



benefits of HSCCC, many researchers have isolated compounds from various natural materials with saving time and costs. In this study, preparative HSCCC apparatus was performed for successful isolation of major flavonoids in OFIEs. Isolated compounds were evaluated their protective activities in hepatocytes against oxidative stress due to ethanol exposure.

## MATERIALS AND METHODS

### Preparation of *Opuntia ficus-indica* fruit extracts

The fruits of *O. ficus-indica* were collected from Jeju Island (Republic of Korea). A voucher specimen (DW-1) has been deposited at the Herbarium of Medicinal Plant Garden, College of Pharmacy, Seoul National University (Goyang, Korea). The dry powdered fruit of *O. ficus-indica* (5 kg) was extracted with 70% ethanol (10 L) three times. After removal of the solvents by evaporation, the residue (385 g) was suspended in distilled water and then successively fractionated with methylene chloride to remove lipids. The water-soluble fraction was fractionated with *n*-butanol (1.5 L, repeat 3 times). Major flavonoids were in *n*-butanol fraction while high-polar compounds were removed to water fraction. The *n*-butanol fraction was evaporated, lyophilized, and stored in a refrigerator before use.

### Determination of *K* (partition coefficient) values using high-performance liquid chromatography

Approximately 5 mg of *n*-butanol fraction was weighed in a 20 ml test vial into which 3 ml of each phase of the preequilibrated two-phase solvent system was added. The test vial was shaken violently for several minutes to get equilibrium. The upper and lower phases were separated and evaporated under N<sub>2</sub> gas, respectively. Dried residues of each phase were dissolved in methanol of 1 mL and analyzed by high-performance liquid chromatography ultraviolet (HPLC-UV). The *K* value was obtained by the ratio of peak area between upper and lower phase. The analytical HPLC-UV system consisted of a Finnigan Surveyor HPLC system with a pump, an autosampler, a PDA detector and the Finnigan LCQ Advantage MAX with the Xcalibur<sup>®</sup> software (Thermo Fisher Scientific Inc., MA, USA). All of analytes were analyzed by an Ascentis<sup>®</sup> Express C<sub>18</sub> column (150 × 4.6 mm, I.D., 2.7 μm, Supelco Analytical, Bellefonte, PA, USA) with the following gradient system: water with 0.5% formic acid (v/v) (A) and acetonitrile (B); gradient program: 10% B to 90% B, 0–25 min; 90% B to 10% B. Flow rate was set to be at 0.3 mL/min, and all samples were injected with the volume of 10 μL.

### Preparation of two-phase solvent system and sample solution

After determination of *K* value by HPLC-UV, the two-phase solvent system was selected: methylene chloride-methanol-*n*-butanol-water (5:4:3:5, v/v/v/v). The solvent system was thoroughly equilibrated in a separation funnel by repeatedly vigorously shaking at room temperature, and then separated shortly before use. The *n*-butanol fraction was dissolved in 5 mL of each phase of selected solvent system for HSCCC separation (1000 mg). The fraction was dissolved before use.

### High-speed countercurrent chromatography separation procedure

The mobile phase of solvent system was selected to organic lower phase. The multilayer coil column was filled first with the stationary phase. Then, the mobile upper phase was pumped into the column by a chromatographic pump (L-6200, Hitachi, Japan) at a flow rate of 3 mL/min while preparative HSCCC apparatus (TBE-1000A, Shanghai

Tauto Biotech, Co., Ltd., China) was rotated at a revolution speed of 500 rpm. After hydrodynamic equilibrium was established, prepared sample solution was subjected to HSCCC apparatus. The monitoring of HSCCC peak fractions was performed by combining effluent line of the HSCCC apparatus to the UV detector (UV/VIS-151, Gilson Inc., Middleton, WI, USA) at 330 nm. The eluent from the UV detector was collected by a fraction collector (FC-204, Gilson Inc.) in 3 min per each test tube. HSCCC systems were kept the internal column temperature at 25°C by circulatory temperature regulator (RW-0525G, Jeio Tech., Korea).

### Identification of isolated peak fractions

HSCCC peak fractions were identified by comparing their <sup>1</sup>H, <sup>13</sup>C NMR, and ESI-MS spectroscopic data with literature values. The optical rotation value was measured to determine the absolute configuration of compound 2 by polarimeter (FT-6500, JASCO Corp., Tokyo, Japan). <sup>1</sup>H and <sup>13</sup>C NMR spectroscopic data were obtained on a JEOL LA 300 spectrometer at 300 MHz and 75 MHz, respectively. ESI-MS spectroscopic data were obtained on the Finnigan LCQ advantage MAX with the Xcalibur<sup>®</sup> chromatographic software.

### Evaluation of rat hepatocyte protective activities

Male Sprague Dawley rats were purchased from Koatech Co., Ltd. (Yongin, Korea) with body weights of 200–250 g, housed in conventional cages (two per each cage) at room temperature under a 12 h light-dark cycle and fed *ad libitum* with free access to water. Isolated rat hepatocytes were prepared from male Sprague Dawley rats by collagenase perfusion technique of Berry and Friend, by means of minor modifications.<sup>[17]</sup> The isolated rat hepatocytes were plated onto each cell culture dishes (35 mm × 10 mm, 4 × 10<sup>5</sup> cells/dishes). After 18 h, the cultured cells were treated with or without each sample for 2 h. Then, the hepatocytes were exposed to 200 mM ethanol for 48 h to induce hepatotoxicity.<sup>[18]</sup> Cell viability was detected by MTT assay which relies on mitochondrial metabolic capacity of viable cells. Prepared MTT solution (0.2 mL, 5 mg/mL in PBS) was added into each plate after 48 h incubation. After 2 h, the supernatant was removed and 0.6 mL of DMSO was added to each plate to dissolve the formazan crystals. The absorbance was measured by spectrophotometer (Spectra Max340PC, USA) at 540 nm. Data were expressed as the value of relative protection (%) calculated as 100 × (value for the ethanol-treated – value of sample)/(value for the ethanol-treated – value of control). Quercetin was chosen as a positive control against ethanol-intoxicated oxidative stress.<sup>[18]</sup> All animal procedures were approved by the Institutional Animal Care and Use Committee at Seoul National University and conducted with special care taken to avoid any undue animal pain or suffering.

### Cellular glutathione content and activities of related enzyme assay

Cells from culture plates were pooled in 2 mL of 0.1 M phosphate buffer (pH 7.4) and homogenized. The homogenate was centrifuged for 25 min at 3000 g at 4°C and the supernatant was collected for the measurements of antioxidant enzyme and glutathione contents. Total glutathione (GSH + oxidized glutathione [GSSG]) in the supernatant was determined spectrophotometrically using the enzymatic cycling method.<sup>[19]</sup> Glutathione reductase (GR) activity was measured according to the method based on the reduction of GSSG by GR in the presence of NADPH. The activity of glutathione peroxidase (GPx) was determined by quantifying the rate of oxidation of GSH to GSSG using cumene hydroperoxide.<sup>[20,21]</sup> Each value was shown the mean ± standard deviation (SD) of three experiments.

## Statistical analysis

One-way analysis of variance procedures were used to assess significant differences among control groups and treatment groups. For each significant effect of treatment, the Student's *t*-test was used for comparisons of multiple group means. The criterion for statistical significance was set at  $P < 0.05$ , 0.01, or 0.001. The calculations were performed with the SAS 9.1 software (SAS Institute Inc., Cary, NC, USA).

## RESULTS AND DISCUSSION

### Selection of high-speed countercurrent chromatography solvent system

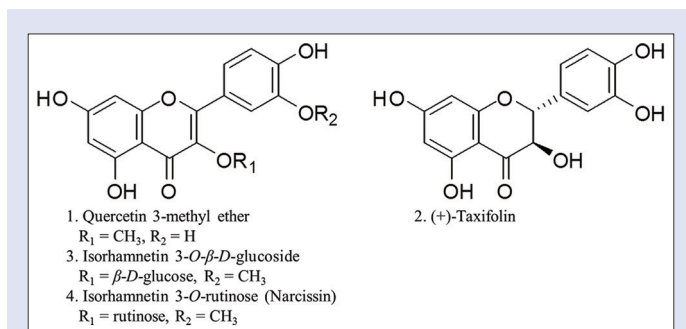
Through several trials with the change solvent systems, as shown in Table 1, the *K* values of flavonoids were in the range of  $0.2 < K < 2$  in the solvent system comprising methylene chloride-methanol-*n*-butanol-water (5:4:3:5, v/v/v/v). Chemical structures of flavonoids are shown in Figure 1.

### High-speed countercurrent chromatography separation

Four flavonoids were successfully separated by HSCCC. Quercetin 3-*O*-methyl ether (1, 312–251 min, 18 mg), (+)-taxifolin (2, 381–396 min, 11 mg), isorhamnetin 3-*O*- $\beta$ -*D*-glucoside (3, 411–450 min, 7 mg), and narcissin (4, 462–522 min, 17 mg) were obtained [Figure 2]. The purity of isolated flavonoids was >90% as evaluated by HPLC-UV at 330 nm [Figure 3]. The structures of all compounds were confirmed by ESI-MS, <sup>1</sup>H, and <sup>13</sup>C NMR spectra by comparing previous literature.<sup>[22,23]</sup> The measured optical rotation value of compound 2 was + 40.1 (25°C, *c* = 0.3 in methanol) that confirm its structure to (+)-taxifolin. Spectroscopic data of quercetin 3-*O*-methyl ether (1), (+)-taxifolin (2), isorhamnetin 3-*O*- $\beta$ -*D*-glucoside (3), and narcissin (4) were in agreement with published data (T1, 2 and F1 in Supplementary material).

### Evaluation of rat hepatocyte protective activities

Before evaluation of hepatoprotective activities, OFIEs showed no cytotoxic effect comparing with the control group (at 100  $\mu$ g/mL of



**Figure 1:** Chemical structures of isolated flavonoids in *Opuntia ficus-indica* fruit extracts

the extract, not shown). The OFIEs, its *n*-butanol fraction, and all of isolated flavonoids exhibited protective activities [Figure 4]. Among them, (+)-taxifolin (2) was showed the most significant protective activity (55.3% at 50  $\mu$ M), which is higher than the level of 100  $\mu$ M quercetin (47.3%). To elucidate the biochemical mechanism of the hepatoprotective activity of the (+)-taxifolin, their effects on the level of cellular GSH and activities of related enzymes (Gpx and GR) were measured. As shown in Table 2, (+)-taxifolin preserved activities of Gpx and GR and elevated the level of reduced GSH against ethanol.

## CONCLUSION

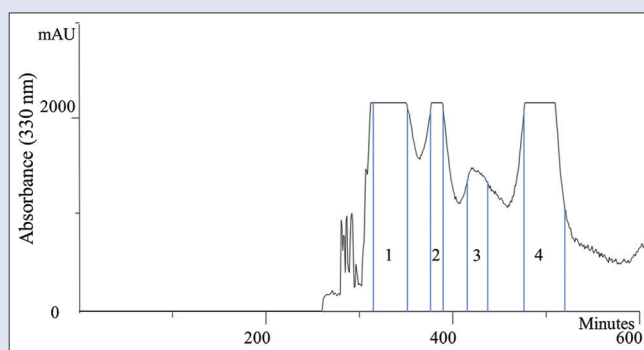
Major flavonoids in OFIEs, quercetin 3-*O*-methyl ether (1), (+)-taxifolin (2), isorhamnetin 3-*O*- $\beta$ -*D*-glucoside (3), and narcissin (4) were successfully separated by HSCCC. Among isolated compounds, (+)-taxifolin exhibited significant protection on hepatocytes against alcoholic oxidative stress. Effects of cellular glutathione and related enzymes indicate that the hepatoprotective activity of (+)-taxifolin may be due to maintaining the level of GSH. This result would be attributed to the powerful anti-oxidative activity of taxifolin.<sup>[24,25]</sup> To sum, OFIEs and its active compound, (+)-taxifolin were suggested to reduce hepatic damage by alcoholic oxidative stress. Biological relevance of the (+)-taxifolin *in vivo* needs to be studied further.

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### Conflicts of interest

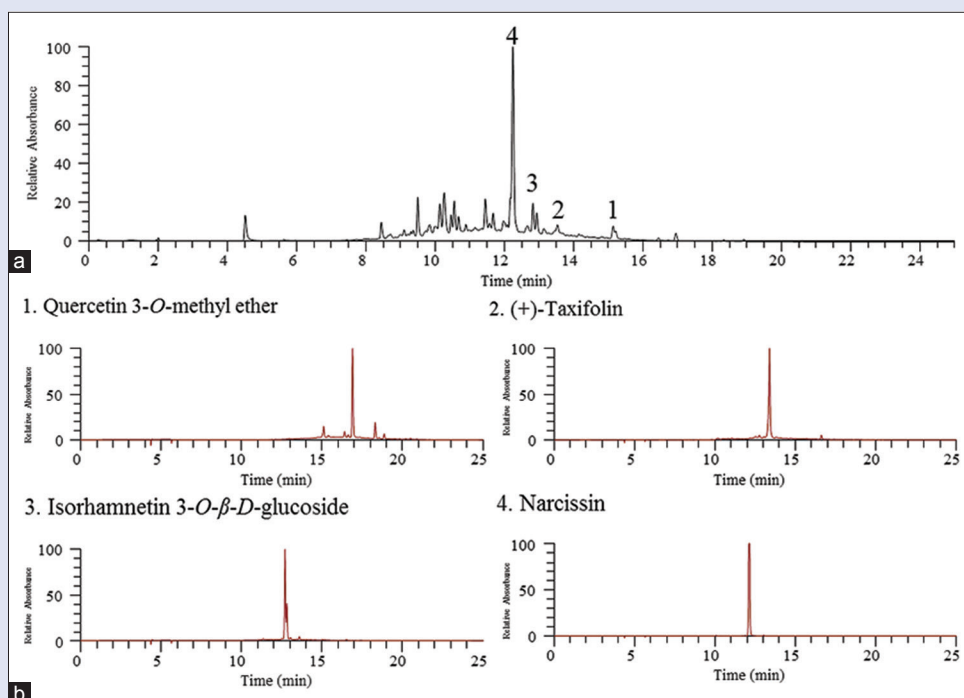
There are no conflicts of interest.



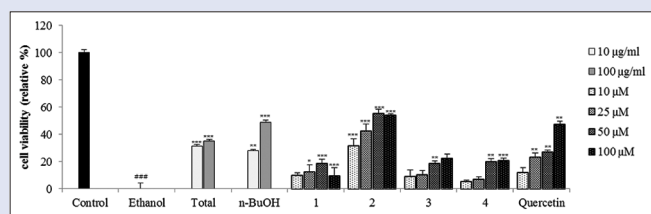
**Figure 2:** High-speed countercurrent chromatography chromatogram of the *Opuntia ficus-indica* fruit extracts *n*-butanol fraction. Solvent system consists of methylene chloride-methanol-*n*-butanol-water (5:4:3:5, v/v/v/v). 1,000 mg of *n*-BuOH fraction (dissolved in 5 mL of each phase of the solvent system) was injected; flow rate of 3 mL/min; rotation speed of 500 rpm; retention of stationary phase in (A) and (B) were approximately over 50%

**Table 1:** The *K* (partition coefficient) values of compound 1-4 in different solvent systems

Solvent system	Volume ratio	Settling time (s)	1	2	3	4
Chloroform-methanol-water	4:1:2	8	0.32	0.20	0.06	0.05
	3:4:2	14	0.54	0.43	0.29	0.23
Methylene chloride-methanol- <i>n</i> -butanol-water	3:1:1:3	27	0.78	0.67	0.29	0.44
	3:1:1:3	25	0.92	0.79	0.48	0.47
	3:1:1:3	22	1.27	0.78	0.54	0.34
	3:1:1:3	25	1.78	1.43	1.08	0.75



**Figure 3:** High-performance liquid chromatography chromatograms of the (a) crude n-butanol fraction and (b) collected compounds by high-speed countercurrent chromatography. High-performance liquid chromatography experimental condition: an Ascentis<sup>®</sup> Express C18 column (150 mm × 4.6 mm, I.D., 2.7 μm, Supelco Analytical, USA), with the following gradient system was used: water with 0.5% formic acid (v/v) (a) and acetonitrile (b); gradient program: 10% B to 90% B, 0–25 min; 90% B to 10% B. Flow rate was set to be at 0.3 mL/min. All samples were injected with the volume of 10 μL



**Figure 4:** Protective effects of *Opuntia ficus-indica* fruit extracts and n-BuOH fraction, isolated compounds (1–4), and quercetin (positive control) on intoxicated hepatocytes by ethanol exposure. Each value represents the mean ± standard deviation ( $n = 9$ ). \*\*\*Differs significantly from the untreated, control group at the level of  $P < 0.001$ . \*\*\*,\*\*\*\*Differs significantly from ethanol-treated group at the level  $P < 0.05, 0.01, \text{ and } 0.001$ , respectively (analysis of variance test)

**Table 2:** Effect of (+)-taxifolin 50 μM on the oxidized glutathione/glutathione ratio and activities of glutathione reductase, and glutathione peroxidase on hepatocytes damaged by alcoholic oxidative stress

Group	GR (mU/mg protein)	GPx (mU/mg protein)	GSSG/total GSH (ratio)
Control	130.079±7.833	0.126±0.002	0.226±0.007
Ethanol	74.610±7.969***	0.119±0.000**	0.464±0.017***
Compound 2	115.814±5.776**	0.189±0.005***	0.309±0.011***

Primary cultured rat hepatocytes were exposed to 200 mM ethanol with or without 50 μM of (+)-taxifolin (2). Each value represents the mean±SD ( $n=3$ ). \*\*\*,\*\*\*\* Differs significantly from the untreated, control group at the level of  $P<0.01$  and 0.001, respectively; \*\*\*\*\*,\*\*\*\*\*Differs significantly from ethanol-treated group at the level of  $P<0.01$  and 0.001, respectively (ANOVA test). GSH: Glutathione; GSSG: Oxidized glutathione; GR: Glutathione reductase; GPx: Glutathione peroxidase; SD: Standard deviation

## REFERENCES

- Ahn DK. Illustrated Book of Korean Medicinal Herbs. Seoul: KyoHak Publishing Co., Ltd.; 1998.
- Trejo-González A, Gabriel-Ortiz G, Puebla-Pérez AM, Huizar-Contreras MD, Munguía-Mazariegos MR, Mejía-Arreguín S, *et al.* A purified extract from prickly pear cactus (*Opuntia fuliginosa*) controls experimentally induced diabetes in rats. *J Ethnopharmacol* 1996;55:27-33.
- Alimi H, Hfaiedh N, Bouoni Z, Hfaiedh M, Sakly M, Zourgui L, *et al.* Antioxidant and antilcerogenic activities of *Opuntia ficus indica* f. inermis root extract in rats. *Phytomedicine* 2010;17:1120-6.
- Vazquez-Ramirez R, Olguin-Martinez M, Kubli-Garfias C, Hernandez-Munoz R. Reversing gastric mucosal alterations during ethanol-induced chronic gastritis in rats by oral administration of *Opuntia ficus-indica* mucilage. *World J Gastroenterol* 2006;12:4318-24.
- Kim SH, Jeon BJ, Kim DH, Kim TI, Lee HK, Han DS, *et al.* Prickly pear cactus (*Opuntia ficus indica* var. saboten) protects against stress-induced acute gastric lesions in rats. *J Med Food* 2012;15:968-73.
- Lim KT. Inhibitory effect of glycoprotein isolated from *Opuntia ficus-indica* var. saboten MAKINO on activities of allergy-mediators in compound 48/80-stimulated mast cells. *Cell Immunol* 2010;264:78-85.
- Kim JM, Kim DH, Park SJ, Park DH, Jung SY, Kim HJ, *et al.* The n-butanolic extract of *Opuntia ficus-indica* var. saboten enhances long-term memory in the passive avoidance task in mice. *Prog Neuropsychopharmacol Biol Psychiatry* 2010;34:1011-7.
- Wiese J, McPherson S, Odden MC, Shlipak MG. Effect of *Opuntia ficus indica* on symptoms of the alcohol hangover. *Arch Intern Med* 2004;164:1334-40.
- Alimi H, Hfaiedh N, Mbarki S, Bouoni Z, Sakly M, Ben Rouma K. Evaluation of *Opuntia ficus indica* f. inermis fruit juice hepatoprotective effect upon ethanol toxicity in rats. *Gen Physiol Biophys* 2012;31:335-42.
- Galati EM, Mondello MR, Lauriano ER, Taviano MF, Galluzzo M, Miceli N. *Opuntia ficus indica* (L.) Mill. fruit juice protects liver from carbon tetrachloride-induced injury. *Phytother Res* 2005;19:796-800.
- Morán-Ramos S, Avila-Nava A, Tovar AR, Pedraza-Chaverri J, López-Romero P, Torres N.

- Opuntia ficus indica* (nopal) attenuates hepatic steatosis and oxidative stress in obese Zucker (fa/fa) rats. *J Nutr* 2012;142:1956-63.
12. Ncibi S, Ben Othman M, Akacha A, Krifi MN, Zourgui L. *Opuntia ficus indica* extract protects against chlorpyrifos-induced damage on mice liver. *Food Chem Toxicol* 2008;46:797-802.
  13. Stintzing FC, Schieber A, Carle R. Identification of betalains from yellow beet (*Beta vulgaris* L.) and cactus pear [*Opuntia ficus-indica* (L.) Mill.] by high-performance liquid chromatography-electrospray ionization mass spectrometry. *J Agric Food Chem* 2002;50:2302-7.
  14. Lee EH, Kim HJ, Song YS, Jin C, Lee KT, Cho J, *et al.* Constituents of the stems and fruits of *Opuntia ficus-indica* var. *saboten*. *Arch Pharm Res* 2003;26:1018-23.
  15. Saleem M, Kim HJ, Han CK, Jin C, Lee YS. Secondary metabolites from *Opuntia ficus-indica* var. *saboten*. *Phytochemistry* 2006;67:1390-4.
  16. Ito Y. Efficient preparative countercurrent chromatography with a coil planet centrifuge. *J Chromatogr* 1981;214:122-5.
  17. Berry MN, Friend DS. High-yield preparation of isolated rat liver parenchymal cells: A biochemical and fine structural study. *J Cell Biol* 1969;43:506-20.
  18. Kim JW, Yang H, Cho N, Kim B, Kim YC, Sung SH. Hepatoprotective constituents of *Firmiana simplex* stem bark against ethanol insult to primary rat hepatocytes. *Pharmacogn Mag* 2015;11:55-60.
  19. Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: Applications to mammalian blood and other tissues. *Anal Biochem* 1969;27:502-22.
  20. Carlberg I, Mannervik B. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J Biol Chem* 1975;250:5475-80.
  21. Flohé L, Günzler WA. Assays of glutathione peroxidase. *Methods Enzymol* 1984;105:114-21.
  22. Li YL, Li J, Wang NL, Yao XS. Flavonoids and a new polyacetylene from *Bidens parviflora* Willd. *Molecules* 2008;13:1931-41.
  23. Lee YS, Lee S, Lee HS, Kim BK, Ohuchi K, Shin KH. Inhibitory effects of isorhamnetin-3-O-beta-D-glucoside from *Salicornia herbacea* on rat lens aldose reductase and sorbitol accumulation in streptozotocin-induced diabetic rat tissues. *Biol Pharm Bull* 2005;28:916-8.
  24. Yun BS, Lee IK, Kim JP, Chung SH, Shim GS, Yoo ID. Lipid peroxidation inhibitory activity of some constituents isolated from the stem bark of *Eucalyptus globulus*. *Arch Pharm Res* 2000;23:147-50.
  25. Marozienne A, Kliukiene R, Sarlauskas J, Cenas N. Inhibition of phthalocyanine-sensitized photohemolysis of human erythrocytes by polyphenolic antioxidants: Description of quantitative structure-activity relationships. *Cancer Lett* 2000;157:39-44.