrite · HPLC

Introduction

Euphorbia supina Rafin belonging to the family "Euphorbiaceae" is an annual herbaceous plant growing on the roadside or on the ground in Korea. This plant is more commonly found in Korea than the taxonomically related plants, *E. humifusa* and *E. maculata* (Cha et al. 1996; Ko and Jeon 2003). The medicinal herb of *E. supina* has been known to be effective to treat bronchitis, jaundice, hemorrhage, and the gastrointestinal disease including gastritis or gastric ulcers, peptic ulcer, diarrhea, and hemorrhoid (An et al. 2007; Cha et al. 1996). An et al. (2007) noted the isolation of the phenolic compounds, scopoletin, *p*-hydroxybenzaldehyde, methyl gallate, gallic acid, quercetin, avicularin, juglanin, and astragalin.

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Peroxynitrite ($ONOO^-$) is one of reactive nitrogen species (RNS) that is formed through the combination of superoxide anion radical ($\cdot O_2^-$) and nitric oxide radical ($\cdot NO$). $ONOO^-$ is highly reactive oxidant, although it is not a radical but it is an ion (Pryor and Squadrito 1996). The excess production of $ONOO^-$ results in the peroxidation of lipids and proteins, cytotoxicity, and neurotoxicity and further hypercholesterolemia, atherosclerosis, obesity, or diabetes mellitus (Patcher et al. 2005; Drel et al. 2007; Korda et al. 2008).

Recently, it is known that the NO production in the gastric epithelial cells and *Helicobacter pylori*-induced NO production can result in gastrointestinal ulcer disease (Jang et al. 2009). $ONOO^-$ is protonated in very low pH conditions within the stomach to yield peroxynitrous acid (ONOOH) which is readily cleaved into nitrite ion ($\cdot NO_2^-$) and hydroxyl radical ($\cdot OH$). Of these, hydroxyl radical is considered to be the important factor for ulceration due to its high capability to induce tissue injury (Radi et al. 1991; Rachmilewitz et al. 1994). Therefore, the development of $ONOO^-$ scavengers is required because endogenous enzymes to scavenge $ONOO^-$ are lacking in the body (Kim et al. 2004).

Although medicinal drugs such as H_2-antagonist, e.g., cimetidine, ranitidine, and famotidine, and the proton pump inhibitors, e.g., omeprazole and rabeprazole are clinically used to treat peptic ulcers, the research to develop the $ONOO^-$ scavengers to treat this disease have been continued. In particular, it is reported that ellagic acid abundant in nuts, strawberry, raspberry, black raspberry, and grapefruit exhibits anti-ulcer activity through the mechanism to inhibit NO production and cytokine-induced reactive oxygen radical (ROS) generation (Alvarez-Suarez et al. 2011; Silva Beserra et al. 2011). Yasuhiro et al. (1997) also suggested a role of peroxynitrite in the pathogenesis of gastric lesion. Therefore, the utilization of the plant extract with potent $ONOO^-$ scavenging effect is beneficial for treatment or prevention of gastrointestinal ulcers.

Ellagic acid is a dimer esterified between the two gallic acids, and so it has four hydroxyl groups. Galloylated flavonoid glycosides refer to the flavonoid glycoside in which gallic acid esterifies to the sugar moiety. Because ellagic acid and two galloylated flavonoid glycosides, isoquercitrin 6″-gallate and astragalin 6″-gallate, were isolated from *E. supina* in the present study, we aim to identify the composition of polyphenols including those three substances. In this report, we describe the identification of polyphenols, quantitative analysis, analytical method, validation, and peroxynitrite-scavenging activity. In addition, structure–activity relationship (SAR) on $ONOO^-$-scavenging activity vs. polyphenols is also discussed.

Materials and methods

Plant material

The herbs of *E. supina* (Euphorbiaceae) were collected in Wonju city, Gangwon province, Korea on August, 2012. These herbs were dried in a dark area, finely cut, and used for the plant material. This plant was identified by Prof. S. C. Lim (Department of Horticulture and Landscape, Sangji University, Korea) and confirmed by comparing its characteristics with the description in the Korean Plants Encyclopedia (Lee 1983). The voucher specimen (natchem-#32) was deposited in the Laboratory of Natural Products Chemistry, Department of Pharmaceutical Engineering, Sangji University, Korea).

Reagents and instruments

The stationary phases of column chromatography were Silica gel 60 (70–230 mesh, Merck, Germany) and ODS (octadecylsilane) (12 nm, S-75 μm, YMC Co., Ltd., Japan), and TLC plates were silica gel 60 and RP-18 purchased from Merck Co. (Germany). HPLC system was the Varian HPLC system consisted of Prostar 210 pumps, Prostar 325 UV–Vis detector, Shiseido Capcell PAK C18 column (5 μm, 4.6 × 250 mm, Japan). Column temperature was constantly maintained using a MetaTherm temperature controller. The solvents as mobile phases, MeOH, CH_3CN, and H_2O were the HPLC grade purchased from J.T. Baker Co. (Phillipsburg, NJ, USA). The collected data was processed using the Varian Star Workstation. The reagents of diethylenetriaminepentaacetic acid (DTPA) (Sigma Co., St. Louis, MO, USA), dihydrorhodamine 123 (DHR 123) (Molecular Probes, Eugene, OR, USA), and peroxynitrite (Cayman Chemicals Co., Ann Arbor, MI, USA) were used for the $ONOO^-$-scavenging assay.

Extraction and fractionation

The dried herbs of *E. supina* (800 g) were extracted three times with 6 L MeOH for 5 h under reflux. This extracted solution was filtered, evaporated on a rotary evaporated under reduced pressure, and then further dried using a freeze-dryer to give a solid MeOH extract (195 g). For fractionation, this MeOH extract was suspended in H_2O and partitioned three times with 800 mL diethyl ether (ether). The ether layer was concentrated to give an ether fraction (47.9 g). In the same way, the water layer was successively fractionated with EtOAc and BuOH to deliver the EtOAc fraction (27.5 g) and BuOH fraction (32.9 g).

Isolation and identification

EtOAc fraction (25.0 g) was chromatographed on silica gel column chromatography (SiO_2 450 g, ø 60 mm × 35 cm) with $CHCl_3$-MeOH-H_2O (7:3:1, lower layer) to isolate polyphenolic compounds. Aliquots (#1-105) were collected by 50 mL (each), divided into EA-1 (#01-15), EA-2 (#16-24), EA-3 (#25-55), EA-4 (#56-95), EA-5 (#96-105) after TLC checking, grouping, and concentrating. These five fractions were used for further purification.

EA-2 (730 mg) was subjected to ODS column chromatography (150 g, ø 25 mm × 35 cm) with MeOH-H_2O (55:45), collected by 10 mL (each), grouped and concentrated to give EA-2a (#03-04), EA-2b (#09-11), EA-2c (#17-19), EA-2d (#25-30), and EA-2e (#49-51). Recrystallization of the five fraction from MeOH yielded the five compounds, gallic acid (compound **1**, 27 mg) from EA-2a, methyl gallate (compound **2**, 20 mg) from EA-2b, avicularin (compound **6**, 52 mg) from EA-2c, astragalin (compound **7**, 65 mg) from EA-2d, and juglanin (compound **8**, 15 mg) from EA-2e. These five compounds were identified by comparisons of ^{1}H-NMR and ^{13}C-NMR spectroscopic data with the literature data (An et al. 2007).

EA-4 was chromatographed on ODS column chromatography (150 g, ø 25 mm × 35 cm) with MeOH-H_2O (50:50) and collected by 20 mL (each). Aliquots were checked by TLC, grouped, and concentrated to yield the subfractions, EA-4a (#05-08), EA-4b (#13-16), EA-4c (#26-40). Recrystallization of the three fractions from MeOH yielded isoquercitrin 6″-gallate (compound **3**) from EA-4a, astragalin 6″-gallate (compound **5**) from EA-4b, and ellagic acid (compound **4**) from EA-4c. These were also identified by comparisons of physicochemical and spectroscopic data (^{1}H- and ^{13}C-NMR) with literature data (Liu et al. 1997; Srivastava et al. 2007). Those eight compounds were used for HPLC analysis to determine their quantities in *E. supina*. The purities of the standard compounds were more than 99.0 % as determined by calculating the peak area percentage.

Compound 3 (isoquercitrin 6″-gallate)

Yellowish powder from MeOH, mp 220–222 °C, UV (MeOH) λ_{max} nm (log ε): 265.5 (4.294), 213.0 (4.539); IR ν_{max} (KBr, cm^{-1}): 3,375 (br., O–H), 2,913 (aromatic C–H), 1,655 (α,β-unsaturated ketone), 1,082 (glycosidic C–O); ESI-HR-MS *m/z*: 617.1152 ([M+H]$^+$, cald. 616.1064 for $C_{28}H_{24}O_{16}$); ^{1}H-NMR (600 MHz, DMSO-d_6) δ: quercetin—6.18 (1H, d, J = 1.8 Hz, H-6), 6.37 (1H, d, J = 1.8 Hz, H-8), 7.44 (1H, d, J = 1.8 Hz, H-2′), 6.73 (1H, d, J = 8.4 Hz, H-5′), 7.57 (1H, dd, J = 1.8, 8.4 Hz, H-6′); D-glc—5.44 (1H, d, J = 7.2 Hz, H-1″), 3.43 (1H, m, H-2″), 3.29 (1H, m, H-3″), 3.36 (1H, m, H-4″), 3.29 (1H, m, H-5″), 4.27 (1H, dd-like, H_a-6″), 4.18 (1H, dd, J = 4.8, 12.0 Hz, H_b-6″); galloyl—6.90 (2H, s, H-2‴, 6‴); ^{13}C-NMR (150 MHz, DMSO-d_6) δ: 156.78 (C-2), 133.85 (C-3), 177.76 (C-4), 161.40 (C-5), 99.01 (C-6), 164.54 (C-7), 94.01 (C-8), 156.92 (C-9), 104.39 (C-10), 121.47 (C-1′), 116.26 (C-2′), 145.18 (C-3′), 148.83 (C-4′), 115.74 (C-5′), 122.35 (C-6′); D-glc—101.82 (C-1″), 74.72 (C-2″), 74.51 (C-3″), 70.02 (C-4″), 76.73 (C-5″), 63.56 (C-6″); galloyl—119.84 (C-1‴), 109.07 (C-2‴, 6‴), 145.84 (C-3‴, 5‴), 138.81 (C-4‴), 166.16 (C-7‴).

Compound 5 (astragalin 6″-gallate)

Yellowish powder from MeOH, mp 212–214 °C, UV (MeOH) λ_{max} nm (log ε): 265.5 (4.442), 210.0 (4.574); IR ν_{max} (KBr, cm^{-1}): 3,370 (br., O–H), 2,901 (aromatic C–H), 1,655 (α,β-unsaturated ketone), 1,081 (glycosidic C–O); ESI-HR-MS *m/z*: 601.1203 ([M+H]$^+$, cald. 600.1115 for $C_{28}H_{24}O_{15}$); ^{1}H-NMR (600 MHz, DMSO-d_6) δ: kaempferol—6.21 (1H, d, J = 1.8 Hz, H-6), 6.41 (1H, d, J = H-8), 7.95 (2H, d, J = 9.0 Hz, H-2′, 6′), 6.77 (2H, d, J = 9.0 Hz, H-3′, 5′); D-glc—5.47 (1H, d, J = 7.8 Hz, H-1″), 3.42 (1H, m, H-2″), 3.26 (1H, m, H-3″), 3.35 (1H, m, H-4″), 4.27 (1H, dd-like, H_a-6″), 4.18 (1H, dd, J = 3.6, 9.6 Hz, H_b-6″); galloyl—6.91 (1H, s, H-2‴, 6‴); ^{13}C-NMR (150 MHz, DMSO-d_6) δ: 156.88 (C-2), 133.62 (C-3), 177.78 (C-4), 161.62 (C-5), 99.19 (C-6), 164.56 (C-7), 94.18 (C-8), 157.25 (C-9), 104.43 (C-10), 121.27 (C-1′), 131.24 (C-2′, 6′), 115.50 (C-3′, 5′), 160.30 (C-4′); D-Glc—101.89 (C-1″), 74.52 (C-2″, 3″), 69.72 (C-4″), 76.52 (C-5″), 63.22 (C-6″); galloyl—119.82 (C-1‴), 109.00 (C-2‴), 145.84 (C-3‴), 138.74 (C-4‴), 145.84 (C-4‴), 109.00 (C-6‴), 166.10 (C-7‴).

Preparation of sample- and standard solutions

The standard stock solution (1,000 μg/mL) was prepared by dissolving each standard compound in MeOH and preserved at less than 4 °C. The working standard solution was prepared by serial dilutions of the standard stock solution. Regression equation was determined by plotting the peak area (y) vs. concentration (x, μg/mL) at six concentration solutions. MeOH extract and its fractions were employed for HPLC quantification analysis. Sample solutions were made by dissolving small amounts of the lyophilized samples and were melted and mixed on an ultrasonicator. Sample solutions were filtered using a disposable syringe filter (0.50 μm, Dismic-25JP, Advantec, Japan) prior to injection to HPLC system.

HPLC analytical method

The two solvents, solvent A and solvent B, were used for the mobile phases of HPLC. Solvent A was H_2O added

with 0.05 %-trifluoroacetic acid (TFA) and solvent B was MeOH-CH_3CN (60:40, v/v) added with 0.05 %-TFA. The gradient elution was progressed at a flow rate of 1.0 mL/min under the program of (A)/(B) = 85/15 (0 min) → 35/65 (45 min) → 10/90 (46 min, hold for 4 min) → 85/15 (52 min, hold for 5 min). UV wavelength of the detector was fixed at 254 nm and monitored for 45 min. Column temperature was maintained constantly at 40 °C.

Validation experiment of the HPLC method

The experiments on the validation of the HPLC method were performed based on the ICH (International Conference on Harmonization) guidelines in terms of linearity, sensitivity, precision, and accuracy. Linearity was assessed by calculating the R^2 values of the regression equations determined from the analysis at six concentrations of standard compounds. LOD (limit-of-detection) and LOQ (limit-of-quantification) were determined for the sensitivity. The values of LOD and LOQ were determined by signal-to-noise (S/N) method, in which an S/N ratio of 3 for LOD and an S/N ratio of 10 for LOQ were applied.

Precision and accuracy of the analytical method were investigated based on the intermediate evaluation method which measures the intra-day variability and the inter-day variability. The intra-day variability was examined through analysis made within a day and the inter-day variability was evaluated by injecting to HPLC system five times a day for a consecutive of four days. Relative standard deviation (RSD) values were determined by evaluating the retention times and peak areas during the five times of analysis. RSD was considered as a measure of precision and accuracy. To evaluate accuracy, recovery tests were performed through the method of spiking the standard compounds in the sample solution. Recovery rate (%) was determined by the rate of the spiked extract solution vs. the non-spiked extract solution.

Assay for peroxynitrite-scavenging activity

The $ONOO^-$ scavenging assay was measured using the method described by Kooy et al. (1994). This method is to monitor the highly fluorescent rhodamine formed from non-fluorescent DHR 123 under $ONOO^-$. Rhodamine buffer (pH 7.4) consists of 50 mM sodium phosphate dibasic, 50 mM sodium phosphate monobasic, 90 mM sodium chloride, 5 mM potassium chloride, and 100 μM DTPA. The final concentration of DHR 123 was 5 μM. This buffer solution was prepared and preserved on the ice-bath prior to use. The samples including plant extracts, fractions and the compounds were dissolved in 10 % DMSO (final conc., 5 μg/mL). The final fluorescent intensity was measured with or without the treatment of 10 μM $ONOO^-$ in 0.3 N NaOH. The fluorescence intensity of oxidized DHR 123 was measured at the excitation and emission of 480 and 530 nm using the microplate fluorescence reader FL 500 (Bio-Tek Instruments Inc., Winooski, VT, USA).

Through the detection of the oxidation of DHR-123, $ONOO^-$ scavenging activity was calculated by subtracting the background fluorescence from the final fluorescent intensity. L-penicillamine with great $ONOO^-$-scavenging activity was used as a positive control. The data were expressed as mean ± SEM (standard error of the mean).

Results

Identification of the isolated compounds

The MeOH extract obtained by the reflux extraction method was divided into ether-, EtOAc-, and BuOH fractions by the solvent fractionation and used for HPLC analysis and $ONOO^-$ scavenging assay. From the EtOAc fraction, eight compounds were isolated by column chromatographic procedures and identified as gallic acid, methyl gallate, avicularin, astragalin, and juglanin (An et al. 2007) together with isoquercitrin 6″-gallate, astragalin 6″-gallate (Liu et al. 1997) and ellagic acid (Srivastava et al. 2007) by comparisons of the physicochemical data and spectroscopic data (^{1}H- and ^{13}C-NMR). These compounds were also used in the HPLC analysis and $ONOO^-$ scavenging assay. The structures of eight isolated compounds and four additional compounds (kaempferol, quercetin, isoquercitrin, and hyperoside) used for the assay were shown in Fig. 1.

Compounds **3** and **5** were galloylated flavonoid glycosides esterifying gallic acid to the sugar hydroxyl. Compound **5** was identified as astragalin 6″-gallate because the ^{13}C-NMR data were in agreement with the literature data described by Liu et al. (1997). ^{1}H- and ^{13}C-NMR data of compound **3** was very similar to the data of compound **5**, but the aglycone of compound **3** was identified as quercetin, for which the B-ring had a catechol moiety characterized by values of δ 7.44 (1H, d, J = 1.8 Hz, H-2′), 6.73 (1H, d, J = 8.4 H, H-5′), 7.57 (1H, dd, J = 1.8, 8.4 Hz, H-6′). The assignment of ^{13}C-NMR data of isoquercitrin 6″-gallate are shown in the "Materials and Methods" section.

Content of polyphenols in the extract and fractions

The contents of the eight compounds in MeOH extract and its fractions are shown in Table 1. HPLC chromatograms of the MeOH extract and its fractions were shown in Fig. 2. In the MeOH extract the contents of the three compounds, ellagic acid (15.64 mg/g), isoquercitrin 6″-gallate (16.99 mg/g), and

Fig. 1 Structure of polyphenols used for HPLC analysis or peroxynitrite-scavenging assay

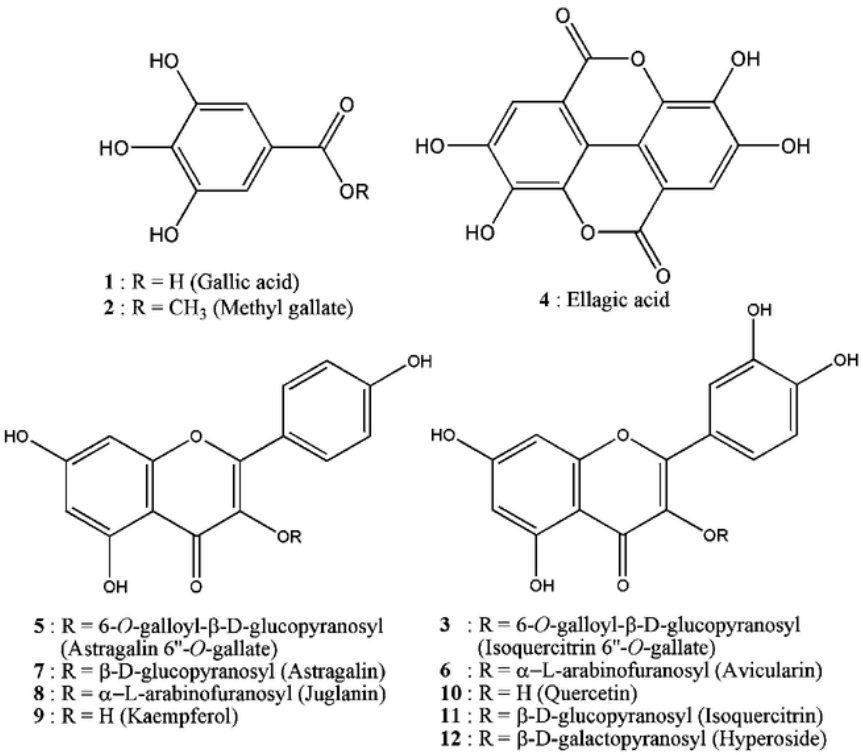

Table 1 Content of polyphenols in the MeOH extract of *E. supina* and its fractions (mg/g)

Analyte	Extract and fractions			
	MeOH	Ether	EtOAc	BuOH
Gallic acid	5.40 (1.33)	8.50 (0.51)	24.92 (0.86)	9.52 (0.39)
Methyl gallate	3.12 (0.77)	10.76 (0.64)	13.36 (0.46)	5.21 (0.21)
Isoquercitrin 6″-gallate	16.99 (4.17)	3.53 (0.21)	46.49 (1.60)	41.90 (1.73)
Ellagic acid	15.64 (3.84)	10.37 (0.62)	59.60 (2.05)	36.12 (1.49)
Astragalin 6″-gallate	13.72 (3.37)	8.14 (0.49)	66.31 (2.28)	7.53 (0.31)
Avicularin	2.99 (0.73)	2.56 (0.15)	6.66 (0.23)	0.84 (0.03)
Astragalin	2.10 (0.51)	0.68 (0.04)	11.00 (0.38)	1.87 (0.08)
Juglanin	0.99 (0.24)	0.89 (0.05)	4.45 (0.15)	0.29 (0.01)
Total	60.94 (14.96)	45.42 (2.71)	232.8 (8.01)	103.3 (4.25)

Values in the parentheses are content of analytes in the dried plant materials (mg/g)

astragalin 6″-gallate (13.72 mg/g), were comparatively high. The content of gallic acid and methyl gallate are shown to be 5.40 and 3.12 mg/g, respectively. The contents of other three flavonoids, avicularin, astragalin, and juglanin, are low, and isoquercitrin were not detected in this study. Of the fractions, EtOAc fraction (232 mg/g) exhibited the highest content of polyphenols. The content of the BuOH fraction (103.3 mg/g) was lower than of EtOAc fraction, but much higher than the ether fraction. The composition of polyphenols in the two fractions was also dominated by ellagic acid, isoquercitrin 6″-gallate, and astragalin 6″-gallate.

$ONOO^-$ scavenging activity of the extract, its fractions, and the polyphenols

$ONOO^-$ scavenging activities which were measured at the concentration of 0.08, 0.40, 2.00 and 10.0 mg/mL are shown in Table 2. The IC_{50} values of the MeOH extract

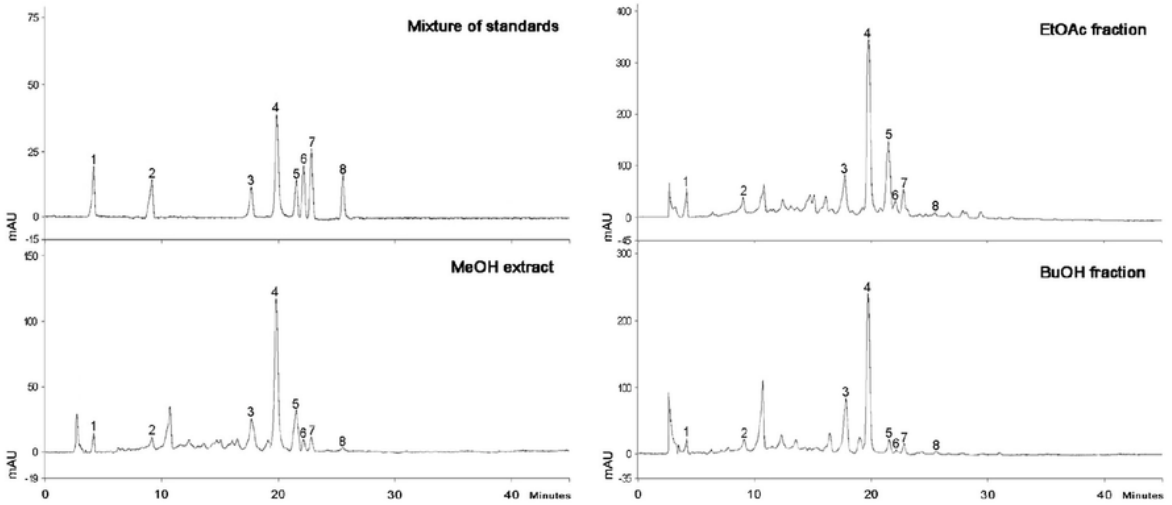

Fig. 2 HPLC chromatograms of a mixture of standards, MeOH extract, and fractions of *E. supina*

Table 2 Peroxynitrite-scavenging effect of the MeOH extract of *E. supina* and its fractions

Sample	Peroxynitrite-scavenging (%)				IC_{50} (μg/mL)
	0.08 μg/mL	0.40 μg/mL	2.00 μg/mL	10.0 μg/mL	
MeOH extract	3.75 ± 6.08[a]	4.35 ± 1.71	36.11 ± 2.45	81.76 ± 0.45	4.43
Ether fraction	0.98 ± 1.56	3.29 ± 1.65	16.11 ± 5.20	62.14 ± 3.32	7.88
EtOAc fraction	7.87 ± 3.14	12.99 ± 0.05	65.21 ± 4.34	95.60 ± 0.35	1.53
BuOH fraction	9.80 ± 0.02	15.71 ± 2.81	63.16 ± 3.74	91.17 ± 0.23	1.55
L-Penicillamine	–	31.01 ± 0.08	51.79 ± 2.50	76.05 ± 1.05	1.86

[a] Value represents mean ± SD ($n = 2$), – (not tested)

Table 3 Peroxynitrite-scavenging effect of the polyphenols in *E. supina*

Type	Compound	Peroxynitrite-scavenging (%)				IC_{50}	
		0.08 μg/mL	0.40 μg/mL	2.0 μg/mL	10 μg/mL	μg/mL	μM
Gallic acid-type	Gallic acid	5.66 ± 1.13[a]	30.53 ± 4.80	68.01 ± 0.97	96.77 ± 0.37	1.23	7.23
	Methyl gallate	18.82 ± 6.89	33.15 ± 4.17	79.85 ± 1.07	97.68 ± 1.22	0.97	5.27
	Ellagic acid	18.01 ± 4.62	52.12 ± 7.31	78.76 ± 6.99	94.44 ± 1.12	0.27	0.89
Kaempferol-type	Kaempferol	3.58 ± 1.88	28.95 ± 6.18	92.50 ± 1.45	99.41 ± 0.11	0.92	3.21
	Astragalin	23.77 ± 6.29	36.66 ± 4.64	42.50 ± 2.02	74.38 ± 1.18	3.88	8.66
	Juglanin	11.03 ± 2.40	19.76 ± 1.00	35.04 ± 2.21	51.39 ± 2.86	9.32	22.3
	Astragalin 6″-gallate	25.69 ± 4.29	40.16 ± 2.62	73.93 ± 1.58	96.11 ± 1.48	0.86	1.43
Quercetin-type	Quercetin	6.88 ± 4.22	47.44 ± 8.90	82.68 ± 3.79	94.82 ± 1.49	0.51	1.69
	Hyperoside	9.97 ± 1.58	26.94 ± 3.29	75.62 ± 4.85	95.70 ± 0.27	1.15	2.48
	Isoquercitrin	3.97 ± 2.57	25.17 ± 4.11	76.71 ± 3.55	94.58 ± 1.49	1.17	2.52
	Avicularin	15.95 ± 4.13	19.63 ± 4.93	59.24 ± 0.20	90.48 ± 1.53	1.62	3.73
	Isoquercitrin 6″-gallate	32.01 ± 7.31	36.55 ± 14.04	69.42 ± 1.31	96.49 ± 0.58	1.05	1.75
Cysteine-type	L-Penicillamine	–	44.89 ± 3.85	72.47 ± 2.12	90.85 ± 1.89	0.69	4.62

[a] Value represents mean ± SD ($n = 2$)

and fractions were indicated as follows: ether fraction (7.88 μg/mL) > MeOH extract (4.43 μg/mL) > BuOH fraction (1.55 μg/mL) > EtOAc fraction (1.53 μg/mL). The IC_{50}s of EtOAc- and BuOH fractions were lower than of L-penicillamine of the positive control.

Four compounds, kaempferol, quercetin, hyperoside, and isoquercitrin, were used in the assay in addition to the isolated eight compounds. The four compounds were tested to discuss the structural activity relationship (SAR). The activities of twelve compounds were shown in Table 3, by being grouped into two types: quercetin type and kaempferol type. The unit of μg/mL was noted so that it could be compared with the activity unit of extracts or fraction, the unit of μM was also used so that it could be discussed for SAR.

Discussion

Optimization and validation of the HPLC method

The experiments on the optimization of HPLC method was designed by considering four parameters, the composition of mobile phases, gradient elution, column temperature, and UV wavelength. The mixed solvents, H_2O, MeOH, and CH_3CN, were investigated for better separations, optimal mobile phase, and environment-friendly solvents. Better separations and peak shapes were displayed when the two solvents, H_2O as solvent A and MeOH-CH_3CN (60:40, v/v) as solvent B were used. Furthermore, addition of 0.05 % TFA in solvent A and solvent B improved the resolution, which is probably due to the deionization of polyphenol compounds.

Gradient elution was employed to detect various peaks depending on a wide range of polarity. During the evaluation of the HPLC condition, the following condition was selected: (A)/(B) = 85/15 (0 min) → 35/65 (45 min) → 10/90 (46 min, hold for 4 min) → 85/15 (52 min, hold for 5 min) as the gradient elution program with a flow rate of 1.0 mL/min. This condition contributed to the better separation and constant retention time. The detection wavelength, 254 nm, was selected because it was more sensitive than other wavelengths for detection of flavonoids and phenolic acids simultaneously.

As shown in Table 4, the present HPLC method was verified by examining the linearity, sensitivity, precision, and accuracy. The R^2 values determined from the analysis for the six concentrations verified the linearity of the regression equations because they were more than 0.9997. As shown in Table 5, this method was also sufficiently precise from the RSD values over 0.26–1.83 % of the peak areas in the intra-day variability tests, and sufficiently accurate considering the recovery rates over 94.02–102.38 %. Conclusively, it was considered that this method could be used for the simultaneous HPLC analysis on the extract of *E. supina*.

Content of polyphenols and $ONOO^-$ scavenging activity

Ellagic acid is a polyphenol that is the esterified dimer between the two gallic acids, and further isoquercitrin 6″-gallate and astragalin 6″-gallate are galloylated flavonoid glycosides. Ellagic acid exhibited the most potent activity (IC_{50} 0.89 μM) of the tested twelve compounds. The two galloylated flavonoid glycosides (IC_{50}, isoquercitrin 1.75 μM; astragalin 1.43 μM) were considerably more active than isoquercitrin (2.52 μM) and astragalin (8.66 μM), respectively, and gallic acid (7.23 μM). These results indicated that esterification of gallic acid to the sugar moiety of flavonoid glycosides enhances the $ONOO^-$ scavenging activity, as reported by Karioti et al. (2010) that acylated flavonoid glycosides possess dramatically increased antioxidant and antibacterial effects when compared to their corresponding glycosides. (−)-Epigallocatechin gallate, (−)-epigallocatechin which were the constituents of green tea are the examples of galloylated flavonoid with potent antioxidant activity (Hong et al. 2013).

Table 4 Linearity of standard curves and detection/quantification limits for the standard compounds

No.	Standard compounds	Calibration equation (linear model)[a]	Linear range (μg/mL)	R^{2b}	LOD[c] (μg/mL)	LOQ[d] (μg/mL)
1	Gallic acid	$y = 145.021x + 33.587$	3.13–100.00	0.9997	0.80	2.68
2	Methyl gallate	$y = 228.517x + 56.229$	3.13–100.00	0.9997	0.41	1.37
3	Isoquercitrin 6″-gallate	$y = 170.598x + 42.468$	3.13–100.00	0.9999	0.63	2.10
4	Ellagic acid	$y = 589.979x + 72.816$	3.13–100.00	0.9998	0.13	0.44
5	Astragalin 6″-gallate	$y = 255.632x + 54.871$	3.13–100.00	0.9999	0.37	1.24
6	Avicularin	$y = 247.289x + 65.751$	1.56–50.00	0.9999	0.34	1.14
7	Astragalin	$y = 460.649x + 65.398$	1.56–50.00	0.9999	0.18	0.61
8	Juglanin	$y = 182.691x + 58.731$	1.56–50.00	0.9998	0.36	1.19

[a] *y*, peak area at 254 nm; *x*, concentration of the standard (μg/ml); [b] R^2, correlation coefficient for six data points in the calibration curves ($n = 4$); [c] *LOD* limit of detection (S/N = 3); [d] *LOQ* limit of quantification (S/N = 10)

Table 5 Precision and recovery data of each analyte

Analyte	Precision test					Recovery test					
	t_R (min)	Intra-day variability RSD (%)		Inter-day variability RSD (%)		Initial conc. (μg/mL)	Amount added (μg)	Concentration after addition (μg/mL)		Recovery (%)	RSD (%)
		t_R	Area	t_R	Area			Expected	Measured		
Gallic acid	4.16	0.33	0.85	0.84	2.83	7.019	6.250	13.27	13.58	102.38	0.89
Methyl gallate	9.11	0.20	1.60	1.05	2.59	4.057	6.250	10.31	9.86	95.65	1.98
Isoquercitrin 6″-gallate	17.73	0.03	0.71	1.40	1.07	22.09	25.00	47.09	45.83	97.32	0.93
Ellagic acid	19.79	0.06	0.26	1.34	1.20	20.33	25.00	45.33	44.92	99.09	0.40
Astragalin 6″-gallate	21.56	0.13	0.72	1.29	1.21	17.83	25.00	42.83	40.23	94.02	0.77
Avicularin	22.18	0.12	1.48	1.31	2.33	3.892	3.125	7.017	6.89	98.12	0.76
Astragalin	22.87	0.11	1.37	1.27	2.88	2.724	4.375	7.099	6.90	97.20	0.52
Juglanin	25.50	0.15	1.83	0.81	4.71	1.281	1.563	2.843	2.77	97.34	1.00

Relative standard deviation (RSD) values were calculated for both retention time (t_R) and peak area of five experiments. Recovery tests were performed in the MeOH extract spiked with each standard compound

Generally, quercetin type (quercetin, isoquercitrin, avicularin) was more active than kaempferol type (kaempferol, astragalin, and juglanin). Those two types are structurally differentiated by existence of a catechol in their B-ring. Compounds with catechol in the B-ring showed a stronger activity to scavenge the peroxynitrite. This fact was supported by the report of Heijnen et al. (2001) that the aromatic hydroxyl groups are the reactive centers of phenolic compounds in peroxynitrite-scavenging activity. An additional hydroxyl group at C-2 of phenol (catechol) clearly increases the activity.

It has been reported that excess production of $ONOO^-$ could cause hypercholesterolemia, atherosclerosis, obesity, and diabetes mellitus (Patcher et al. 2005; Drel et al. 2007; Korda et al. 2008) in addition to gastrointestinal diseases, e.g., gastric ulcer (Alvarez-Suarez et al. 2011; Silva Beserra et al. 2011) or Crohn's disease (Rosillo et al. 2011). In particular, ellagic acid is known to be effective against several models of gastric ulcer in animal experiments (Silva Beserra et al. 2011) and even Crohn's disease (Rosillo et al. 2011).

The $ONOO^-$ scavenging activity and polyphenols contents of the MeOH extract and its fractions were indicated as follows: ether fraction (IC_{50}: 7.88 μg/mL, 45.42 mg/g) < MeOH extract (4.43 μg/mL, 60.94 mg/g) < BuOH fraction (1.55 μg/mL, 103.3 mg/g) < EtOAc fraction (1.53 μg/mL, 232.8 mg/g). Among them, EtOAc fraction exhibited the strongest $ONOO^-$ scavenging activity with the highest content of polyphenols. Their polyphenols compositions were dominated by the three active compounds i.e. gallic acids (25.60 %), isoquercitrin 6″-gallate (19.97 %), and astragalin 6″-gallate (28.48 %). The potent activity of this fraction is considered to be dependent of the high content of galloylated flavonoid glycosides and ellagic acid that are believed to be beneficial for the treatment or preventions of such gastrointestinal disease by highly scavenging $ONOO^-$. The legendary use of *E. supina* could be attributed to the high content of polyphenols, particularly ellagic acid, isoquercitrin 6″-gallate, and astragalin 6″-gallate with potent activities.

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