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백년초 종자의 간세포 보호활성 성분

Hepatoprotective constituents of *Opuntia ficus-indica* seeds against ethanol-intoxicated rat primary hepatocytes

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Abstract

Alcoholic liver disease (ALD) is a common liver disease with high mortality. Although the pathogenesis of ALD remains to be clarified, chronic alcohol consumption plays the key role on genesis and progress of disease. Therefore, the protection of hepatocytes from alcohol-induced cellular damage has been considered as an effective treatment for delaying development of ALD. In the course of searching for hepatoprotective substances from natural products, it was found that an 80 % ethanol extract of *Opuntia ficus-indica* (Cactaceae) seeds showed the protective activity against ethanol-intoxicated rat primary hepatocytes. *O. ficus-indica* is a perennial plant which is commonly called “prickly pear” or “tuna,” and its fruits and seeds are known to contain flavonoids, lignans, phenolic compounds, fatty acids and sterols. As a folk medicine in Korea, *O. ficus-indica* has been used for treatment of diabetes, hypertension, asthma, burns, scars, edema and indigestion. Recently many studies have reported the biological activities of *O. ficus-indica* such as antioxidant, antiulcer, neuroprotective and hepatoprotective activity. To the best of my knowledge, however, its hepatoprotective constituents against ethanol-induced toxicity were not uncovered yet. In the present study, bioassay-guided fractionation of an 80 % ethanol extract of *O. ficus-indica* seeds resulted in the isolation of nine flavonoids (**1-9**), six lignans (**10-15**) and a phenolic compound (**16**). Among isolated compounds, lignans having furofuran ring moiety and flavonoids possessing 3',4'-dihydroxyl B ring in the structure showed the potent protective activities against ethanol-intoxicated rat primary hepatocytes. Through the study of the structure and activity relationship, furofuran ring skeleton of lignans, dihydroxylation at C-3' and 4' in B ring

and the presence of a hydroxyl group instead of methoxy or sugar moiety at C-3 of flavonoids were important for protective activity against ethanol-injured rat primary hepatocytes.

Keywords : *Opuntia ficus-indica*, alcoholic liver disease, lignans, flavonoids, rat primary hepatocytes, ethanol

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Contents

List of Abbreviations

$[\alpha]_D$: specific rotation

AcCN: acetonitrile

n-BuOH: *n*-butanol

c.c.: column chromatography

$CHCl_3$: chloroform

COSY: correlation spectroscopy

d: doublet

dd: doublet of doublet

DMEM: Dulbecco's modified eagle's medium

DMSO: dimethylsulfoxide

dt: doublet of triplet

EC_{50}: the half maximal effective concentration

ESIMS: electron spray impact mass spectroscopy

EtOAc: ethyl acetate

EtOH: ethanol

ext.: extract

fr.: fraction

HMBC: heteronuclear multi-bond correlation

Hz: hertz

IR: infrared spectroscopy

m: multiplet

M: methanol

MeOH: methanol

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NMR: nuclear magnetic resonance

R_f: migration distance relative to solvent front in TLC

RP: reverse phase

s: singlet

t: triplet

R_t: retention time

TLC: thin layer chromatography

UV: ultraviolet absorption spectroscopy

W: water

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I. Introduction

Alcoholic liver disease

Alcoholic liver disease (ALD) is one of major disease with significant morbidity and mortality in Europe and the U.S. It also commonly occurs in Korea and takes 20% of proportion of whole chronic liver disease following to Hepatitis B and C (Kim, 2008). ALD represents a variety of clinical symptoms and morphological changes that range from fatty liver to alcoholic hepatitis and severely to alcoholic cirrhosis. Generally it is considered that alcoholic liver disease is caused by excessive and long-lasting alcohol intake, but the drinking habits are not the only reason of pathogenesis. The genetic and environmental backgrounds like gender, race, age, nutrition and viral infection are also involved in it. The chronic heavy drinkers, in fact, mostly develop fatty liver, but the only 10-35 % of them advance to hepatitis and 8-20 % of them worsen to cirrhosis (Gramenzi et al., 2006). Nevertheless, the excessive alcohol consumption is obviously the key factor of generation and progression of ALD. Therefore, the major mechanisms of ALD are suggested as oxidative stress from metabolic consequence of alcohol consumption, activation of Kupffer cell by endotoxin, adaptive immune responses and the genetic control of immunologic mechanisms (Figure 1) (Stewart et al., 2001).

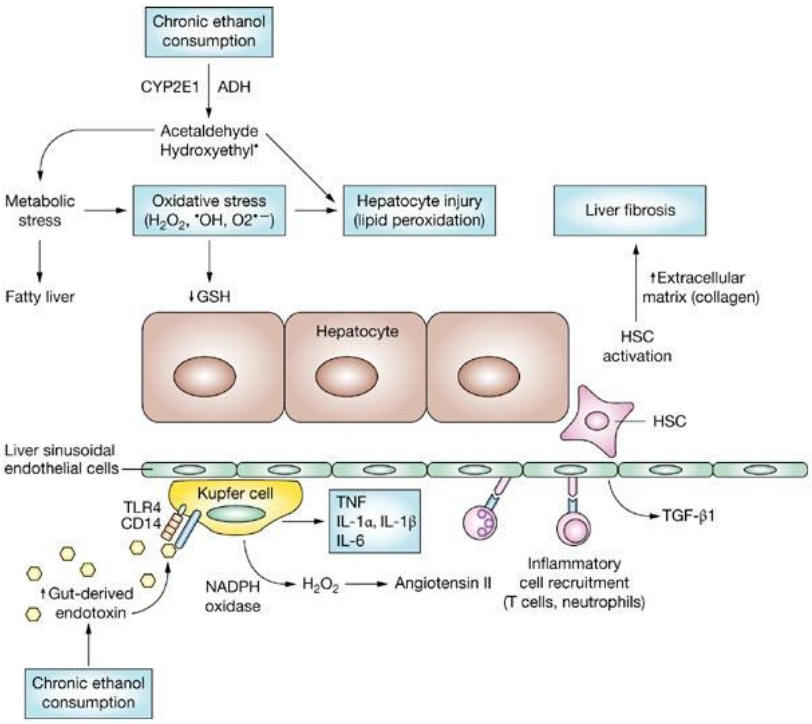


Figure 1. The pathogenesis of alcohol-induced liver disease (Tilg and Day, 2007)

The oxidative stress generating from alcohol metabolism is related to several pathways of alcoholic liver damages and it plays a pivotal role in development of ALD. Oxidation of ethanol in the liver is mediated by three main hepatic enzymes including alcohol dehydrogenase (ADH) in cytoplasm, cytochrome P450 2E1 (CYP2E1) in smooth endoplasmic reticulum of mitochondria and catalase in peroxisomal membrane (Figure 2). In this metabolic pathway, ethanol is converted to acetaldehyde and then acetic acid. Also, reactive oxygen species (ROS) and adducts are additionally generated in this process, and they lead to formation of highly reactive free radicals. Thus, excessive alcohol consumption promotes formation of ROS and causes oxidative stress and eventually cellular damages in the liver.

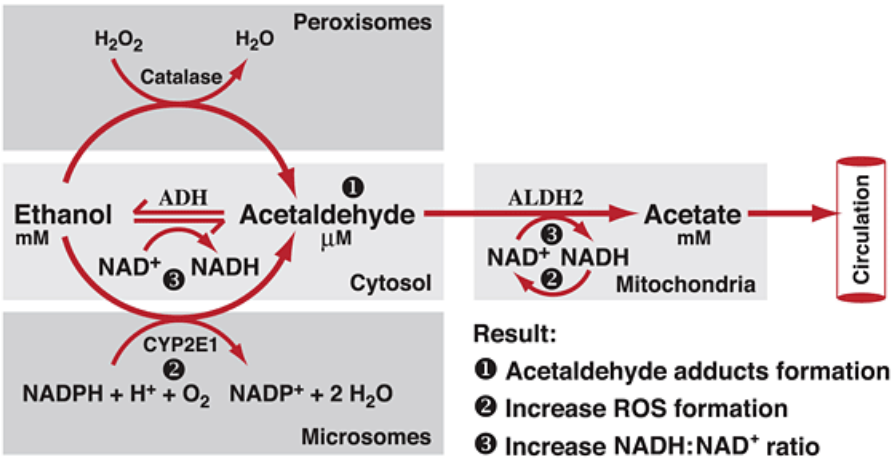


Figure 2. Oxidative pathways of alcohol metabolism (Zakhari, 2006)

Rat primary hepatocytes

The liver is the largest organ in the body and hepatocyte is the most abundant cell type by mass and number in the liver. Hepatocyte fulfills complex functions at the same time by effectively functioning structures and organelles. It comprises synthesis and storage of protein, transformation of carbohydrate, synthesis of cholesterol and phospholipid, and formation and secretion of bile. Besides, hepatocyte processes absorbed nutrients and xenobiotics (Dufour and Clavien, 2010).

Isolated rat primary hepatocytes are physiologically and biochemically similar to human hepatocytes, and often used to screen hepatic activities *in vitro*. Isolated rat primary hepatocytes are useful screening system for studying metabolism of xenobiotics because they closely resemble the *in vivo* situation (Jones et al., 1978). Also, they have a great advantage on metabolic studies because of their capability of synthesizing the necessary cofactors that are involved in enzymatic activities of liver (Holtzman et al., 1972).

The development of the therapeutic agents of alcoholic liver disease

In a few decades, there have been numerous therapies in an attempt to reverse the progression of acute alcoholic liver disease. These include corticosteroids, oxypentifylline (pentoxifylline), propylthiouracil (PTU), nutritional support, antioxidants and anti-TNF-α agents (Chae, 2009). Among the trials, corticosteroids, pentoxifylline and PTU were proved the beneficial effect on progression of alcoholic liver disease (Tome and Lucey, 2004). Corticosteroids are well-known to have an effect on the immune responses, decrease production of cytokines, suppress the generation of acetaldehyde adducts and inhibit the formation of collagen. Pentoxifylline is a phosphodiesterase inhibitor which improves a response of red blood cells and inhibits release of TNF-α by regulating the transcription of the TNF-α gene. PTU relieves alcohol-induced damages by reducing hepatic oxygen consumption of hepatocytes.

***Opuntia ficus-indica* (L.) Mill.**

Opuntia ficus-indica (L.) Mill. (Cactaceae) is a perennial plant which is commonly called "prickly pear" or "tuna." *O. ficus-indica* is considered to be native to Mexico, and these days cultivated in Mexico and the Mediterranean regions like Spain, Sicily, Italy and Greece (Griffith, 2004). Also in Korea, it is growing up in Jeju island and Southern coasts. The fruits and seeds of *O. ficus-indica* were known to contain flavonoids, lignans, phenolic compounds, fatty acids and glycosides. As a traditional Korean medicine, *O. ficus-indica* has been used to treat diabetes, hypertension, asthma, burns, scars, edema and indigestion (Lee et al., 2003a). Recently, a number of studies have reported the biological acitivities of *O. ficus-indica* (Table 1).

Table 1. Pharmacological studies of *O. ficus-indica*

Therapeutic target	***in vitro/vivo* assay**	**active materials**	**references**
Alcohol hangover relieving activity	clinical	plant extracts	(Wiese et al., 2004)
Ameliorative effect	*in vivo*; rat erythrocytes	fruits	(Alimi et al., 2012a)
Antiallergic activity	*in vivo*; mice, in vitro; RBL-2H3 cells	glycoprotein	(Lim, 2010)
Antiatherosclerotic effect	*in vivo*; hyperlipidemic rats	fruits and stems	(Choi et al., 2002b)
Antigenotoxic activity	*in vivo*; mice	cladodes	(Zorgui et al., 2009)
Anti-inflammation	*in vivo*; carrageenan-induced edema in rats	stems	(Park et al., 1998)
Antimicrobial activity	*in vitro*	plant extracts	(Jun and Yoon, 2002)
Antioxidant activity	*in vitro*	stems	(Lee et al., 2002)
Antioxidant activity	*in vitro*	betalins	(Gentile et al., 2004)
Antioxidant and antiulcerogenic activity	*in vitro*	fruits	(Galati et al., 2003)
Antioxidant and antiulcerogenic activity	*in vivo*; rats	roots	(Alimi et al., 2010)
Antioxidant and antiulcerogenic activity	*in vitro*; DPPH/ *in vivo*; EtOH-induced gastric ulcer in rats	flowers	(Alimi et al., 2011)
Antiulcer activity	*in vivo*; rats	cladodes	(Galati et al., 2001)
Diabetes and diabetes complications managing activity	*in vitro*	aromadendrin from roots	(Jeon et al., 2011)
Diabetes and diabetes complications managing activity	*in vitro*	roots	(Kang and Lim, 2011)
Diuretic activity	*in vivo*; rats	cladodes, flowers, fruits	(Galati et al., 2002b)

Gastric mucus production increment activity	*in vivo*; EtOH-induced ulcer in rats	cladodes	(Galati et al., 2002a)
Gastric mucosa protective activity	*in vivo*; EtOH-induced ulcer in rats	mucilage and pectin	(Galati et al., 2007)
Gastric lesion protection activity	*in vivo*; stress-induced acute gastric lesions in rats	fruits	(Kim et al., 2012)
Hepatoprotective effect on CCl_4-induced toxicity	*in vivo*; rats	fruits	(Galati et al., 2005)
Hepatoprotective effect on chlorpyrifos-induced toxicity	*in vivo*; mice	cladodes	(Ncibi et al., 2008)
Hepatoprotective effect on EtOH toxicity	*in vivo*; rats	plant extracts	(Alimi et al., 2012b)
Hepatic steatosis attenuation activity	*in vivo*; obese Zucker (fa/fa) rats	plant extracts	(Morán-Ramos et al., 2012)
Hypoglycemic effect	*in vivo*; alloxan-induced diabetic rats	fruits	(Abd El-Razek and Hassan, 2011)
Hypoglycemic effect	*in vivo*; mice	polysaccharides	(Javier et al., 2003)
Hypolipidemic effect	*in vivo*; dietary induced hyperlipidemia in rats	fruits and stems	(Choi et al., 2002a)
Immunological activity enhancer	*in vitro*; anti-complement activity	plant extracts	(Kim and Oh, 2002)
Learning and memory improving activity	*in vivo*; passive avoidance tests	plant extracts	(Jin et al., 2012)
Lip-moisturizing effect	*in vitro*; PPAR-λ activity assay in CV-1 cell	plant extracts	(Hwang et al., 2011)
Lipoxygenase activity	*in vitro*	fruits	(De Gregorio et al., 2010)
Long-term memory improving activity	*in vivo*; mice	plant extracts	(Kim et al., 2010)

Neuroprotective effect	*in vitro*; oxidative injuries induced in rat primary cortical cell	quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether	(Dok-Go et al., 2003) (Lee et al., 2003b)
Neuroprotective effect	*in vitro*; microglial cells	stems	(Lee et al., 2006)
Neuroprotective effect on cerebral ischemia	*in vitro*; cultured mouse cortical cell/ *in vivo*; mice	plant extracts	(Kim et al., 2006)
Supplemented diet activity	*in vivo*; rats	seeds	(Ennouri et al., 2006)
Wound healing activity	*in vivo*; rats	stems	(Park and Chun, 2001)
Wound healing activity	*in vivo*; rats	cladodes	(Trombetta et al., 2006)

II. Materials and Methods

1. Isolation of chemical constituents from *O. ficus-indica* seeds

1.1. Plant material

Seeds of *Opuntia ficus-indica* (L.) Mill. (Cactaceae) were provided from DAEWON Pharm Co., Ltd. in July 2012 and air-dried in the Medicinal herb garden, College of Pharmacy, Seoul National University.

1.2. Reagents and equipments

1.2.1. Reagents

Analytical TLC: Kiesgel 60 F_{254}, Art. 5715, Merck, Germany

First grade solvent for extraction, fractionation and isolation: Dae Jung Pure chemical Eng. Co. Ltd., Korea

HPLC grade solvent: Fisher Scientific, Pittsburgh, PA, USA

ODS gel: YMC-GEL ODS-A, 12nm S-150 μmm, AA12SA5, YMC, Japan

Sea sand: 15-20 mesh, SAMCHUN pure chemical Co. Ltd., Korea

Sephadex LH-20: bead size 25-100 μm, Pharmacia, Sweden

Silica gel: Kiesgel 60, 40-63 μmm, 230-400 mesh, Art. 9385, Merck, Germany

1.2.2. Equipments

Analytical balance: Mettler AE 50, Switzerland

Drying oven: CO-2D-1S, Wooju Sci. Co., Korea

FT-IR: Perkin Elmer 1710 spectrophotometer, USA

Freeze-dryer: DURA-DRY, Fts system Inc., USA

HPLC system (Preparative):

- Gilson 321 pump, USA
- Gilson UV/Vis-151 detector, USA
- Column: Kromasil 100-10-C-18, K41025, Sweden

HPLC-DAD-ESIMS system:

- Finnigan Surveyor MS pump plus
- Finnigan Surveyor PDA detector
- Finnigan LCQ advantage Max
- Column: Ascentis Express C18 HPLC Column (4.6 x 150 mm, 2.7 μm)

MPLC: Reveleris 100922T, GRACEVYDAC, MD, USA

MPLC column: Reveleris C18, PN 5152991, GRACE, MD, USA

NMR: JEOL LA 300 Spectrometer (300 MHz), Japan

Bruker GPX 400 Spectrometer (400 MHz), Germany

Bruker AMX 500 Spectrometer (500 MHz), Germany

Bruker Avance 600 Sepctrometer (600 MHz), Germany

Polarimeter: JASCO, DIP-1000, Japan

Rotary evaporator: Shimadzu 2101, Japan

EYELA, Tokyo Rikakikai Co., Japan

Sonicator: Branson 5200, 5210, UK

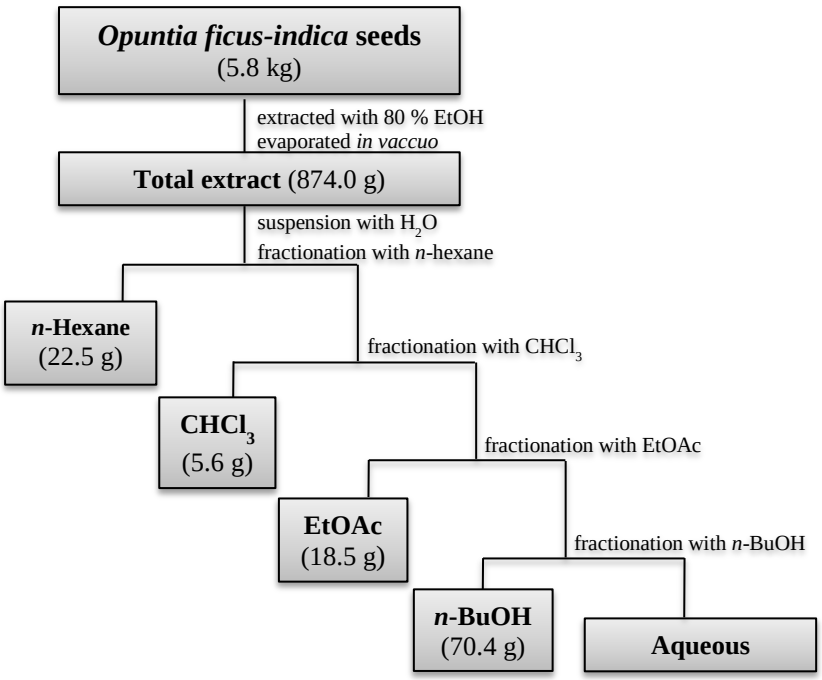
UV lamp: UVP UVGL-58, U.S.A.

UV spectrometer: Shimadzu UV-2101 Spectrophotometer, Japan

1.3. Methods

1.3.1. Extraction and fractionation of *O. ficus-indica*

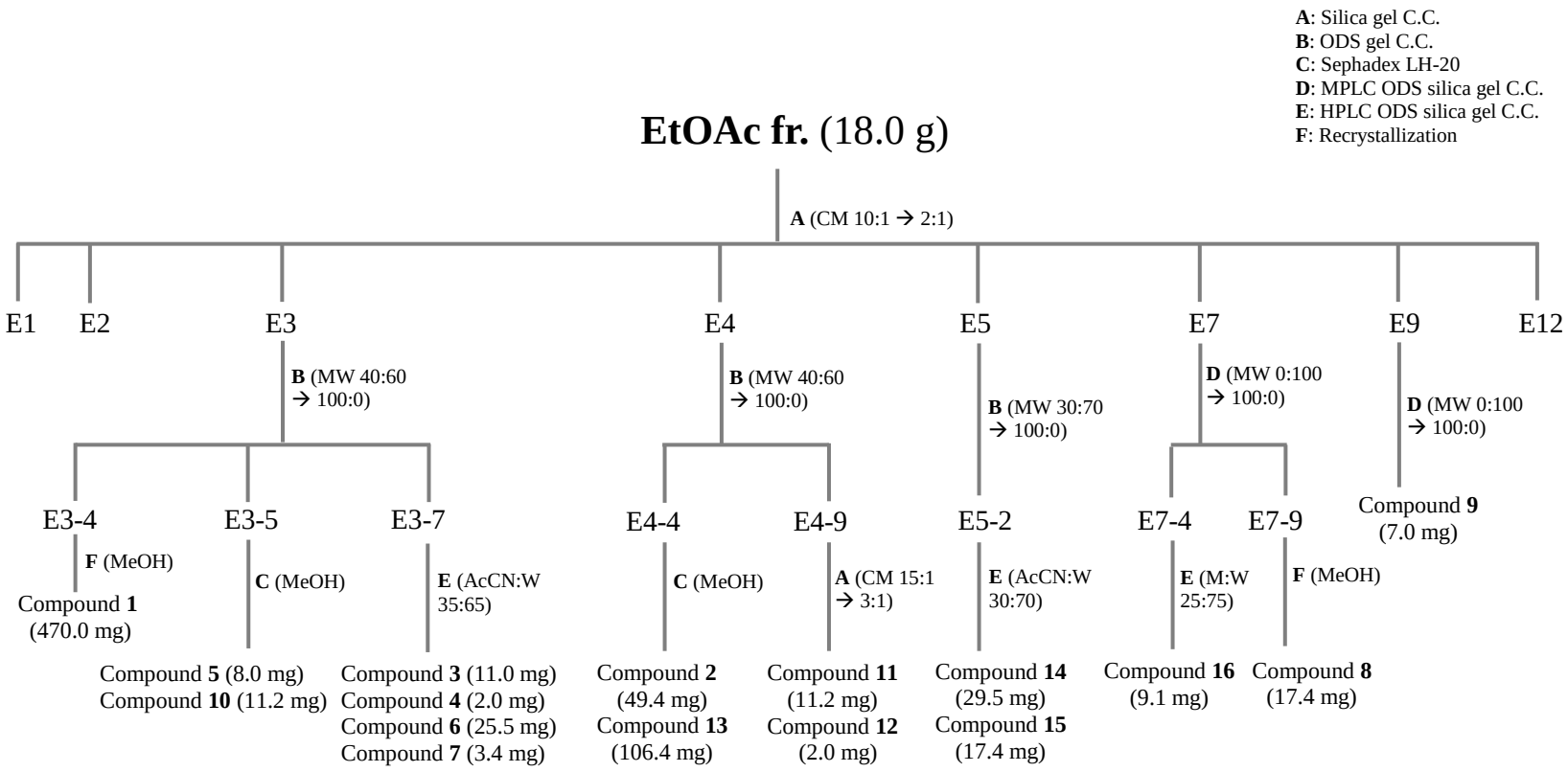
Dried seeds (5.8 kg) of *O. ficus-indica* were extracted with 80 % EtOH (12 L, 90 min x 5) in an ultrasonic apparatus. After removal of the solvent *in vaccuo*, the 80 % EtOH extract (874.0 g) was suspended in H_2O and successively partitioned into *n*-hexane fraction (22.5 g), $CHCl_3$ fraction (5.6 g), EtOAc fraction (18.5 g), and *n*-BuOH fraction (70.4 g), respectively (Scheme 1). Among these fractions, EtOAc fraction showed significantly protective activity against ethanol-intoxicated rat primary hepatocytes (25.1 ± 2.9 % at 10 μg/mL and 100.2 ± 12.3 % at 100 μg/mL). Besides, EtOAc fraction did not exhibit cytotoxicity on normal rat primary hepatocyte cells, so it was subjected to repeated column chromatography and HPLC to give bioactive compounds.



Scheme 1. Extraction and fractionation of *O. ficus-indica* seeds

1.3.2. Isolation of compounds from EtOAc fraction

The EtOAc fraction was subjected to silica gel column chromatography (c.c.) (45 cm x 10 cm) eluted with mixtures of $CHCl_3$-MeOH (from 10:1 to 1:1; v/v ratio) to yield 12 fractions (E1~E12) (Scheme 2). E3 (2.4 g) subjected to ODS gel c.c. with mixtures of MeOH-Water (from 40:60 to 100:0) to yield seven fractions (E3-1~E3-7). Compound **1** (470.0 mg) obtained from E3-4 by recrystallization. Compound **5** (8.0 mg) and **10** (11.2 mg) were isolated from E3-5 by c.c. on Sephadex LH-20 using MeOH. Compound **3** (11.0 mg), **4** (2.0 mg), **6** (25.5 mg), **7** (3.4 mg) were obtained from E3-7 through preparative HPLC (C_{18}, 250 x 21.2 mm, AcCN-Water, 35:65, 8 mL/min, UV 254 nm). E4 (2.4 g) was further separated into nine fractions (E4-1~E4-9) using ODS gel c.c. with mixtures of MeOH-Water (from 40:60 to 100:0). Compound **2** (49.4 mg) and **13** (106.4 mg) were obtained from E4-4 on Sephadex LH-20 using MeOH. Compound **11** (11.2 mg) and **12** (2.0 mg) were isolated from E4-9 by silica gel c.c. with mixtures of $CHCl_3$-MeOH (from 15:1 to 3:1). E5 (0.8 g) was subjected to ODS gel c.c. with mixtures of MeOH-Water (from 30:70 to 100:0) to give six fractions (E5-1~E5-6). Compound **14** (29.5 mg) and **15** (17.4 mg) obtained from E5-2 through preparative HPLC (C_{18}, 250 x 21.2 mm, AcCN-Water, 30:70, 8 mL/min, UV 275 nm). E7 (2.1 g) was subjected to MPLC (C_{18}, 120g, MeOH-Water, from 0:100 to 95:5, 40 mL/min) to yield twelve fractions (E7-1~E7-12). Compound **16** (9.1 mg) was isolated from E7-4 through preparative HPLC (C_{18}, 250 x 21.2 mm, MeOH-Water, 25:75, 8 mL/min, UV 254 nm). Compound **8** (17.4 mg) was obtained from E7-9 by recrystallization. E9 (1.1 g) was subjected to MPLC (C_{18}, 120g, MeOH-Water, from 0:100 to 100:0, 50 mL/min) to give eleven fractions (E9-1~E9-11) and Compound **9** (7.0 mg) was obtained from E9-9 by recrystallization. The overall procedures of isolation of constituents were summarized in Scheme 2.



Scheme 2. Isolation of compounds from EtOAc fraction of *O. ficus-indica* seeds

1.3.3. HPLC-DAD-ESIMS chemical fingerprint analysis

The EtOAc fraction of *O. ficus-indica* seeds was resolved in MeOH as 1.5 mg/mL and analyzed by HPLC-DAD-ESIMS for the identification of peaks of sixteen isolated compounds. The injection sample was prepared by filtering through nylon membrane filter (0.2 μm). Chromatographic separation was achieved on an Ascentis Express C18 column (4.6 mm i.d. x 150 mm, 2.7 μm) and the column temperature was set at 30 ℃. The mobile phase consisted of (A) 0.1% formic acid and (B) acetonitrile at a flow rate of 0.3 mL/min, with gradient elution as follows: 0 min, 20% (v/v) B; 25 min, 80% B; 25.1 min, 20% B; 30 min, 20% B. The injection volume was 10 μL. Peaks of compounds in the sample were identified by comparison of retention times and spectral data of each peak with those of corresponding isolated compounds.

Compound **1**

pale yellowish amorphous powder

$C_{15}H_{12}O_6$

$[\alpha]^{25}_{D}$: +58.6 (*c*=0.30, MeOH)

R_f: 0.70 (Silica TLC, C:M=7:1), 0.70 (RP TLC, M:W=2:1)

ESIMS: *m/z* 287 $[M-H]^-$

^{1}H NMR (DMSO-d_6, 400 MHz): see Table 2

^{13}C NMR (DMSO-d_6, 100 MHz): see Table 5

Compound **2**

pale yellowish amorphous powder

$C_{15}H_{12}O_7$

$[\alpha]^{25}_{D}$: +40.1 (*c*=0.30, MeOH)

R_f: 0.20 (RP TLC, M:W=3:2)

ESIMS: *m/z* 303 $[M-H]^-$

^{1}H NMR (DMSO-d_6, 400 MHz): see Table 2

^{13}C NMR (DMSO-d_6, 75 MHz): see Table 5

Compound **3**

yellowish amorphous powder

$C_{15}H_{10}O_6$

R_f: 0.30 (RP TLC, M:W=2:1)

ESIMS: *m/z* 285 $[M-H]^-$

^{1}H NMR (DMSO-d_6, 400 MHz): see Table 3

^{13}C NMR (DMSO-d_6, 75 MHz): see Table 5

Compound **4**

yellowish amorphous powder

$C_{16}H_{12}O_6$

R_f: 0.70 (RP TLC, M:W=3:2)

ESIMS: *m/z* 299 $[M-H]^-$

^{1}H NMR (DMSO-d_6, 500 MHz): see Table 3

^{13}C NMR (DMSO-d_6, 125 MHz): see Table 5

Compound **5**

yellowish amorphous powder

$C_{15}H_{10}O_7$

R_f: 0.20 (RP TLC, M:W=3:2)

ESIMS: *m/z* 301 $[M-H]^-$

^{1}H NMR (DMSO-d_6, 400 MHz): see Table 3

^{13}C NMR (DMSO-d_6, 75 MHz): see Table 5

Compound **6**

yellowish amorphous powder

$C_{16}H_{12}O_7$

R_f: 0.40 (RP TLC, M:W=3:2)

ESIMS: *m/z* 315 $[M-H]^-$

^{1}H NMR (DMSO-d_6, 500 MHz): see Table 3

^{13}C NMR (DMSO-d_6, 125 MHz): see Table 5

Compoud **7**

yellowish amorphous powder

$C_{16}H_{12}O_7$

R_f: 0.40 (RP TLC, M:W=3:2)

ESIMS: *m/z* 315 $[M-H]^-$, 361 $[M+HCOOH-H]^-$

^{1}H NMR (DMSO-d_6, 500 MHz): see Table 3

^{13}C NMR (DMSO-d_6, 125 MHz): see Table 5

Compound **8**

pale yellowish amorphous powder

$C_{22}H_{22}O_{12}$

R_f: 0.40 (Silica TLC, C:M:W=15:4:1)

ESIMS: *m/z* 315 $[M-Glc]^-$, 477 $[M-H]^-$

^{1}H NMR (DMSO-d_6, 500 MHz): see Table 3

^{13}C NMR (DMSO-d_6, 125 MHz): see Table 5

Compound **9**

pale yellowish amorphous powder

$C_{28}H_{32}O_{16}$

IR ν_{max} (MeOH) (cm^{-1}): 3370, 2917, 1656, 1605, 1512, 1358, 1205, 1062

R_f: 0.30 (Silica TLC, C:M:W=15:4:1)

ESIMS: *m/z* 315 [M-Rha-Glc]$^-$, 623 [M-H]$^-$, 668 [M+HCOOH-H]$^-$

^{1}H NMR (CD_3OD, 500 MHz): see Table 3

^{13}C NMR (CD_3OD, 125 MHz): see Table 5

Compound **10**

white amorphous powder

$C_{18}H_{16}O_6$

$[\alpha]^{25}_{D}$: +46.9 (*c*=0.30, MeOH)

CD (*c*=0.025, MeOH) $\Delta\varepsilon$ (nm): +54.32 (230), +57.71 (244)

IR ν_{max} (MeOH) (cm^{-1}): 3296, 2928, 2853, 2749, 2257, 1664, 1604, 1506, 1441, 1286, 1130, 1024, 815

R_f: 0.25 (RP TLC, M:W=3:2)

ESIMS: *m/z* 327 [M-H]$^-$, 373 [M+HCOOH-H]$^-$

^{1}H NMR (DMSO-d_6, 600 MHz): Table 6

^{13}C NMR (DMSO-d_6, 150 MHz): Table 7

Compound **11**

white amorphous powder

$C_{18}H_{16}O_7$

CD (*c*=0.025, MeOH) $\Delta\varepsilon$ (nm): -35.05 (225), -33.8 (243)

IR ν_{max} (MeOH) (cm^{-1}): 3343, 1879, 1684, 1607, 1441, 1272, 1198, 815

R_f: 0.60 (RP TLC, M:W=65:35)

ESIMS: *m/z* 389 [M+HCOOH-H]$^-$, 687 [2M-H]$^-$

^{1}H NMR (CD_3OD, 600 MHz): see Table 6

^{13}C NMR (CD_3OD, 150 MHz): see Table 7

Compound **12**

white amorphous powder

$C_{19}H_{20}O_6$

CD (*c*=0.025, MeOH) $\Delta\varepsilon$ (nm): -37.07 (224), -35.41 (244)

IR ν_{max} (MeOH) (cm^{-1}): 3273, 2928, 2257, 1869, 1656, 1507, 1449, 1378, 1276, 1116, 819

R_f: 0.55 (RP TLC, M:W=2:1)

ESIMS: *m/z* 343 [M-H]$^-$, 687 [2M-H]$^-$

^{1}H NMR (DMSO-d_6, 600 MHz): see Table 6

^{13}C NMR (DMSO-d_6, 150 MHz): see Table 7

Compound **13**

colorless amorphous powder

$C_{18}H_{18}O_6$

R_f: 0.75 (RP TLC, M:W=3:2)

ESIMS: *m/z* 329 $[M-H]^-$, 375 $[M+HCOOH-H]^-$

^{1}H NMR (DMSO-d_6, 500 MHz): see Table 6

^{13}C NMR (DMSO-d_6, 125 MHz): see Table 7

Compound **14**

brownish amorphous syrup

$C_{27}H_{26}O_9$

$[\alpha]^{25}_D$: +25.7 (*c*=0.30, MeOH)

CD (*c*=0.025, MeOH) $\Delta\varepsilon$ (nm): +50.79 (240), +25.46 (256)

IR ν_{max} (MeOH) (cm^{-1}): 3273, 2963, 2935, 2876, 2258, 1606, 1509, 1281, 1023, 819

R_f: 0.65 (RP TLC, M:W=2:1)

ESIMS: *m/z* 493 $[M-H]^-$, 987 $[2M-H]^-$

^{1}H NMR (DMSO-d_6, 600 MHz): see Table 6

^{13}C NMR (DMSO-d_6, 150 MHz): see Table 7

Compound **15**

brownish amorphous syrup

$C_{27}H_{26}O_9$

$[\alpha]^{25}_{D}$: -5.3 (*c*=0.30, MeOH)

CD (*c*=0.025, MeOH) $\Delta\varepsilon$ (nm): +57.29 (240), +29.51 (257)

IR ν_{max} (MeOH) (cm^{-1}): 3283, 2961, 2928, 2875, 2257, 1607, 1508, 1281, 1023, 819

R_f: 0.60 (RP TLC, M:W=2:1)

ESIMS: *m/z* 493 $[M-H]^-$, 987 $[2M-H]^-$

^{1}H NMR (DMSO-d_6, 600 MHz): see Table 6

^{13}C NMR (DMSO-d_6, 150 MHz): see Table 7

Compound **16**

Pale yellowish amorphous syrup

$C_{16}H_{20}O_9$

R_f: 0.80 (RP TLC, M:W=2:1)

IR ν_{max} (MeOH) (cm^{-1}): 3370, 2932, 2260, 1714, 1597, 1517, 1274, 1074, 825

ESIMS: *m/z* 355 $[M-H]^-$, 401 $[M+HCOOH-H]^-$

^{1}H NMR (DMSO-d_6, 500 MHz): see Table 8

^{13}C NMR (DMSO-d_6, 125 MHz): see Table 8

Table 2. ^{1}H NMR spectral data of compounds **1-2**

Position	**1**	**2**
	δ_H (J in Hz)	
2	5.05, d (11.4)	4.97, d (11.2)
3	4.58, dd (11.5, 4.8)	4.50, dd (11.2, 6.2)
6	5.86, d (2.0)	5.85, d (2.0)
8	5.91, d (2.0)	5.90, d (2.0)
2'	7.31, d (8.5)	7.31, d (1.4)
3'	6.78, d (8.5)	
5'	6.78, d (8.5)	6.74, s
6'	7.31, d (8.5)	6.74, s

^{1}H data were measured at 400 MHz in DMSO-d_6.

Table 3. ^{1}H NMR spectral data of compounds **3-7**

Position	**3**[a]	**4**[b]	**5**[a]	**6**[b]	**7**[b]
	δ_H (J in Hz)				
6	6.19, d (2.0)	6.19, d (2.1)	6.18, d (1.8)	6.18, d (2.0)	6.19, d(1.9)
8	6.43, d (2.0)	6.43, d (1.9)	6.40, d (1.9)	6.40, d (1.9)	6.47, d (1.9)
2'	8.03, dd (11.6, 2.8)	7.93, d (8.9)	7.67, d (2.1)	7.55, d (2.2)	7.75, d (1.9)
3'	6.93, dd (9.8, 2.7)	6.94, d (8.8)			
5'	6.93, dd (9.8, 2.7)	6.94, d (8.8)	6.88, d (8.5)	6.90, d (8.5)	6.94, d (8.4)
6'	8.03, dd (11.6, 2.8)	7.93, d (8.9)	7.53, dd (8.4, 2.2)	7.44, dd (8.5, 2.2)	7.69, dd (8.5, 2.0)
OCH_3		3.77, s		3.78, s	3.84, s

^{1}H data were measured at [a] 400 and [b] 500 MHz in DMSO-d_6, respectively.

Table 4. ^{1}H NMR spectral data of compounds **8-9**

Position	**8**[a]	**9**[b]
	δ_H (J in Hz)	
6	6.21, d (1.9)	6.21, d (2.0)
8	6.44, d (1.9)	6.41, d (2.0)
2'	8.02, d (1.9)	7.94, d (2.0)
5'	6.90, d (8.6)	6.91, d (8.6)
6'	7.51, dd (8.4, 2.0)	7.62, dd (8.5, 2.0)
OCH_3	3.85, s	3.94, s
	***β*-D-glucose**	***β*-D-glucose**
1''	5.50, d (7.7)	5.24, d (7.4))
2''	3.56, m	3.47, m
3''	3.37, m	3.45, m
4''	3.68, m	3.24, m
5''	3.40, m	3.36, m
6''	3.50, 3.36, m	3.81, 3.41, m
		***α*-L-rhamnose**
1'''		4.52, d (1.1)
2'''		3.48, m
3'''		3.60, m
4'''		3.25, m
5'''		3.41, m
6'''		1.09, d (6.2)

^{1}H data were measured at [a] 500 MHz in DMSO-d_6 and [b] 500 MHz in CD_3OD, respectively.

Table 5. ^{13}C NMR spectral data of compounds **1-9**

position	**1**[a]	**2**[b]	**3**[b]	**4**[c]	**5**[b]	**6**[c]	**7**[c]	**8**[c]	**9**[d]
2	82.9	83.1	146.8	155.6	147.7	155.6	178.8	156.2	159.7
3	71.4	71.6	135.6	137.6	135.7	137.7	135.8	133.1	136.3
4	197.9	197.7	175.8	177.9	175.8	177.9	175.9	177.4	180.2
5	163.3	163.3	160.7	161.2	160.7	161.3	160.7	161.2	163.8
6	96.0	96.0	98.2	98.6	98.2	98.6	98.2	98.2	100.8
7	166.8	166.8	163.9	164.3	163.9	164.1	163.9	164.1	166.8
8	95.0	95.0	93.5	93.7	93.3	93.6	93.6	93.6	95.7
9	162.6	162.6	156.1	156.4	156.1	156.3	156.1	156.3	159.3
10	100.5	100.5	103.0	104.1	103.0	104.2	103.0	104.0	106.1
1'	127.6	128.1	121.6	120.6	121.9	120.8	122.0	121.0	123.8
2'	129.5	115.1	129.5	130.1	115.0	115.4	111.7	113.5	115.4
3'	114.9	144.9	115.4	115.7	145.0	145.3	147.3	146.9	149.1
4'	157.7	145.8	159.1	160.2	146.7	148.7	146.6	149.4	151.7
5'	114.9	115.4	115.4	115.7	115.6	115.8	115.5	115.1	116.9
6'	129.5	119.4	129.5	130.1	119.9	120.6	121.7	121.8	124.8
OCH_3				59.7		59.7	55.8	55.9	57.6
				***β*-D-glucose**					
1"								101.6	105.2
2"								71.2	76.7
3"								75.9	79.0
4"								67.9	72.4
5"								73.1	78.2
6"								60.3	69.3
				***α*-L-rhamnose**					
1'''									103.3
2'''									73.1
3'''									72.9
4'''									74.6
5'''									70.6
6'''									18.7

^{13}C NMR data were measured at [a] 100 (DMSO-d_6), [b] 75 (DMSO-d_6), [c] 125 (DMSO-d_6) and [d] 125 (CD_3OD) MHz, respectively.

Table 6. ^{1}H NMR spectral data of compounds **10-15**

position	**10**[a]	**11**[b]	**12**[a]	**13**[c]	**14**[a]	**15**[a]
	δ_H (J in Hz)					
2	6.82, d (1.8)	6.85, d (1.9)	6.80, d (1.9)	6.75, d (1.9)	6.80, d (1.9)	6.79, d (2.3)
5	6.77, d (7.8)	6.80, d (7.8)	6.75, d (8.3)	6.70, d (8.1)	6.75, d (8.3)	6.75, d (8.3)
6	6.72, dd (8.2, 1.9)	6.77, dd (7.3, 1.9)	6.69, dd (8.2, 1.9)	6.60, dd (8.2, 1.9)	6.70, dd (8.3, 1.9)	6.69, dd (7.8, 2.3)
7	4.87, d (7.8)	4.83, d (8.3)	4.82, d (7.7)	4.54, d (4.0)	4.81, d (7.7)	4.80, d (7.8)
8	4.15, m	4.05, m	4.04, m	2.94, m	4.01, m	4.01, m
9	3.25, m	3.69, dd (12.4, 2.8)	3.32, m	3.69, m	3.52, dd (12.4, 2.8)	3.51, dd (11.9, 2.3)
	3.55, m	3.48, dd (12.4, 4.6)	3.52, dd (12.2, 2.3)	4.08, m	3.33, dd (12.4, 4.6)	3.31, dd (12.4, 5.0)
2'	7.36, d (1.8)	7.16, d (2.3)	7.0, d (1.8)	6.75, d (1.9)	6.88, d (1.9)	6.91, d (1.8)
5'	7.02, d (8.2)	6.97, d (8.7)	6.88, d (8.2)	6.70, d (8.1)	6.89, d (8.3)	6.86, d (8.2)
6'	7.28, dd (8.7, 2.3)	7.12, dd (8.2, 1.8)	6.95, dd (8.2, 2.2)	6.60, dd (8.2, 1.9)	6.85, dd (8.3, 1.8)	6.83, dd (8.7, 1.9)
7'	7.62, d (16.0)	7.57, d (16.0)	6.48, d (16.1)	4.54, d (4.0)	4.63, d (4.6)	4.62, d (4.6)
8'	6.73, dd (15.1, 1.9)	6.31, d (16.0)	6.18, dt (16.0, 5.9)	2.94, m	2.98, m	2.99, m
9'	9.60, d (7.8)		3.99, dd (6.0, 1.4)	4.08, m 3.69, m	4.09, m 3.72, m	4.10, m 3.72, m
2"					6.73, d (1.9)	6.74, d (1.9)
5"					6.67, d (7.8)	6.68, d (7.8)
6"					6.59, dd (8.3, 1.9)	6.60, dd (7.8, 1.9)
7"					4.53, d (5.0)	4.52, d (5.0)
8"					2.95, m	2.97, m
9"					4.09, m 3.72, m	4.10, m 3.72, m
OCH_3			3.25, s			

^{1}H data were measured at [a] 600 (DMSO-d_6), [b] 600 (CD_3OD) and [c] 500 (DMSO-d_6) MHz, respectively.

Table 7. ^{13}C NMR spectral data of compounds **10-15**

position	**10**[a]	**11**[b]	**12**[a]	**13**[c]	**14**[a]	**15**[a]
1	127.2	130.1	126.5	132.5	127.6	127.6
2	115.0	116.5	113.9	113.8	114.9	114.9
3	145.2	147.6	144.2	144.7	145.1	145.2
4	145.9	148.1	144.7	145.2	145.8	145.7
5	115.5	117.3	114.4	115.4	115.4	115.4
6	118.9	121.3	117.8	117.2	118.8	118.8
7	75.6	78.5	74.6	85.1	75.6	75.5
8	78.6	81.3	77.3	53.8	78.2	78.2
9	60.0	62.9	59.1	71.0	60.2	60.2
1'	127.4	130.2	128.8	132.5	134.5	134.7
2'	116.8	118.3	113.4	113.8	114.5	114.4
3'	143.9	146.5	142.6	144.7	143.4	143.0
4'	146.2	148.1	142.0	145.2	142.5	142.8
5'	117.2	119.3	115.7	115.4	116.5	116.6
6'	123.0	124.1	118.6	117.2	118.9	118.6
7'	153.2	147.0	130.0	85.1	84.7	84.6
8'	126.8	118.2	123.4	53.8	53.7	53.8
9'	194.1	171.6	71.2	71.0	70.9	70.9
1"					132.3	132.3
2"					113.6	113.6
3"					145.2	145.1
4"					144.6	144.6
5"					115.3	115.3
6"					117.0	117.0
7"					85.0	85.0
8"					53.6	53.6
9"					71.0	71.0
OCH_3			56.1			

^{13}C NMR data were measured at [a] 150 (DMSO-d_6), [b] 150 (CD_3OD), [c] 125 (DMSO-d_6) MHz, respectively.

Table 8. ^{1}H and ^{13}C NMR spectral data of compound **16**

Position	**16**	
	δ_H (J in Hz)	δ_C
1		165.4
2	6.48, d (16.0)	113.9
3	7.63, d (15.9)	146.3
1'		125.5
2'	7.34, d (1.6)	111.4
3'		147.9
4'		149.6
5'	6.80, d (8.2)	115.5
6'	7.15, dd (8.2, 1.5)	123.3
OCH_3	3.82, s	55.7
	***β*-D-glucose**	
1"	5.46, d (8.1)	94.2
2"	3.20, m	72.5
3"	3.23, m	76.5
4"	3.07, m	69.5
5"	3.22, m	77.8
6"	3.46, m 3.66, m	60.6

^{1}H NMR data was measured at 500MHz and ^{13}C NMR data was measured at 125MHz in DMSO-d_6, respectively.

2. Evaluation of hepatoprotective activity *in vitro* model

2.1. Animal material

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. 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.

2.2. Reagents and equipments

2.2.1. Reagents

Dulbecco's modified Eagle's media (DMEM), penicillin/streptomycin, dexamethasone, and insulin for cultures of rat primary hepatocyte cells, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium (MTT), dimethylsulfoxide (DMSO), ethanol, trypan blue and urethane were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT, USA). Cell culture dishes were purchased from Corning (New York, NY, USA). For isolation of rat primary hepatocytes, Hank's balanced salt solution (HBSS) was purchased from Gibco Co. (Grand Island, NY, USA), ethylenediaminetetraacetic acid (EDTA), Pronase E and Collagenase type IV were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Collagen type I for dish coating was purchased from BD (Becton, Dickinson and Company) (Franklin Lakes, NJ, USA).

2.2.2. Equipments

Autoclave: Sanyo MLS 3000, Japan

Catheter: BD IV catheter 381423, NJ, USA

Centrifuge: Effendroff, Germany

Clean bench: Sookong, Korea

Elisa reader: Molecular Devices E_{max}, USA

Forceps: KASCO S13-167, Parkistan

Peristaltic pump: MasterFlex 7553-70, Cole-Parmer Instrument Co., IL, USA

pH meter: Phoenix PMS-500, USA

Phase contrast inverted microscope: Olympus CK2-Japan

Scissors: Professional, Parkistan

Silicon tubing: MaterFlex 96440-14, Cole-Parmer Instrument Co., IL, USA

Water bath: EYELA, Tokyo Rikakikai Co., Japan

Water-jacked CO_2 incubator: Forma Scientific Co., USA

2.3. Methods

2.3.1. Isolation of rat primary hepatocytes

Isolated rat primary hepatocytes were prepared from male SD rats by the collagenase perfusion technique of Berry and Friend by means of minor modifications (Berry and Friend, 1969; Kleinman et al., 1979). First of all, several types of buffer solutions were arranged and pre-warmed in the water bath at 43 ℃ to keep the outlet temperature of cannula at 37 ℃: perfusion solution of HBSS without Ca^{2+} and with 0.2 mM EDTA, 0.1 % pronase-E solution of HBSS with 200 mM $CaCl_2$, 0.05 % collagenase solution of HBSS with 200 mM $CaCl_2$. All buffers and instruments were prepared with sterilization by an autoclave technique in advance. For perfusion work, an adult rat (about 250 g body weights) was anesthetized with urethane (1 g/kg body weight), and the abdominal cavity was opened. After placing the viscera to the left outside of the abdominal cavity, liver portal vein was cannulated with catheter and perfusion solution was provided at a flow rate of 30 mL/min. Observing the change of hepatic appearance, the superior vena cava was gripped by a forcep to diminish a perfusion pathway, and the inferior vena cava was cut for relieving pressure. Following this, pronase-E and collagenase solution were serially supplied. After completing collagenase perfusion, the liver was dissected from the body and dispersed allowing the cells to release to HBSS solution within a sterile petri dish. The solution was filtered by passing through sterilized gauze, and centrifuged at 50 g for 4 min. The pellet was washed with HBSS containing 200 mM $CaCl_2$ and centrifuged again at 50 g for 4 min. The pellet was washed again with culture medium made up of DMEM with high glucose, 10 % FBS, 1 % PS, 1 μM dexamethasone and 0.1 μM insulin, and then centrifuged at 50 g for 4 min. The obtained cell suspension was seeded on

collagen-coated culture dishes at the concentration of 2 x 10^5 cells/mL and maintained at 37 ℃ in a humidified incubator containing 5 % CO_2 gas.

2.3.2. Ethanol-induced hepatotoxicity

Stabilizing isolated rat primary hepatocytes, the culture medium was replaced 16 h after from seeding. To induce toxicity on rat primary hepatocytes using ethanol, the experimental condition was firstly examined. Developing proper exposure time of a toxicant, rat primary hepatocytes were treated with ethanol at concentrations of 100 mM and 200 mM for ranging from 2 to 72 h, respectively. Cells were treated with ethanol, also, at concentrations of ranging from 50 to 350 mM for 48 h to determine the final concentration of a toxicant. A condition exposing 200 mM ethanol to rat primary hepatocyte cells for 48 h was used for evaluating the protective activities of substances.

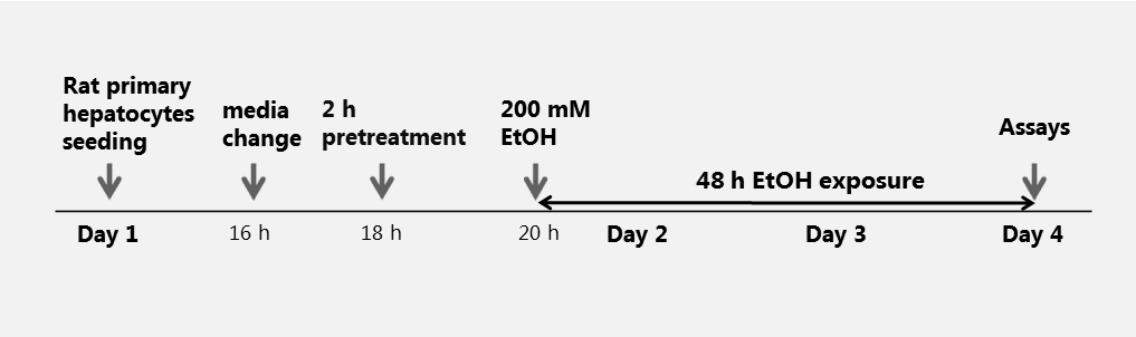


Figure 3. Overall experimental procedures for evaluation of hepatoprotective activity *in vitro*

2.3.3. Measurement of protective activity on cell viability

For the assay, the cells were seeded on 35 π dishes at a density of 2×10^5 cells/mL and incubated for 18 h. Rat primary hepatocytes were pretreated with a vehicle or compounds at the concentration ranging from 10 to 50 μM for 2 h, and followed by administering ethanol to the cultures in the presence of a vehicle or compounds for the further 48 h (Liu et al., 2010). Protective activity of each compound on cell proliferation was assessed by MTT assay (Mosmann, 1983). Rat primary hepatocyte cells were incubated with 2 mg/mL of MTT for 2 h. Reduction of MTT to formazan was assessed in an ELISA plate reader at 540 nm. Data were expressed at percent cell viability relative to control cultures as the mean of three independent experiments.

Cell viability (% of control) = (Abs. of sample-treated) / (Abs. of control) x 100

2.4. Statistical analysis

All data were expressed as means ± S.D. The evaluation of statistical significance was determined by an 'one-way ANOVA' test using computerized statistical package, with $p < 0.05$ *, $p < 0.01$ ** and $p < 0.001$ ***considered to be statistically significant.

III. Result and discussion

1. Structure elucidation of isolated compounds from *O. ficus-indica* seeds

1.1. Compounds **1** and **2**

Compound **1** was obtained as pale yellowish amorphous powder and $[\alpha]^{25}_{D}$ +58.6 (*c*=0.30, MeOH). The molecular formula, $C_{15}H_{12}O_6$, was established by the negative mode ESIMS (*m/z* 287 $[M-H]^-$) and the ^{13}C NMR spectrum. The ^1H NMR spectrum showed the signals for 1,4-substituted aromatic protons [δ_H 7.31 (2H, d, *J* = 8.5, H-2', 6'), 6.78 (2H, d, *J* = 8.5, H-3', 5')], two meta coupling protons [δ_H 5.91 (1H, d, *J* = 2.0, H-8), 5.86 (1H, d, *J* = 2.0, H-6)] and two oxygenated methine protons [δ_H 5.05 (1H, d, *J* = 11.4, H-2), 4.58 (1H, dd, *J* = 11.5, 4.8, H-3)]. Based on above data, compound **1** was identified to (+)-aromadendrin of flavanol moiety with comparison of literature values (Wang et al., 2008).

Compound **2** was isolated as pale yellowish amorphous powder and $[\alpha]^{25}_{D}$ +40.1 (*c*=0.30, MeOH). The molecular formula, $C_{15}H_{12}O_7$, was determined by ion peak at *m/z* 303 $[M-H]^-$ of the negative mode ESIMS. The ^1H NMR spectrum showed the signals for 1,3,4-substituted aromatic protons [δ_H 7.31 (1H, d, *J* = 1.4, H-2'), 6.74 (2H, s, H-5', 6')], two meta coupling protons [δ_H 5.90 (1H, d, *J* = 2.0, H-8), 5.85 (1H, d, *J* = 2.0, H-6)] and two oxygenated methine protons [δ_H 4.97 (1H, d, *J* = 11.2, H-2), 4.50 (1H, dd, *J* = 11.2, 6.2, H-3)]. The ^{13}C NMR spectrum revealed fifteen carbons including aromatic carbons and carbonyl carbon. From these data, the structure of compound **2** was elucidated as (+)-taxifolin by comparison with previously reported literature (Wang et al., 2008).

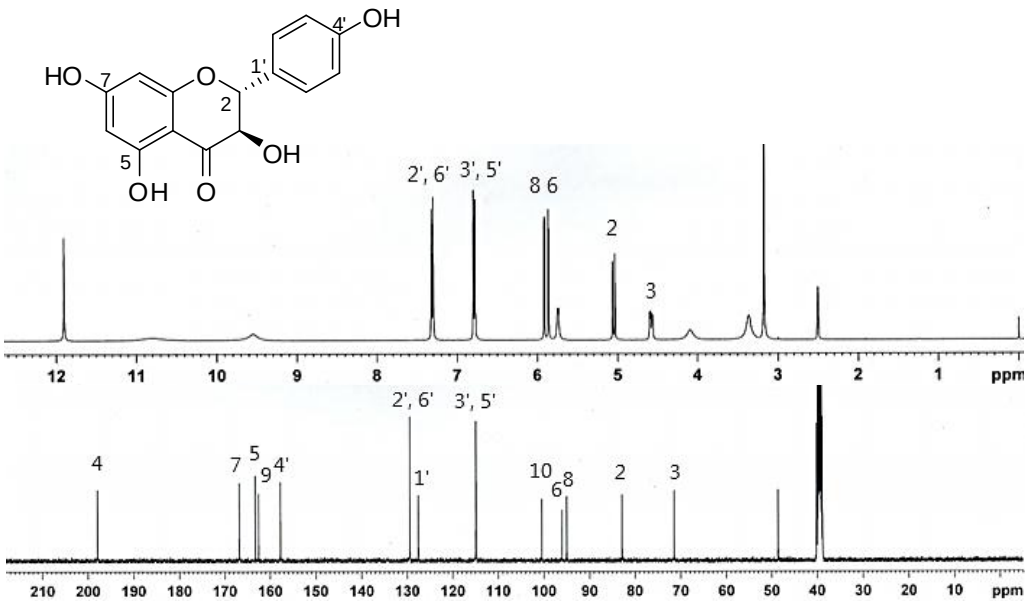


Figure 4. ^{1}H and ^{13}C NMR spectra of compound **1**

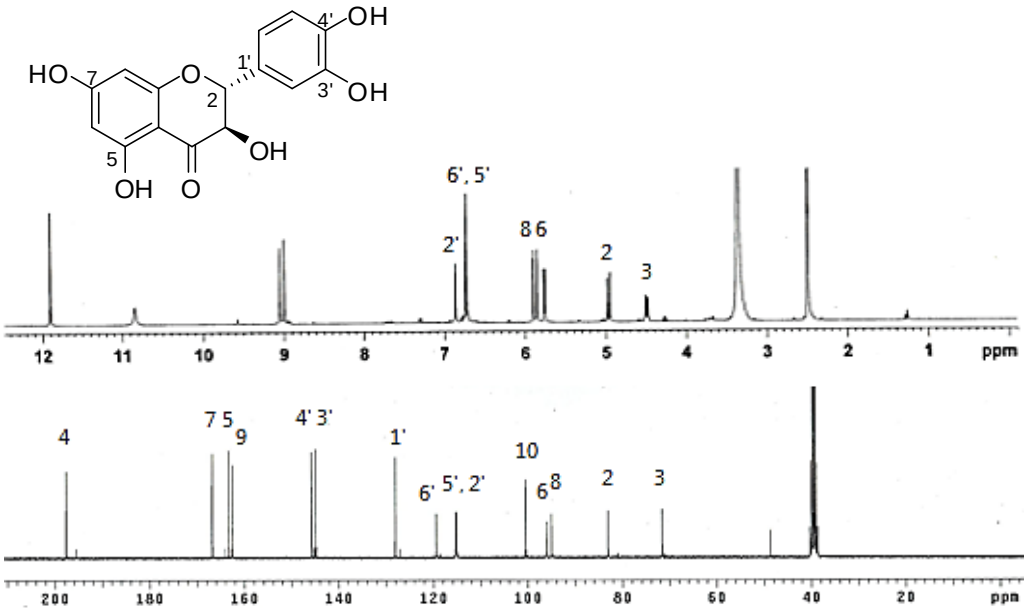


Figure 5. ^{1}H and ^{13}C NMR spectra of compound **2**

1.2. Compounds **3** and **4**

Compound **3** was obtained as yellowish amorphous powder and its molecular formula was identified as $C_{15}H_{10}O_6$ based on the negative mode ESIMS (*m/z* 285 $[M-H]^-$) and the ^{13}C NMR analysis. The ^1H NMR spectrum showed the signals for 1,4-substituted aromatic protons [δ_H 8.03 (2H, dd, *J* = 11.6, 2.8, H-2', 6'), 6.93 (2H, dd, *J* = 9.8, 2.7, H-3', 5')] and two meta coupling protons [δ_H 6.43 (2H, d, *J* = 2.0, H-8), 6.19 (1H, d, *J* = 2.0, H-6)]. From these described spectral data, compound **3** was concluded as kaempferol of flavonol moiety (Sultan et al., 2008).

Compound **4** was isolated as yellowish amorphous powder and its molecular formula was determined as $C_{16}H_{12}O_6$ by the negative mode ESIMS (*m/z* 299 $[M-H]^-$) and the ^{13}C NMR spectral data. The ^1H and ^{13}C NMR spectral data of compound **4** showed similar patterns with those of compound **3** except for a methoxy signal [δ_H 3.77 (3H, s, $-OCH_3$)/ δ_c 59.7 ($-OCH_3$)]. In the HMBC spectrum, it was confirmed that the proton of methoxy group was correlated with C-3 (δ_c 137.6). Through comparison of above data with literature values, compound **4** was identified to kaempferol 3-methyl ether (Wang et al., 1989).

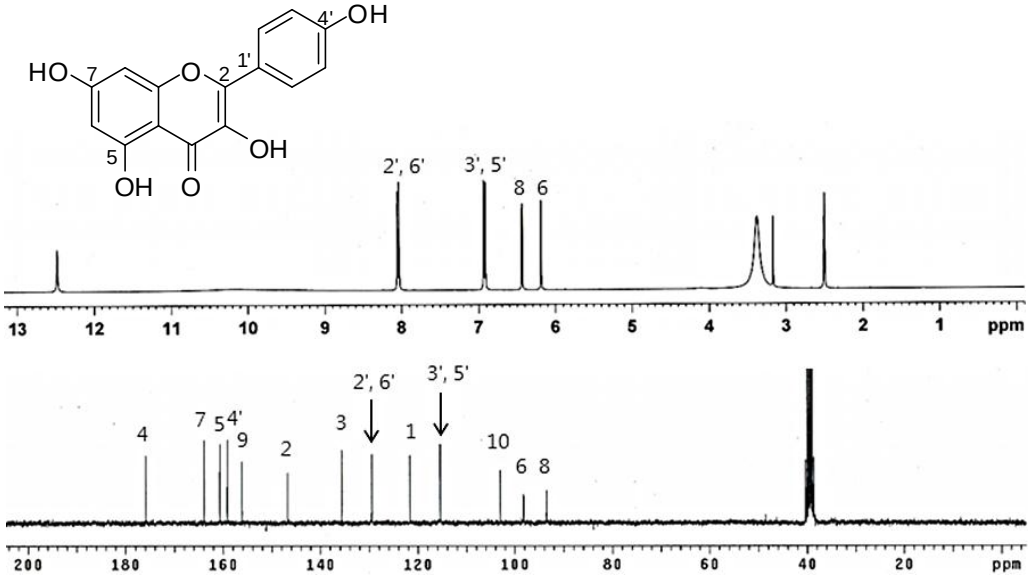


Figure 6. ^{1}H and ^{13}C NMR spectra of compound **3**

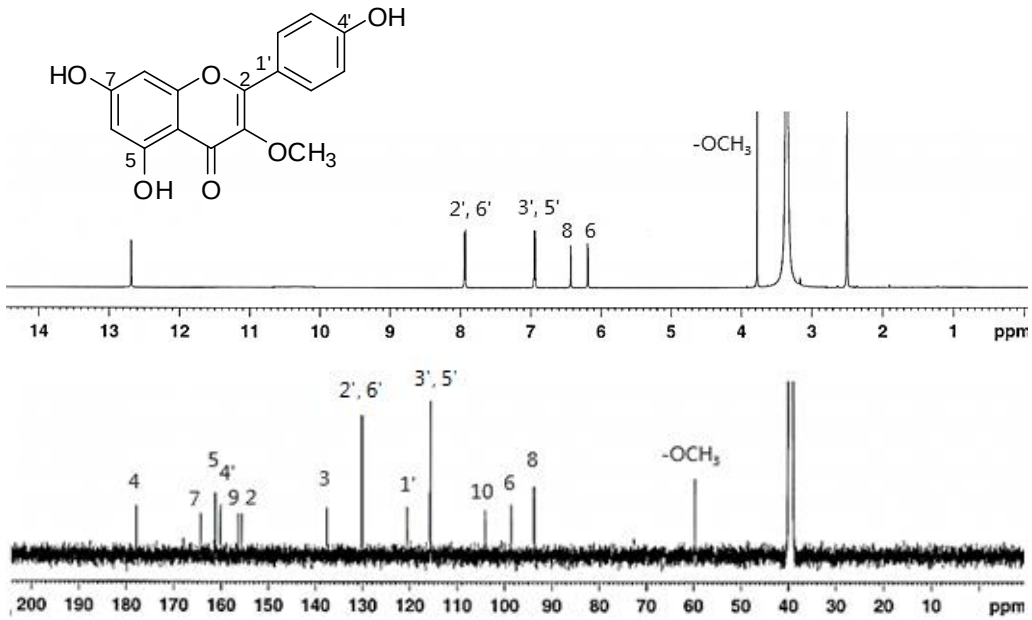


Figure 7. ^{1}H and ^{13}C NMR spectra of compound **4**

1.3. Compounds **5** and **6**

Compound **5** was isolated as yellowish amorphous powder and the molecular formula was determined as $C_{15}H_{10}O_7$ by ion peak at *m/z* 301 $[M-H]^-$ of the negative mode ESIMS and the ^{13}C NMR spectral data. The ^1H NMR spectrum revealed the aromatic protons of ABX system [δ_H 7.67 (1H, d, *J* = 2.1, H-2'), 7.53 (1H, dd, *J* = 8.4, 2.2, H-6'), 6.88 (1H, d, *J* = 8.5, H-5')] and two meta coupling protons [δ_H 6.40 (1H, d, *J* = 1.9, H-8), 6.18 (1H, d, *J* = 1.8, H-6)]. From these ^1H NMR spectrum data, compound **5** was assigned as quecetin of flavonol moiety and it was confirmed with the ^{13}C NMR spectral data (Bukhari et al., 2009).

Compound **6** was obtained as yellowish amorphous powder. The ESIMS spectrum showed an ion peak at *m/z* 315 $[M-H]^-$ indicating its molecular formula of $C_{16}H_{12}O_7$. The ^1H and ^{13}C NMR spectrum displayed similar patterns with those of compound **5** except for the existence of a methoxy group signal [δ_H 3.78 (3H, s, $-OCH_3$)/δ_c 59.7 ($-OCH_3$)]. In the HMBC spectrum, it was revealed that the proton of methoxy group was correlated with C-3 (δ_c 137.7). From comparison of these data with previously reported literature, compound **6** was defined as quercetin 3-methyl ether (Shi et al., 2012).

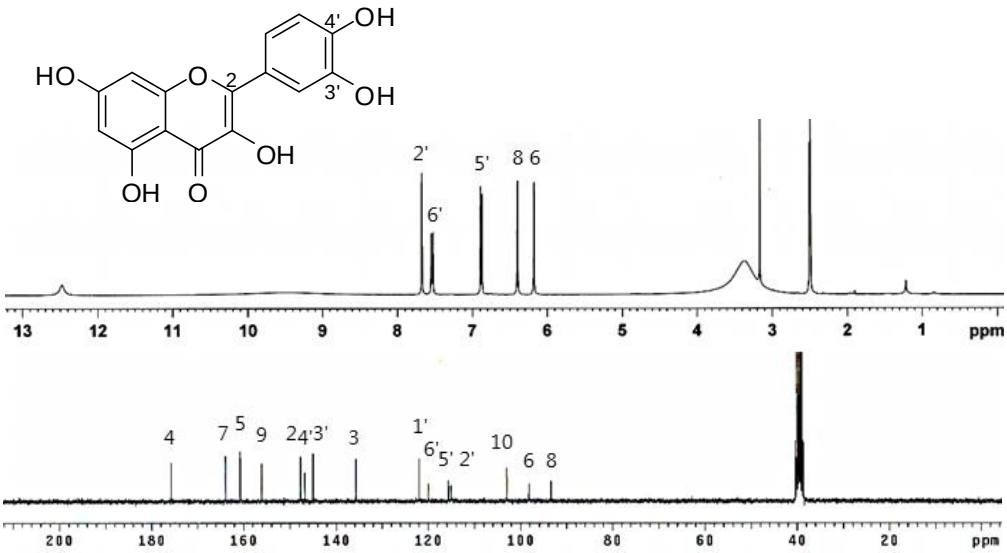


Figure 8. ^{1}H and ^{13}C NMR spectra of compound **5**

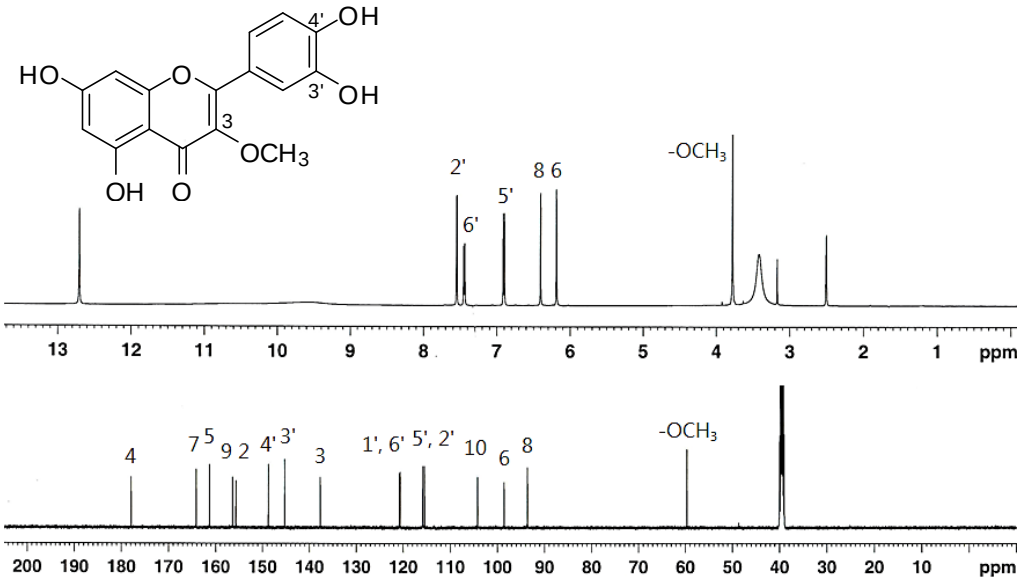


Figure 9. ^{1}H and ^{13}C NMR spectra of compound **6**

1.4. Compounds **7** and **8**

Compound **7** was obtained as yellowish amorphous powder and the molecular formula decided as $C_{16}H_{12}O_7$ by estimation of ion peaks on the negative mode ESIMS (*m/z* 315 $[M-H]^-$, 361 $[M+HCOOH-H]^-$) and analysis of the ^{13}C NMR spectrum. The ^1H NMR spectrum exhibited very similar patterns with those of compound **6** including the presence of a methoxy signal at δ_H 3.84 (3H, s, $-OCH_3$). In the ^{13}C NMR spectrum, however, the methoxy group signal appeared at δ_c 55.8, about 4 ppm downfield compared to those of compound **6**, indicating that the attaching location was different. From the HMBC spectrum analysis, it was revealed that the methoxy proton was correlated with C-3' (δ_c 147.3). Consequently, compound **7** was identified as isorhamnetin by comparison of above data with literature values (Shi et al., 2012).

Compound **8** was isolated as yellowish amorphous powder and its molecular formula was established as $C_{22}H_{22}O_{12}$ based on the negative mode ESIMS (*m/z* 315 $[M-Glc]^-$, 477 $[M-H]^-$) and the ^{13}C NMR analysis. The ^1H and ^{13}C NMR spectrum showed similar patterns with those of compound **7** except for the region of hexose moiety. The coupling constant of anomeric proton [δ_H 5.50 (1H, d, J = 7.7, H-1")] and the chemical shift of anomeric carbon [δ_c 101.6 (C-1")] suggested β-D-glucose. In the HMBC spectrum it was confirmed that the sugar moiety was attached at C-3 of aglycone by the correlation of anomeric proton with C-3 (δ_c 133.1). On the basis of these spectral data, the structure of compound **8** was concluded as isorhamnetin 3-*O*-β-D-glucopyranoside (Olszewska and Roj, 2011).

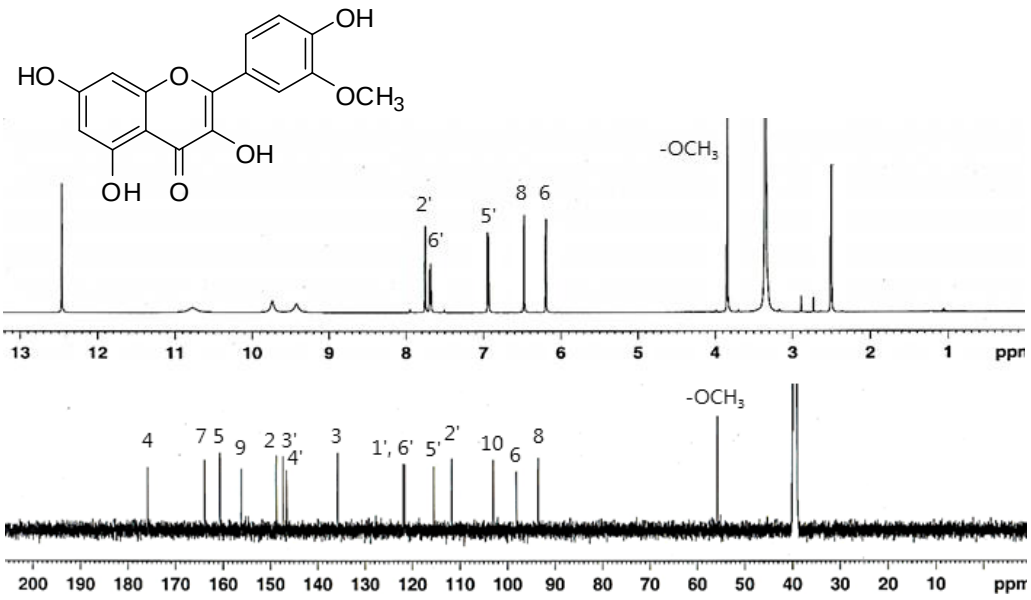


Figure 10. ^{1}H and ^{13}C NMR spectra of compound **7**

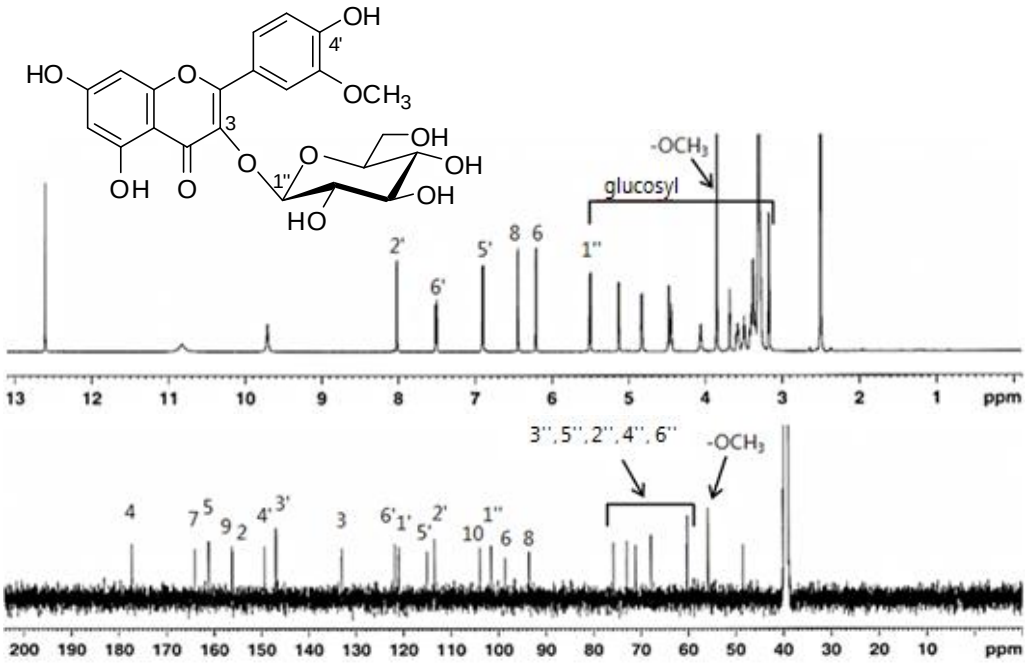


Figure 11. ^{1}H and ^{13}C NMR spectra of compound **8**

1.5. Compound **9**

Compound **9** was obtained as pale yellowish amorphous powder, and displayed ion peaks at *m/z* 315 $[M\text{-}Rha\text{-}Glc]^-$, 623 $[M\text{-}H]^-$ and 668 $[M+HCOOH\text{-}H]^-$ on the negative mode ESIMS suggesting that the molecular formula was $C_{28}H_{32}O_{16}$. The ^1H and ^{13}C NMR spectrum exhibited similar patterns with those of compound **7** except for the region of sugar moiety. The coupling constant of two anomeric protons [δ_H 5.24 (1H, d, J = 7.4, H-1") and δ_H 4.52 (1H, d, J = 1.1, H-1''')] and chemical shift of anomeric carbons [δ_c 105.2 (C-1") and δ_c 103.3 (C-1''')] suggested that D-glucose was attached as β form and L-rhamnose as α form. From the HMBC analysis, the anomeric proton of L-rhamnose (H-1''') was correlated with C-6" of D-glucose indicating that the sugar moiety was a rutinose ($Rha^1 \rightarrow {}^6Glu$). Also, the position of rutinose was established to be at C-3 (δ_c 136.3) of aglycone by the correlation. Thus, the structure of compound **9** was defined as narcissin by comparison with previously reported literature (Lee et al., 2003a).

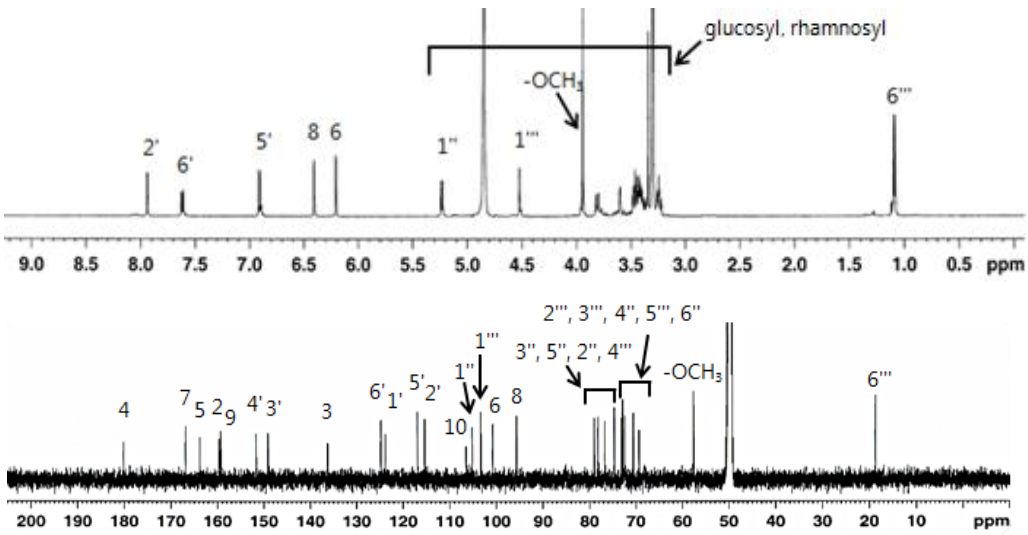


Figure 12. ^1H and ^{13}C NMR spectra of compound **9**

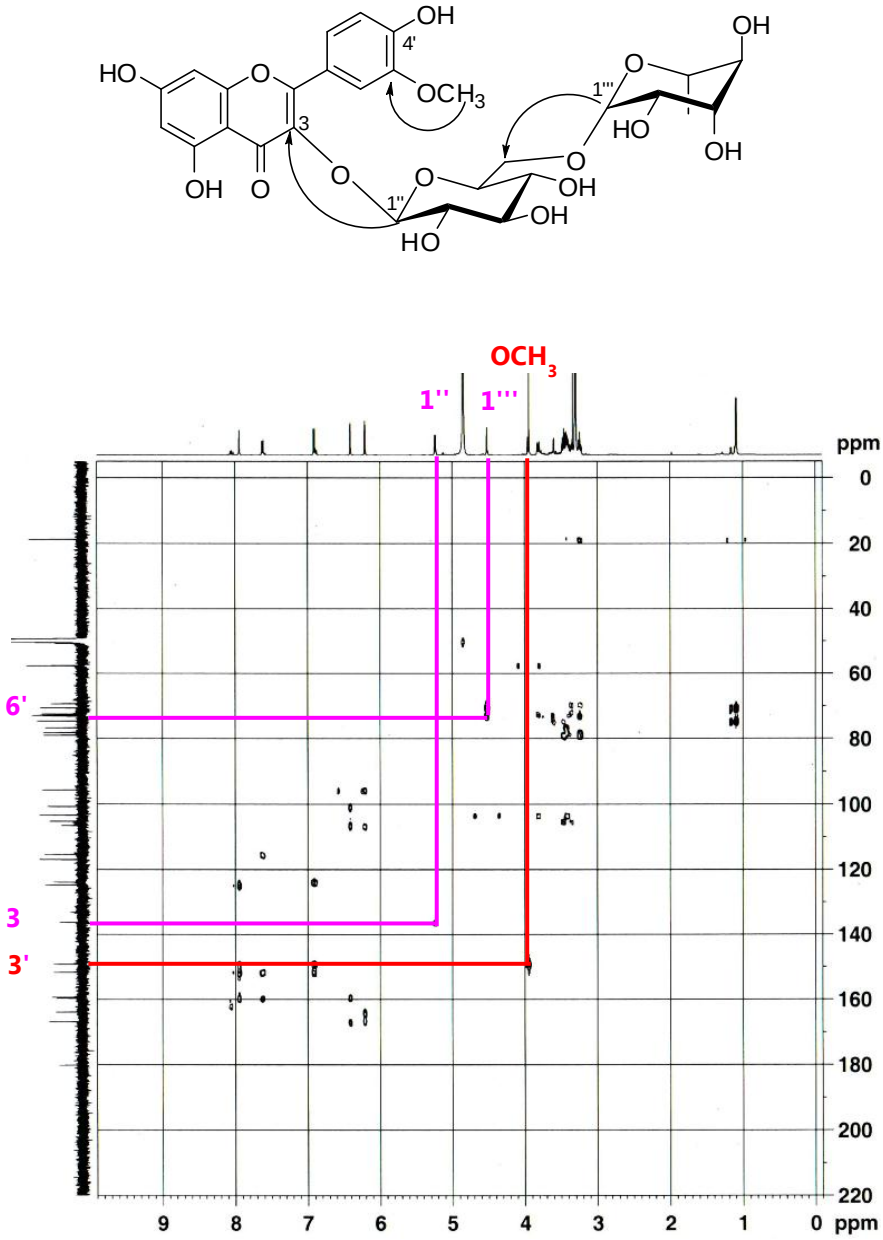


Figure 13 . HMBC spectrum of compound **9**

1.6. Compound **10**

Compound **10** was obtained as white amorphous powder, $[\alpha]^{25}_{D}$ +46.9 (*c*=0.30, MeOH), and demonstrated $C_{18}H_{16}O_6$ as the molecular formula by ion peaks on the negative mode ESIMS (*m/z* 327 $[M-H]^-$, 373 $[M+HCOOH-H]^-$). The IR spectrum showed the presence of hydroxyl (3296 cm^{-1}), aldehyde (2749 cm^{-1}), conjugated carbonyl (1664 cm^{-1}), *trans* double bond (815 cm^{-1}) and aromatic ring (1604, 1441 cm^{-1}) functions. The ^1H NMR spectrum exhibited the signals for an aldehyde proton [δ_H 9.60 (1H, d, *J* = 7.8, H-9')], aromatic protons of two ABX system [δ_H 7.36 (1H, d, *J* = 1.8, H-2'), 7.28 (1H, dd, *J* = 8.7, 2.3, H-6'), 7.02 (1H, d, *J* = 8.2, H-5'); δ_H 6.82 (1H, d, *J* = 1.8, H-2), 6.77 (1H, d, *J* = 7.8, H-5), 6.72 (1H, dd, *J* = 8.2, 1.9, H-6)], double bond protons with *trans* configuration [δ_H 7.62 (1H, d, *J* = 16.0, H-7'), 6.73 (1H, dd, *J* = 15.1, 1.9, H-8')], and two oxymethine protons of 1,4-dioxane ring moiety [δ_H 4.87 (1H, d, *J* = 7.8, H-7), 4.15 (1H, m, H-8)]. The ^1H-^1H COSY spectrum revealed the connectivity of protonated carbons (C-7/C-8/C-9). In the HMBC analysis, the key correlation peaks from δ_H 9.60 (1H, d, *J* = 7.8, H-9') to δ_c 126.8 (C-8'), from δ_H 7.62 (1H, d, *J* = 16.0, H-7') to δ_c 194.1 (C-9'), 123.0 (C-6') and 116.8 (C-2'), from δ_H 4.87 (1H, d, *J* = 7.8, H-7) to δ_c 143.9 (C-3'), 127.2 (C-1), 118.9 (C-6), 115.0 (C-2) and 78.6 (C-8) displayed the positions of an aldehyde carbon (C-9'), two olefinic carbons (C-7' and C-8'), an oxymethylene carbon (C-8), and aromatic ring carbons attaching to 1,4-dioxane ring (C-1, C-2 and C-6). The relative configuration of H-7/H-8 was suggested as *trans* from the large coupling constant (*J* = 7.8) (Lin et al., 2007). The absolute configurations of C-7 and C-8 were elucidated from the characteristic cotton effect in CD spectrum (Arnoldi and Merlini, 1985). From the positive cotton effect at 230 and 244 nm, compound **10** was determined as a kind of neoligan, 7*S*,8*S*-isoamericanin A, with comparison of literature values (Kim et al., 2003; Waibel et al., 2003).

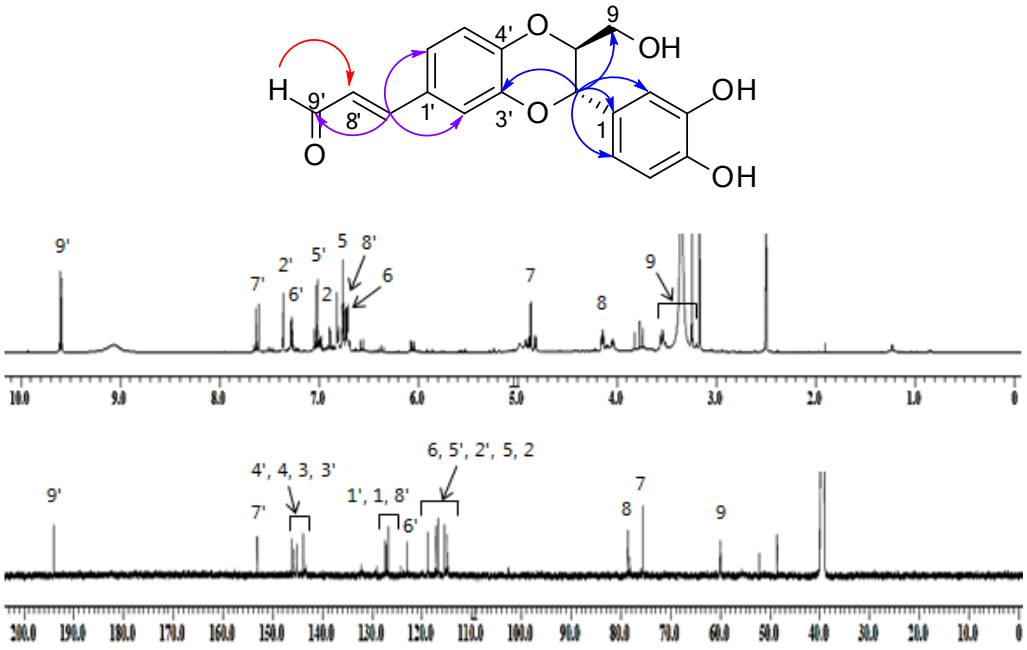


Figure 14. ^{1}H and ^{13}C NMR spectra of compound **10**

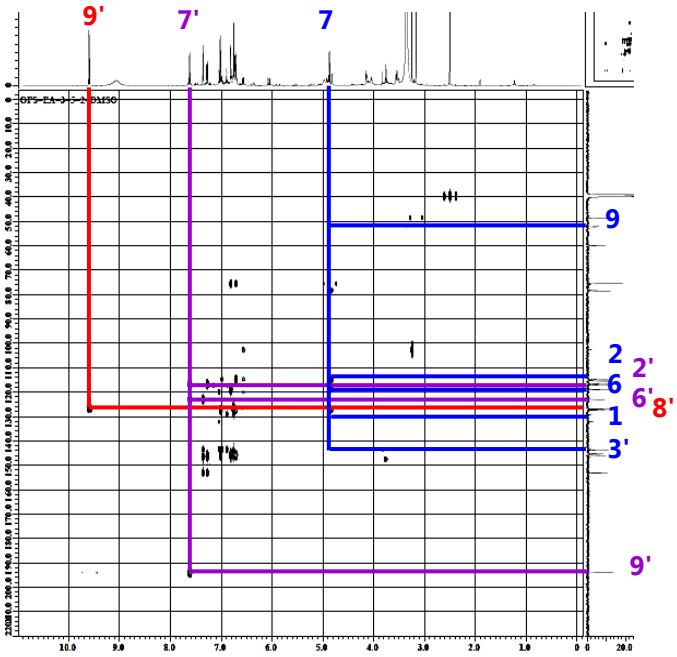


Figure 15. HMBC spectrum of compound **10**

1.7. Compound **11**

Compound **11** was obtained as white amorphous powder and the molecular formula was determined as $C_{18}H_{16}O_7$ from the ion peaks on the negative mode ESIMS (*m/z* 389 $[M+HCOOH-H]^-$, 687 $[2M-H]^-$). The IR spectrum showed the presence of hydroxyl (3343 cm^{-1}), conjugated carbonyl (1684 cm^{-1}), *trans* double bond (815 cm^{-1}), and aromatic ring (1607, 1441 cm^{-1}) functions. The ^1H NMR spectrum revealed the signals for aromatic protons of two ABX system [δ_H 7.16 (1H, d, *J* = 2.3, H-2'), 7.12 (1H, dd, *J* = 8.2, 1.8, H-6'), 6.97 (1H, d, *J* = 8.7, H-5'); δ_H 6.85 (1H, d, *J* = 1.9, H-2), 6.80 (1H, d, *J* = 7.8, H-5), 6.77 (1H, dd, *J* = 7.3, 1.9, H-6)], double bond protons with *trans* configuration [δ_H 7.57 (1H, d, *J* = 16.0, H-7'), 6.31 (1H, d, *J* = 16.0, H-8')], and two oxymethine protons of 1,4-dioxane ring moiety [δ_H 4.83 (1H, d, *J* = 8.3, H-7), 4.05 (1H, m, H-8)]. The HMBC analysis showed the correlation peaks from δ_H 7.57 (1H, d, *J* = 16.0, H-7') to δ_c 171.6 (C-9'), 124.1 (C-6'), 118.3 (C-2') and 130.2 (C-1'), from δ_H 4.83 (1H, d, *J* = 8.3, H-7) to δ_c 146.5 (C-3'), 130.1 (C-1), 121.3 (C-6), 116.5 (C-2), 81.3 (C-8), and 62.9 (C-9) indicating two C_6-C_3 units of neolignan moiety. The relative configuration of H-7/H-8 was suggested as *trans* from the coupling constant of 8.3 Hz. The absolute configuration of C-7/C-8 was determined by CD spectra. From the negative cotton effect at 225 and 243 nm, compound **11** was concluded as 7*R*,8*R*-isoamericanoic acid A by comparison with previously reported literature values (Kim et al., 2003; Lin et al., 2007).

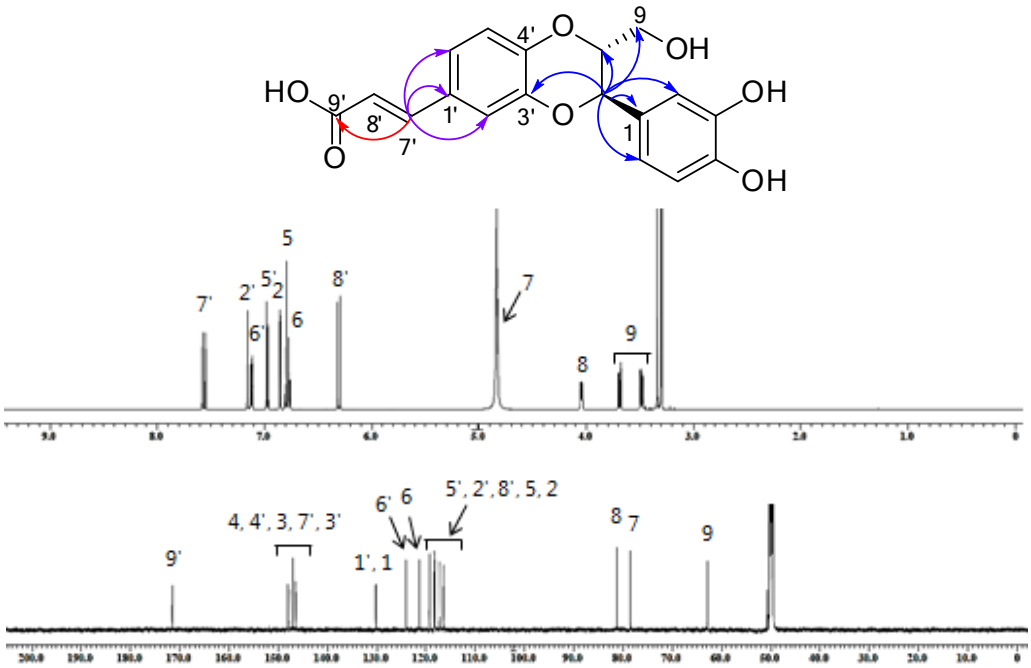


Figure 16. ^{1}H and ^{13}C NMR spectra of compound **11**

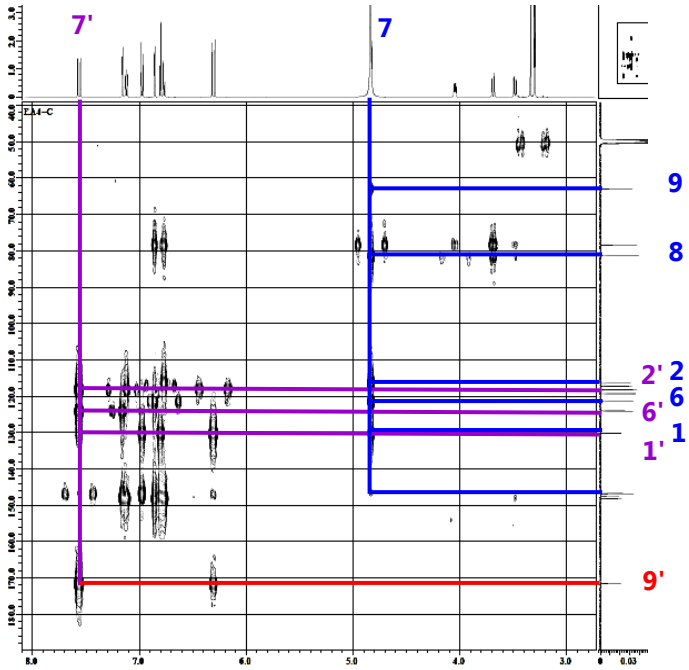


Figure 17. HMBC spectrum of compound **11**

1.8. Compound **12**

Compound **12** was obtained as white amorphous powder and the molecular formula was decided as $C_{19}H_{20}O_6$ from ion peaks on the negative mode ESIMS (*m/z* 343 $[M-H]^-$, 687 $[2M-H]^-$) and the ^{13}C NMR spectrum analysis. The ^1H NMR spectrum showed signals for aromatic protons of two ABX system [δ_H 7.00 (1H, d, *J* = 1.8, H-2'), 6.95 (1H, dd, *J* = 8.2, 2.2, H-6'), 6.88 (1H, d, *J* = 8.2, H-5'); δ_H 6.80 (1H, d, *J* = 1.9, H-2), 6.75 (1H, d, *J* = 8.3, H-5), 6.67 (1H, dd, *J* = 8.2, 1.9, H-6)], double bond protons with *trans* configuration [δ_H 6.48 (1H, d, *J* = 16.1, H-7'), 6.18 (1H, dt, *J* = 16.0, 5.9, H-8')], two oxymethine protons of 1,4-dioxane ring moiety [δ_H 4.82 (1H, d, *J* = 7.7, H-7), 4.04 (1H, m, H-8)], two oxymethylene protons [δ_H 3.99 (2H, dd, *J* = 6.0, 1.4, H-9'), 3.52 (1H, dd, *J* = 12.2, 2.3, H-9a), 3.32 (1H, m, H-9b)], and one methoxy group proton [δ_H 3.25 (3H, s, $-OCH_3$)]. The HMBC analysis displayed the correlation peaks from δ_H 6.48 (1H, d, *J* = 16.1, H-7') to δ_c 128.8 (C-1'), 118.6 (C-6'), 113.4 (C-2') and 71.2 (C-9'), from δ_H 4.82 (1H, d, *J* = 7.7, H-7) to δ_c 142.6 (C-3'), 126.5 (C-1), 117.8 (C-6), 113.9 (C-2), 77.3 (C-8) and 59.1 (C-9) indicating two C_6-C_3 units of neolignan moiety. The relative configuration of H-7/H-8 was deduced as *trans* from the coupling constant of 7.7 Hz. The absolute configuration of C-7/C-8 was assigned as 7*R*/8*R* from the negative cotton effect at 224 and 244 nm. Through comparison of above evidence with literature values, compound **12** was defined as 7*R*,8*R*-9'-*O*-methylisoamericanol A (Jin et al., 2009; Kim et al., 2003).

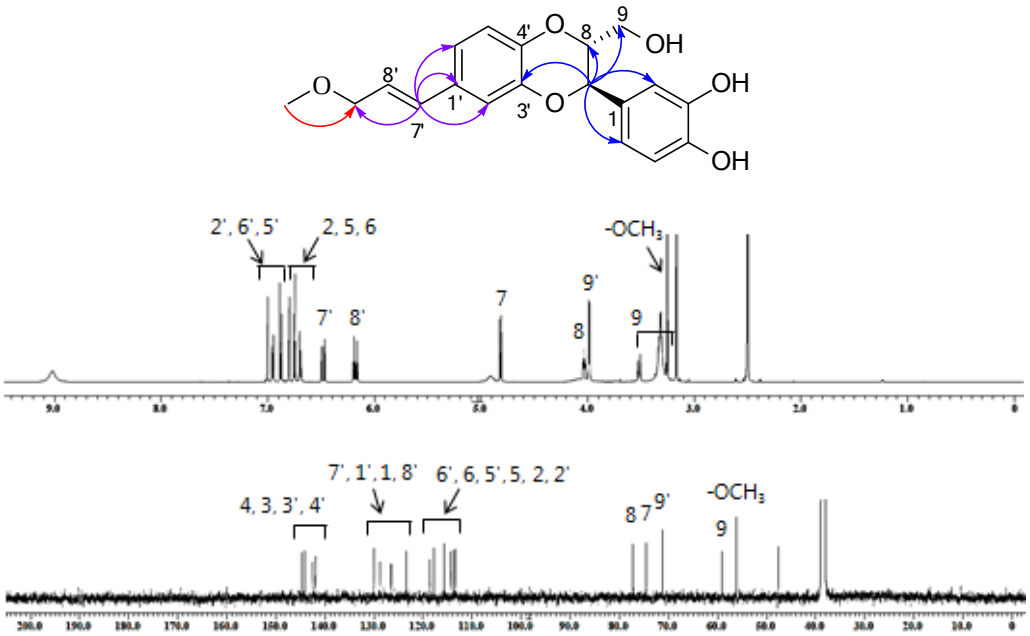


Figure 18 . ^{1}H and ^{13}C NMR spectra of compound **12**

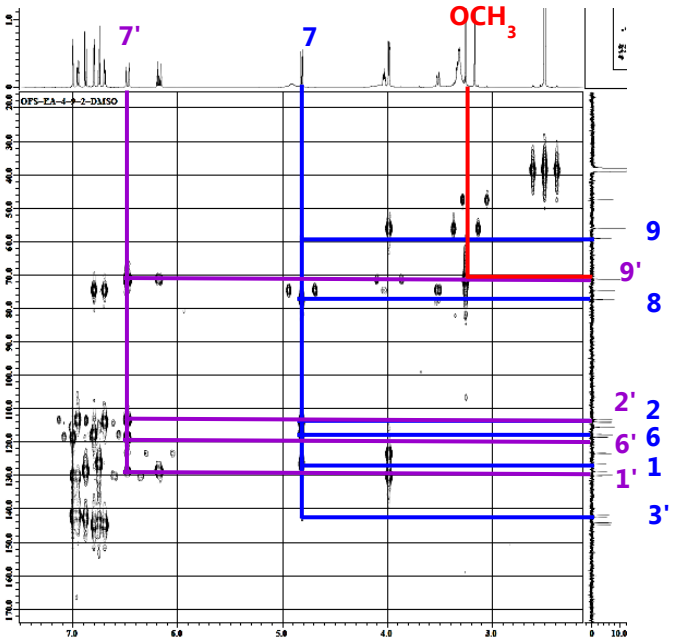


Figure 19. HMBC spectrum of compound **12**

1.9. Compound **13**

Compound **13** was isolated as colorless amorphous powder and revealed nine carbon signals. Considering its ion peaks at *m/z* 329 $[M-H]^-$ and 375 $[M+HCOOH-H]^-$ on the negative mode ESIMS, the molecular formula could be assigned as $C_{18}H_{18}O_6$ and the structure of compound **13** was deduced symmetrical. In the ^1H NMR spectrum, the characteristic signals of aromatic protons of ABX system [δ_H 6.75 (2H, d, J = 1.9, H-2, 2'), 6.70 (2H, d, J = 8.1, H-5, 5'), 6.60 (2H, dd, J = 8.2, 1.9, H-6, 6')], two oxymethine protons [δ_H 4.54 (2H, d, J = 4.0, H-7, 7')], two oxymethylene protons [δ_H 4.08 (2H, m, H-9a, 9'a), 3.69 (2H, m, H-9b, 9'b)] and two methine protons [δ_H 2.94 (2H, m, H-8, 8')] were observed that suggesting the presence of two C_6-C_3 units coupled by forming furofuran ring skeleton. The ^1H-^1H COSY spectrum confirmed the connectivity of protonated carbons (C-7, 7'/C-8, 8'/C-9, 9'). It has been known that the methine protons (H-8, 8') are obtained as *cis* configuration in naturally producing furofuran ring skeleton, and the oxymethine protons (H-7, 7') have different chemical shifts according to the relative configuration of aryl groups: between δ_H 3.75 and 4.70 in diequatorial, and between δ_H 3.25 and 4.0 in diaxial (Casabuono and Pomillo, 1994). The signals for H-7 and H-7' of compound **13** were identical and exhibited at δ_H 4.54 indicating the both aryl groups are equatorial. From the HMBC analysis, the correlations from δ_H 4.54 (H-7, 7') to δ_c 132.5 (C-1, 1'), 117.2 (C-6, 6'), 113.8 (C-2, 2') were confirmed. Through comparison of above data with literature values, thus, compound **13** was identified as 3,3'-bisdemethylpinoresinol (Waibel et al., 2003).

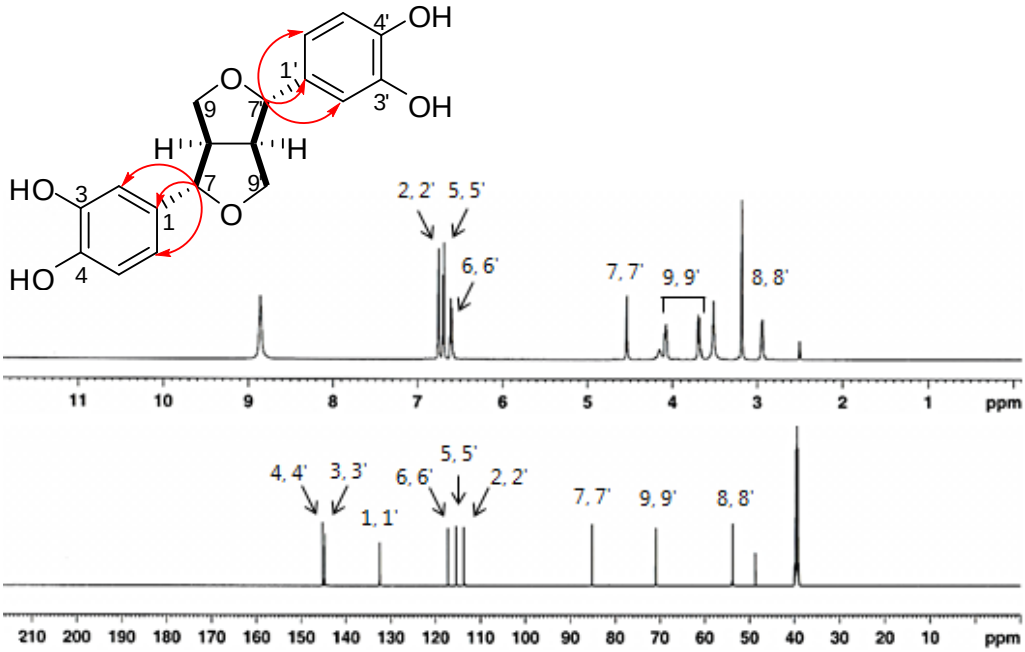


Figure 20. ^{1}H and ^{13}C NMR spectra of compound **13**

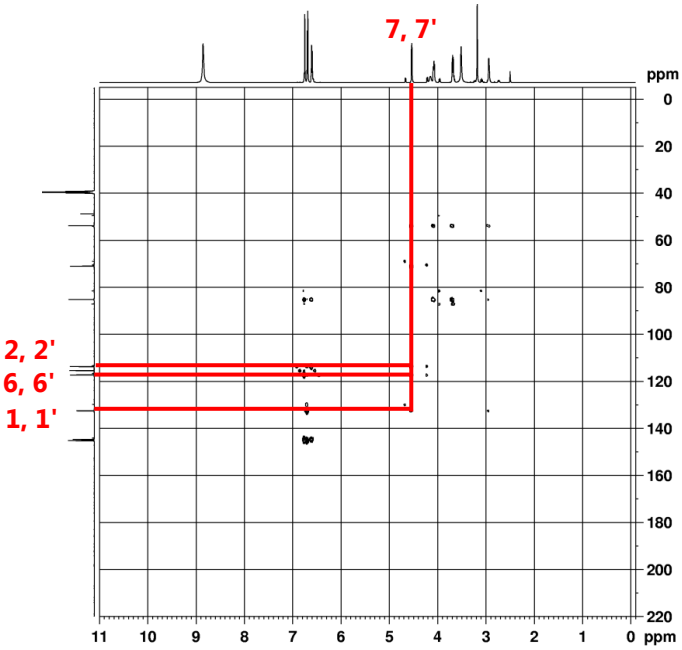


Figure 21. HMBC spectra of compound **13**

1.10. Compound **14**

Compound **14** was obtained as brownish amorphous syrup and $[\alpha]^{25}_{D}$ +25.7 (*c*=0.30, MeOH). The negative mode ESIMS exihibited *m/z* 493 $[M-H]^-$ and 987 $[2M-H]^-$ indicating $C_{27}H_{26}O_9$ as its molecular formula. The ^{1}H NMR spectrum exhibited the signals for aromatic protons of three ABX system [δ_H 6.89 (1H, d, *J* = 8.3, H-5'), 6.88 (1H, d, *J* = 1.9, H-2'), 6.85 (1H, dd, *J* = 8.3, 1.8 H-6'); δ_H 6.80 (1H, d, *J* = 1.9, H-2), 6.75 (1H, d, *J* = 8.3, H-5), 6.70 (1H, dd, *J* = 8.3, 1.9, H-6); δ_H 6.73 (1H, d, *J* = 1.9, H-2"), 6.67 (1H, d, *J* = 7.8, H-5"), 6.59 (1H, dd, *J* = 8.3, 1.9, H-6")], two oxymethine protons of 1,4-dioxane ring moiety [δ_H 4.81 (1H, d, *J* = 7.7, H-7), 4.01 (1H, m, H-8)], and characteristic signals for furofuran ring including two oxymethine protons [δ_H 4.63 (1H, d, *J* = 4.6, H-7'), 4.53 (1H, d, *J* = 5.0, H-7")], four oxymethylene protons [δ_H 4.09 (2H, m, H-9'a, 9"a), 3.72 (2H, m, H-9'b, 9"b)] and two methine protons [δ_H 2.98 (1H, m, H-8'), 2.95 (1H, m, H-8")]. In the HMBC analysis, the key correlation peaks from δ_H 4.81 (1H, d, *J* = 7.7, H-7) to δ_c 142.5 (C-4'), 127.6 (C-1), 118.8 (C-6), 114.9 (C-2), 78.2 (C-8) and 60.2 (C-90), from δ_H 4.63 (1H, d, *J* = 4.6, H-7') to δ_c 134.5 (C-1'), 118.9 (C-6') and 114.5 (C-2'), from δ_H 4.53 (1H, d, *J* = 5.0, H-7") to δ_c 132.3 (C-1"), 117.0 (C-6") and 113.6 (C-2") displayed an isoamericanol-type sesquineolignan structure which was generated by condensation of compound **13** with a phenylpropane unit. The relative configuration of H-7/H-8 was suggested as *trans* from the large coupling constant (*J* = 7.7). The absolute configuration of C-7/C-8 was decided as 7*S*/8*S* from the positive cotton effect at 240 and 256 nm. Therefore, compound **14** was assigned as 7*S*/8*S*-isoprincepin by comparison of above data with literature values (Kamiya et al., 2004).

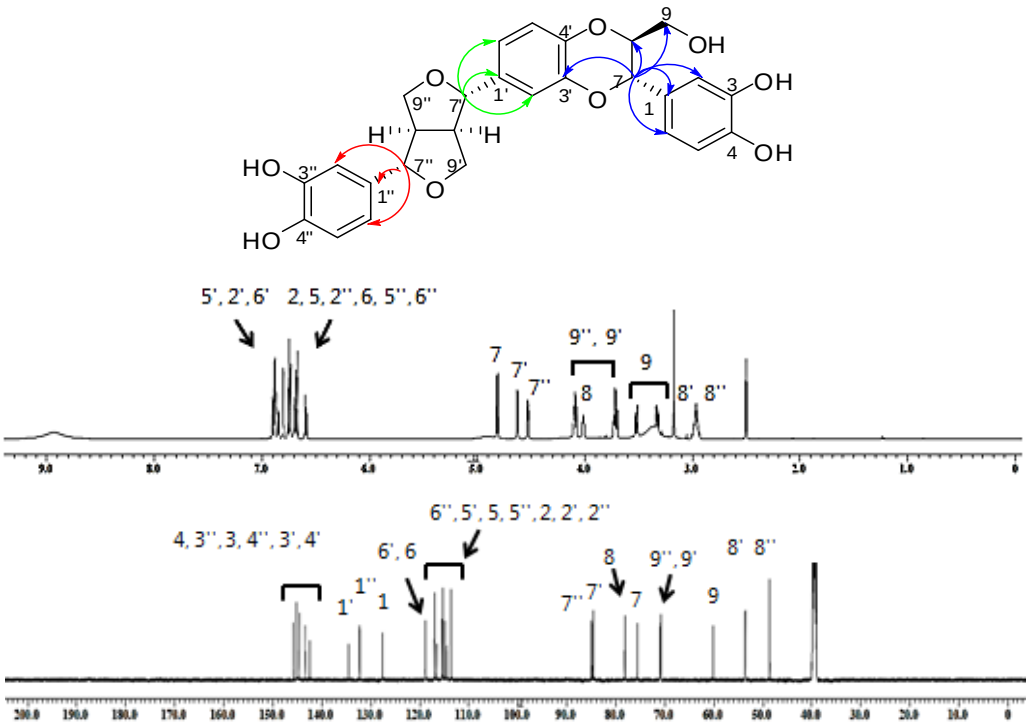


Figure 22. ^{1}H and ^{13}C NMR spectra of compound **14**

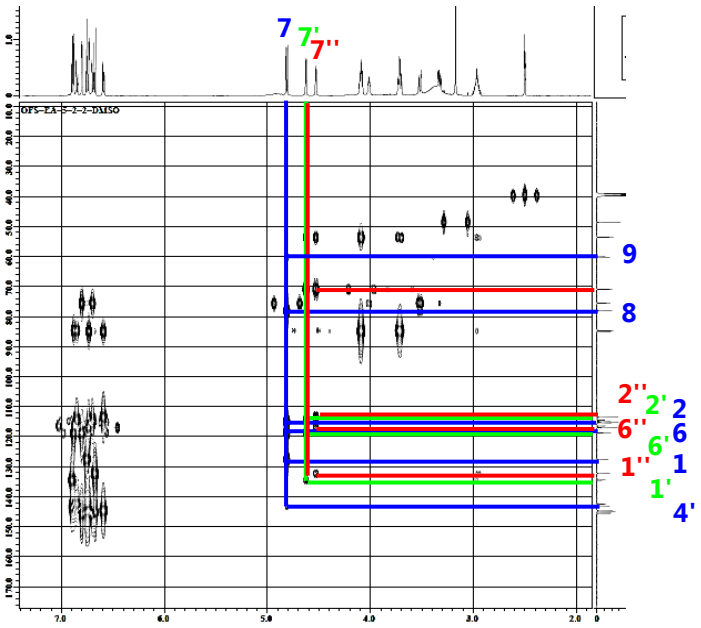


Figure 23. HMBC spectrum of compound **14**

1.11. Compound **15**

Compound **15** was obtained as brownish amorphous syrup, $[\alpha]^{25}_{D}$ -5.3 (*c*=0.30, MeOH) and the molecular formula was determined as $C_{27}H_{26}O_9$ from the ion peaks at *m/z* 493 $[M-H]^-$ and 987 $[2M-H]^-$ on the negative mode ESIMS. The ^1H NMR spectrum showed very similar patterns to those of compound **14** except for the region of H-2' [δ_H 6.91 (1H, d, *J* = 1.8, H-2')] and H-5' [δ_H 6.86 (1H, d, *J* = 8.2, H-5')]. In compound **14,** H-5' (δ_H 6.89) was appeared at more downfield than H-2' (δ_H 6.88), but in **15** it showed an opposite result. These differences were indicative of the distinction of americanol- and isoamericanol-type neolignans (Waibel et al., 2003). From the further analysis of 2D NMR spectral data, compound **15** was revealed as an americanol-type sesquineolignan structure formed by condensation of compound **13** with a phenylpropane unit. The coupling constant of H-7/H-8 was large (*J* = 7.8) deducing the relative configuration as *trans* form (*J* = 7.8). The absolute configuration of C-7/C-8 was concludd as 7*S*/8*S* from the positive cotton effect at 240 and 257 nm. These spectral data indicated that compound **15** was 7*S*,8*S*-princepin by comparison with previously reported literature (Waibel et al., 2003).

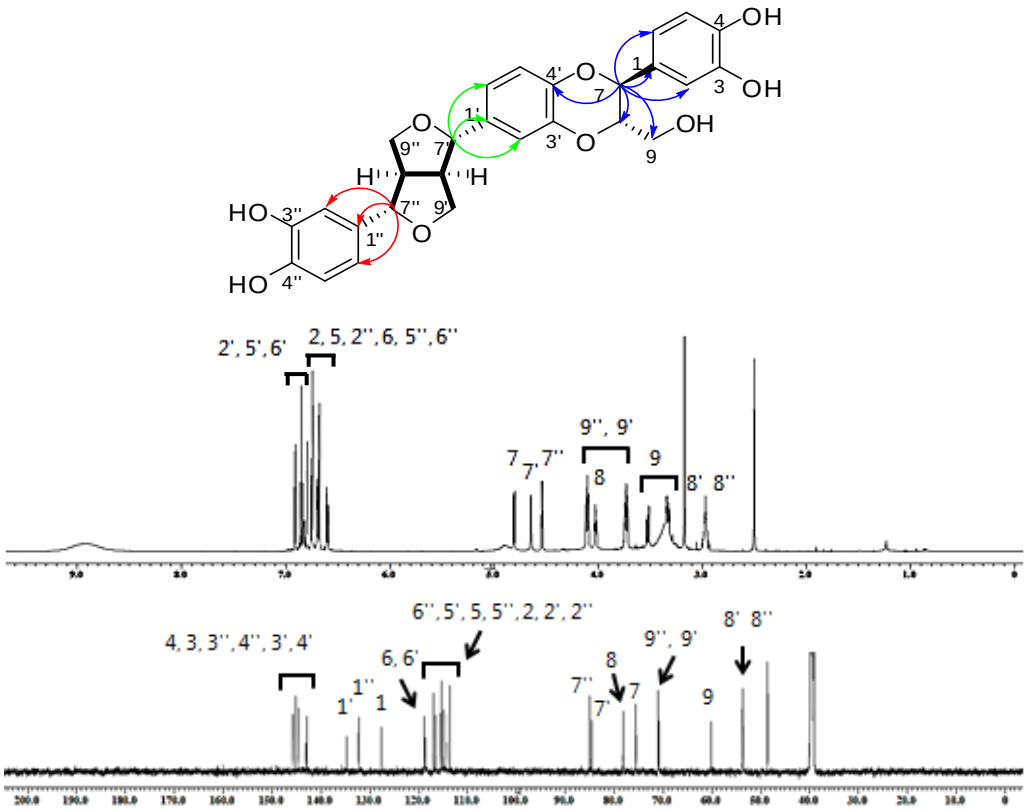


Figure 24. ^{1}H and ^{13}C NMR spectra of compound **15**

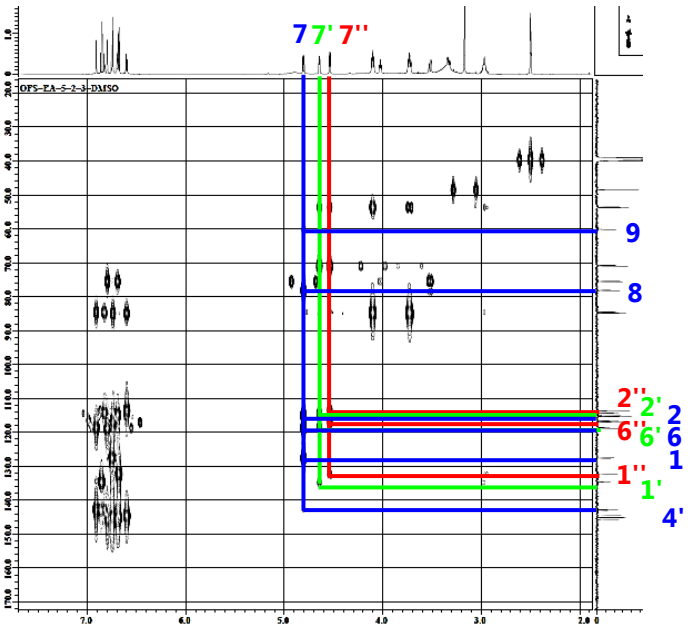


Figure 25. HMBC spectrum of compound **15**

1.12. Compound **16**

Compound **16** was isolated as pale yellowish amorphous syrup and the molecular formula was decided as $C_{16}H_{20}O_9$ by ion peak at *m/z* 355 $[M-H]^-$ and 401 $[M+HCOOH-H]^-$ on the negative ion mode ESIMS. The IR spectrum showed the presence of hydroxyl (3370 cm^{-1}), conjugated carbonyl (1714 cm^{-1}), *trans* double bond (825 cm^{-1}), and aromatic ring (1597 cm^{-1}) functions. The ^1H NMR spectrum revealed the signals for aromatic protons of ABX system [δ_H 7.34 (1H, d, J = 1.6, H-2'), 7.15 (1H, dd, J = 8.2, 1.5, H-6'), 6.80 (1H, d, J = 8.2, H-5')], double bond protons with *trans* configuration [δ_H 7.63 (1H, d, J = 15.9, H-3), 6.48 (1H, d, J = 16.0, H-2)], an aromatic proton of sugar moiety [δ_H 5.46 (1H, d, J = 8.1, H-1'')], and a methoxy proton [δ_H 5.46 (3H, s, $-OCH_3$)]. From the analysis of HMBC spectrum, the correlation peaks from δ_H 7.63 (1H, d, J = 15.9, H-3) to δ_c 165.4 (C-1), 123.3 (C-6') and 111.4 (C-2'), from δ_H 6.48 (1H, d, J = 16.0, H-2) to δ_c 165.4 (C-1) and 125.5 (C-1'), from δ_H 5.46 (1H, d, J = 8.1, H-1'') to δ_c 165.4 (C-1) and from δ_H 3.82 (1H, s, $-OCH_3$) to δ_c 147.9 (C-3') indicated the presence of feruloyl moiety and location of sugar moiety. The sugar moiety was suggested as β-D-glucose from the coupling constant of aromatic proton (J = 8.1) and chemical shift of aromatic carbon (δ_c 94.2). In comparison above data with literature values, compound **16** was identified as 1-*O*-feruloyl-β-D-glucopyranoside (Hashimoto et al., 1992).

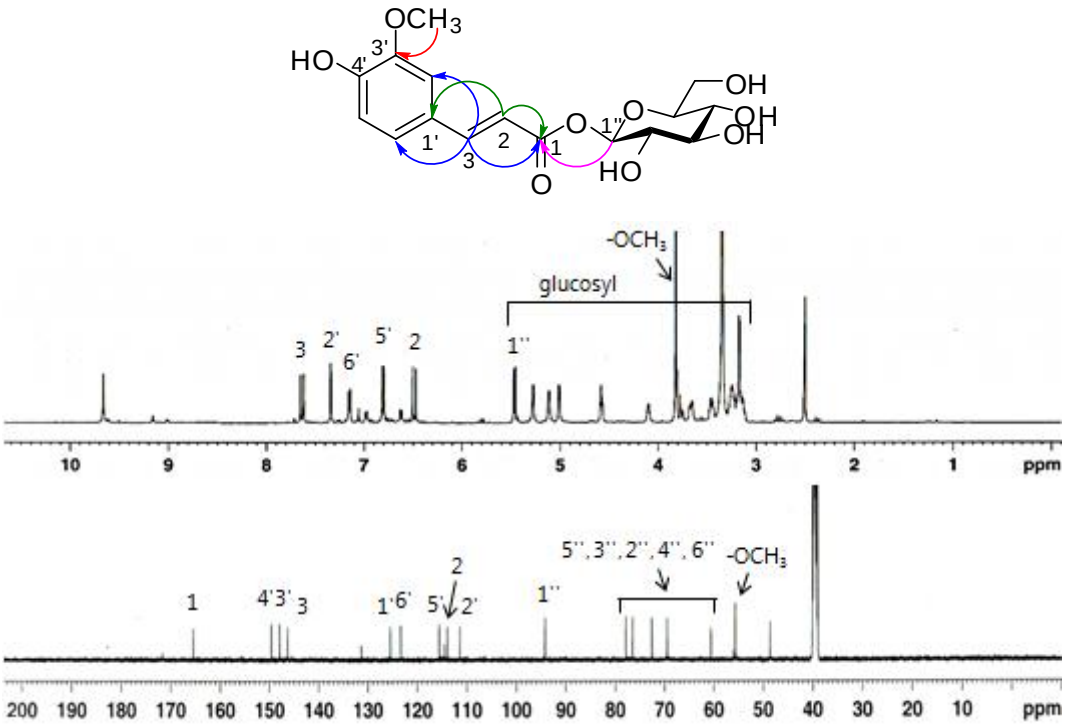


Figure 26. ^{1}H and ^{13}C NMR spectra of compound **16**

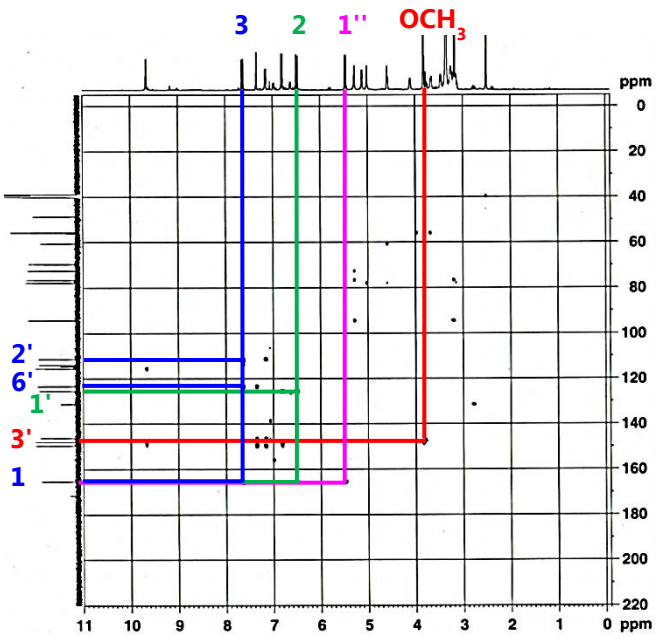
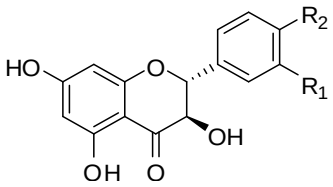
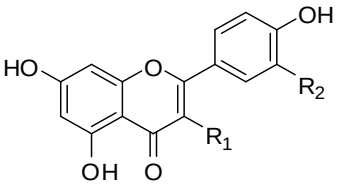


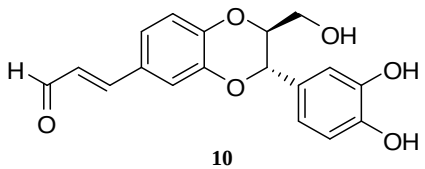
Figure 27. HMBC specturm of compound **16**



	R_1	R_2
1	H	OH
2	OH	OH



	R_1	R_2
3	OH	H
4	OCH_3	H
5	OH	OH
6	OCH_3	OH
7	OH	OCH_3
8	*O*-*β*-D-Glc	OCH_3
9	*O*-*β*-D-Glc(6→1)Rha	OCH_3



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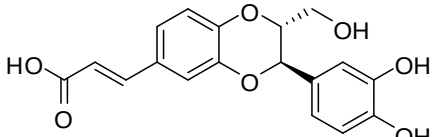

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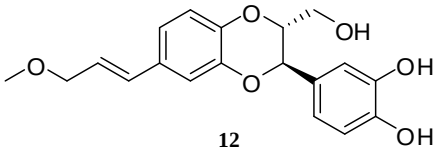

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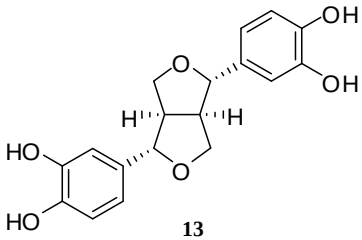

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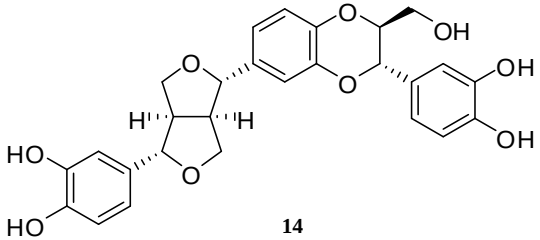

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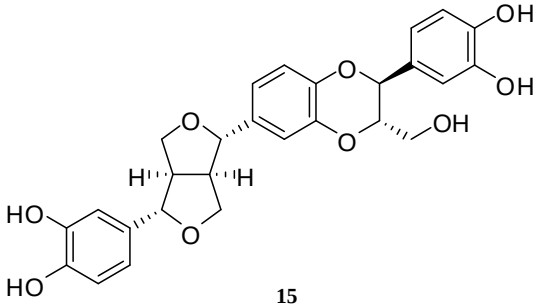

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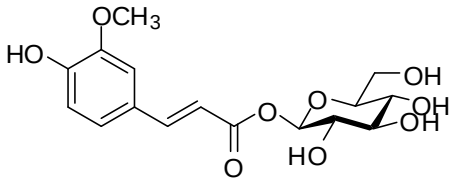

16

Figure 28. The structures of compounds isolated from *O. ficus-indica* seeds

2. Chemical fingerprint of *O. ficus-indica* seeds using HPLC-DAD-ESIMS

The EtOAc fraction of *O. ficus-indica* seeds was analyzed by HPLC-DAD-ESIMS and the HPLC-PDA chromatogram was shown in figure 28. Comparing retention times and spectral data of peaks of the chromatogram with those of each isolated compounds, the corresponding peaks were numbered (Table 9). All peaks of sixteen isolated compounds were identified on HPLC-PDA chromatogram of EtOAc fraction of *O. ficus-indica* seeds.

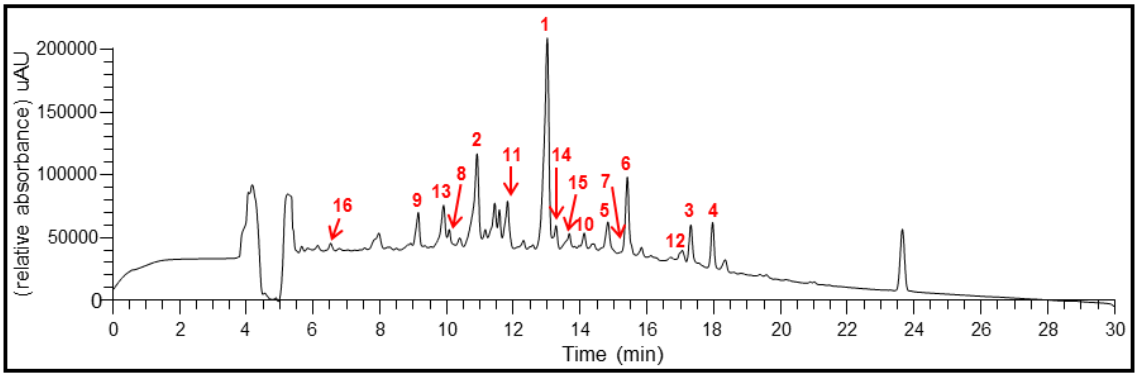

Figure 29. The HPLC-PDA chromatogram of EtOAc fraction of *O. ficus-indica* seeds

The compounds were separated on an Ascentis Express C18 column (4.6 mm i.d. x 150 mm, 2.7 μm) using a gradient solvent system with from 20 % B to 80 % B in 0-25 min and 20 % B in 25.1-30 min. Solvent A: 0.1 % formic acid, solvent B: acetonitrile. Flow rate: 0.3 mL/min.

Table 9. Spectral and chromatographic data of compounds **1-16** detected in EtOAc fraction of *O. ficus-indica* seeds

No.	Compound name	tR (min)	UV λ_{max} (nm)	ESIMS ion peaks (m/z)
1	(+)-aromadendrin	13.01	290	287 $[M-H]^-$
2	(+)-taxifolin	10.90	289	303 $[M-H]^-$
3	kaempferol	17.30	266, 366	285 $[M-H]^-$
4	kaempferol 3-methyl ether	17.96	267, 348	299 $[M-H]^-$
5	quercetin	14.82	258, 371	301 $[M-H]^-$
6	quercetin 3-methyl ether	15.40	255, 356	315 $[M-H]^-$
7	isorhamnetin	15.26	254, 369	315 $[M-H]^-$ 361 $[M+HCOOH-H]^-$
8	isorhamnetin 3-*O*-*β*-D-glucopyranoside	10.03	254, 354	315 $[M-Glc]^-$ 477 $[M-H]^-$
9	narcissin	9.14	254, 353	315 $[M-Rha-Glc]^-$ 623 $[M-H]^-$ 668 $[M+HCOOH-H]^-$
10	(7*S*,8*S*)-isoamericanin A	14.11	286, 334	327 $[M-H]^-$ 373 $[M+HCOOH-H]^-$
11	(7*R*,8*R*)-isoamericanoic acid A	11.82	241, 269	389 $[M+HCOOH-H]^-$ 687 $[2M-H]^-$
12	(7*R*,8*R*)-9'-*O*-methylisoamericanol A	17.00	218, 267	343 $[M-H]^-$ 687 $[2M-H]^-$
13	3,3'-bisdemethylpinoresinol	9.90	281, 341	329 $[M-H]^-$ 375 $[M+HCOOH-H]^-$
14	(7*S*,8*S*)-isoprincepin	13.26	234, 280	493 $[M-H]^-$ 987 $[2M-H]^-$
15	(7*S*,8*S*)-princepin	13.65	238, 275	493 $[M-H]^-$ 987 $[2M-H]^-$
16	1-*O*-feruloyl-*β*-D-glucopyranoside	6.50	220, 330	355 $[M-H]^-$

3. Protective activities of total extract, fractions and the compounds from *O. ficus-indica* seeds against ethanol-intoxicated rat primary hepatocytes

3.1. Ethanol exposure to rat primary hepatocytes

Protection of hepatocytes from ethanol-induced cellular damage has been considered as an effective treatment for delaying progression of alcoholic liver disease. Isolated rat primary hepatocytes are physiologically similar to human hepatocytes and useful for studying metabolism because of their resemblance to the *in vivo* situation. Thus, rat primary hepatocytes were used as a screening tool in the process of searching the therapeutic agents of alcoholic liver disease *in vitro*. To establish the experimental methods for screening protective activity against ethanol-induced hepatotoxicity, exposure time and final concentration of ethanol were firstly investigated. Compared with untreated hepatocytes, those exposed to 200 mM ethanol for 48 h properly decreased cell viability (71.0 ± 4.9 %) to conduct screening protective activity of substances. Therefore, these conditions were used for following work (Figure 29; Figure 30).

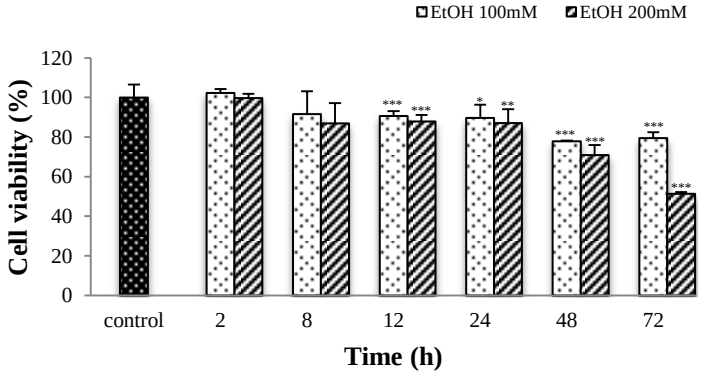

Figure 30. Examination of cytotoxicity on rat primary hepatocytes caused by ethanol exposure at different time and concentrations

Cell viability was measured by MTT assay. Rat primary hepatocytes were treated with ethanol at concentrations of 100 mM and 200 mM for ranging from 2 to 72 h, respectively. Control was incubated with a vehicle alone. Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. *p<0.05, **p<0.005, ***p<0.001 compared with control.

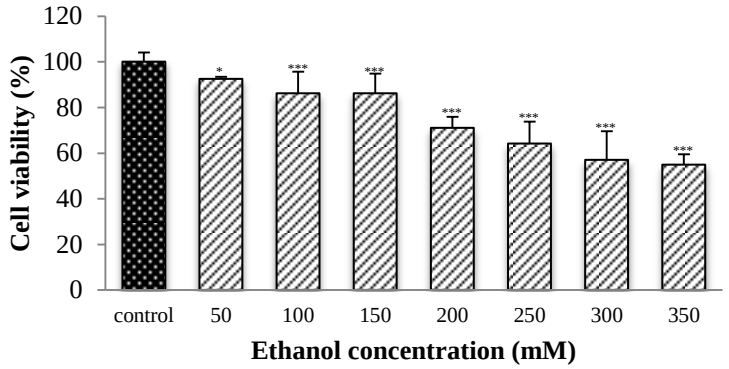

Figure 31. Examination of cytotoxicity on rat primary hepatocytes caused by ethanol exposure at different concentrations for 48 h

Cell viability was measured by MTT assay. Rat primary hepatocytes were treated with ethanol at concentrations of ranging from 50 to 350 mM for 48 h, respectively. Control was incubated with a vehicle alone. Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. *p<0.05, **p<0.005, ***p<0.001 compared with control.

3.2. Protective activities of total extract and fractions against ethanol-intoxicated rat primary hepatocytes

In the course of searching for hepatoprotective substances from natural products, total extract of *O. ficus-indica* seeds showed protective activity against ethanol-intoxicated rat primary hepatocytes (Figure 31). In addition, the EtOAc fraction (82.5 ± 0.9 % at 10 μg/mL and 94.3 ± 6.5 % at 100 μg/mL) exhibited significantly protective activity without cytotoxicity, and compounds **1-16** were isolated from this fraction (Table 10).

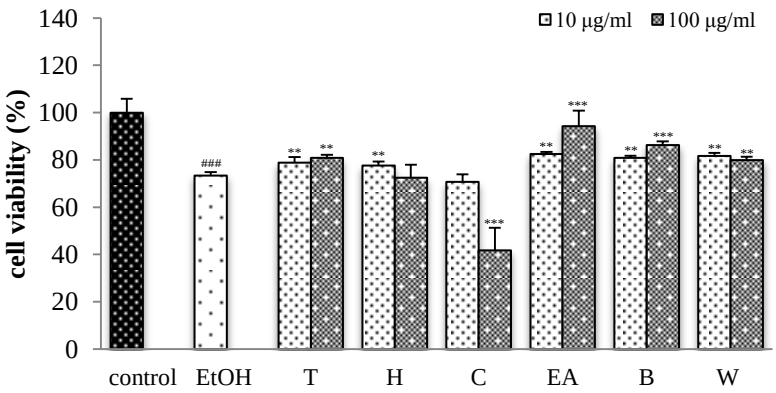

Figure 32. The protective activities of total extract and fractions of *O. ficus-indica* seeds against ethanol-intoxicated rat primary hepatocytes

Cell viability was measured by MTT assay. Ethanol-intoxicated rat primary hepatocytes were 2 h pre-treated with total extract and fractions at concentrations ranging from 10 to 100 μg/mL and incubated for 48 h. Control was incubated with a vehicle alone. Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. #p<0.05, ##p<0.005, ###p<0.001 compared with control. *p<0.05, **p<0.005, ***p<0.001 compared with ethanol group

Table 10. Cytotoxicity of extract and fractions of *O. ficus-indica* on rat primary hepatocytes (%)

	Control	Total ext.	*n*-Hexane	$CHCl_3$	EtOAc	*n*-BuOH	Water
Cell viability (%)	100 ± 5.9	96.1 ± 13.5	81.7 ± 1.8$^{###}$	44.3 ± 5.5$^{###}$	103.4 ± 6.7	93.1 ± 5.6$^{#}$	92.3 ± 1.3$^{#}$

3.3. Protective activities of compounds **1-16** against ethanol-intoxicated rat primary hepatocytes

The protective activities of compounds **1-16** against ethanol-intoxicated rat primary hepatocytes were evaluated (Figure 31). Compounds **2** and **5** were established as positive controls because compound **2** was one of constituents of silymarin complex and compound **5** was also well known hepatoprotective flavonoid (Dixit et al., 2007; Liu et al., 2010). The protective activities of compound **2** were 31.8 % at 10 μM, 42.5 % at 25 μM, 55.3 % at 50 μM and 64.7 % at 100 μM, and compound **5** were 15.1 % at 10 μM, 27.0 % at 25 μM, 34.4 % at 50 μM and 59.4 % at 100 μM, respectively. Among sixteen isolated compounds, lignan group (**10-15**) showed significantly protective activities against ethanol-intoxicated rat primary hepatocytes. They restored the cell viability in dose dependent manners, and especially compounds **13-15**, which containing furofuran ring moiety within the structure, showed the potent protective activity with EC_{50} values of 20.9, 20.4 and 23.0 μM, respectively. Among nine flavonoids (**1-9**), it was found that compounds **2** and **5** having 3',4'-dihydroxyl B ring showed higher protective activities than compounds **1** and **3** having 4'-hydroxyl B ring. On the other hand, compounds **4** and **6** having a methoxy group at C-3 exhibited lower protection than compounds **3** and **5** possessing a hydroxyl group at C-3. The glycosylation in the hydroxyl group at C-3 also affected the activity. Compounds **8** and **9** with glucose group at C-3 showed lower protective activities than compound **7**. Taken together, it was suggested that furofuran ring skeleton of lignans, 3',4'-dihydroxylation in B ring and the lack of methylation or glycosylation on hydroxyl group at C-3 of flavonoids were important for protective activity against ethanol-injured rat primary hepatocytes.

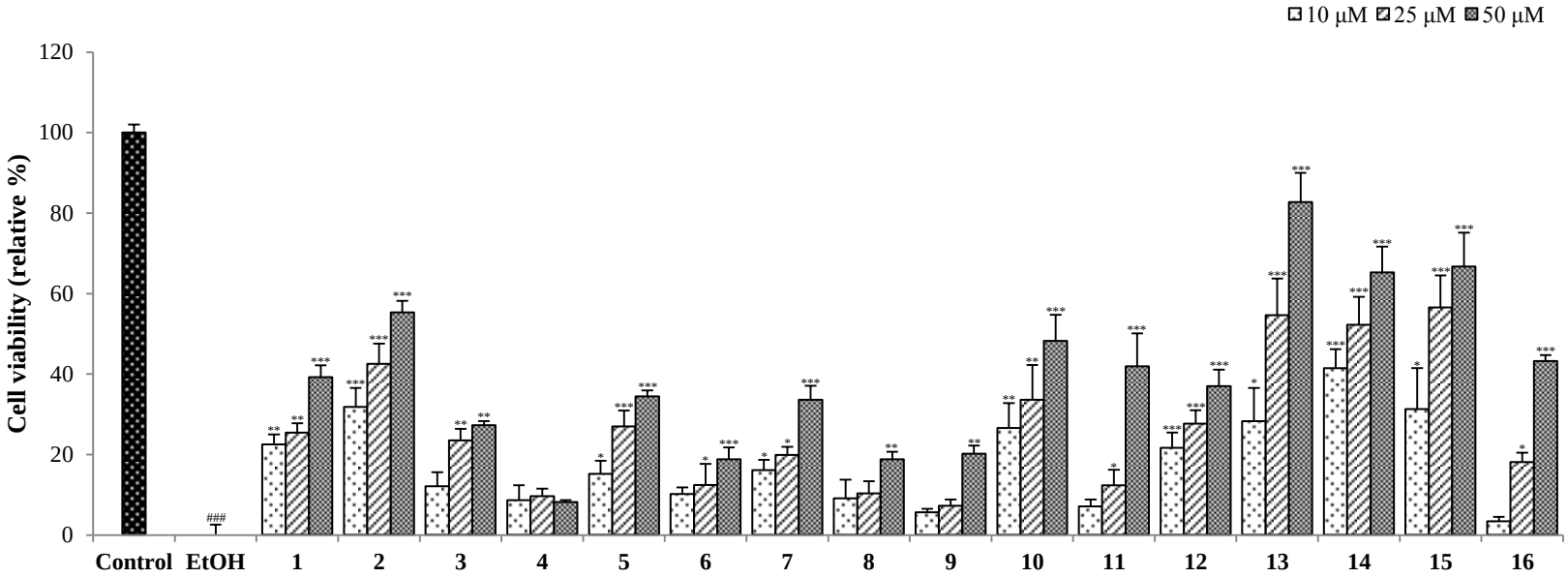

Figure 33. Protective activities of compounds isolated from *O. ficus-indica* seeds against EtOH-intoxicated rat primary hepatocytes

Cell viability was measured by MTT assay. Rat primary hepatocytes were 2 h pre-treated with compounds **1-16** at concentrations ranging from 10 to 50 μM and followed by administering ethanol (200 mM) to the cultures for a further 48 h. Control was incubated with a vehicle alone. Results are expressed as the mean ± S.D. of three independent experiments, each performed using triplicate wells. ###p<0.001, compared with untreated control group; *p<0.05, **p<0.005, ***p<0.001 compared with ethanol group.

IV. Conclusions

1. To develop a new drug candidate for alcoholic liver disease from natural products, the ethanol-intoxicated rat primary hepatocytes were used as an *in vitro* screening model

2. The EtOAc fraction of *Opuntia ficus-indica* (L.) Mill. (Cactaceae) seeds exhibited significantly protective activity against ethanol-intoxicated rat primary hepatocytes.

3. Nine flavonoids (**1-9**), six lignans (**10-15**) and a phenolic compound (**16**) were isolated by the activity-guided isolation over EtOAc fraction.

4. Among these isolated compounds, lignans containing furofuran ring moiety showed the potent protective activity against ethanol-intoxicated rat primary heaptocytes.

5. In the study of structure and activity relationship of flavonoids, 3',4'-dihydroxylation in B ring and the lack of methylation or glycosylation on hydroxyl group at C-3 were suggested as important factors on protective activity against ethanol-induced cellular damage in rat primary hepatocytes.

V. References

Abd El-Razek, F.H. and Hassan, A.A., 2011 Nutritional value and hypoglycemic effect of prickly cactus pear (Opuntia ficus-indica) fruit juice in alloxan-induced diabetic rats. *Australian Journal of Basic and Applied Sciences*, 5(10): 356-377.

Alimi, H., Hfaeidh, N., Bouoni, Z., Sakly, M. and Rhouma, K.B., 2012a. Ameliorative effect of Opuntia ficus indica juice on ethanol-induced oxidative stress in rat erythrocytes. *Experimental and toxicologic pathology,* ahead of print

Alimi, H. et al., 2012b. Evaluation of Opuntia ficus indica f. inermis fruit juice hepatoprotective effect upon ethanol toxicity in rats. *Gen Physiol Biophys*, 31(3): 335-342.

Alimi, H. et al., 2010. Antioxidant and antiulcerogenic activities of Opuntia ficus indica f. inermis root extract in rats. *Phytomedicine*,17(14): 1120-6.

Alimi, H., Hfaiedh, N., Bouoni, Z., Sakly, M. and Ben Rhouma, K., 2011. Evaluation of antioxidant and antiulcerogenic activities of Opuntia ficus indica f. inermis flowers extract in rats. *Environmental toxicology and pharmacology*, 32(3): 406-16.

Arnoldi, A. and Merlini, L., 1985. Asymmetric-Synthesis of 3-Methyl-2-Phenyl-1,4-Benzodioxanes - Absolute-Configuration of the Neolignans Eusiderin and Eusiderin-C and Eusiderin-D. *J Chem Soc Perk*, T 1(12): 2555-2557.

Berry, M.N. and Friend, D.S., 1969. High-Yield Preparation of Isolated Rat Liver Parenchymal Cells - a Biochemical and Fine Structural Study. *J Cell Biol*, 43(3): 506-520.

Bukhari, S.B., Memon, S., Mahroof-Tahir, M. and Bhanger, M.I., 2009. Synthesis, characterization and antioxidant activity copper–quercetin complex. *Spectrochimica Acta Part A*, 71(5): 1901-1906.

Casabuono, A.C. and Pomillo, A.B., 1994. Lignans and a stilbene from Festuca argentina. *Phytochemistry*, 35(2): 479-483.

Chae, H.B., 2009. Alcoholic liver disease. *The Korean Journal of Gastroenterology*, 53: 275-282.

Choi, J. et al., 2002a. Biological activities of the extracts from fruit and stem of prickly pear (Opuntia ficus-indica var. saboten). II. Effects on dietary induced hyperlipidemia. *Saengyak Hakhoechi*, 33(3): 230-237.

Choi, J., Lee, C.K., Moon, Y.I., Park, H.J. and Han, Y.N., 2002b. Biological activities of the extracts from fruits and stem of prickly pear (Opuntia ficus-indica var. saboten) III. Effects on subacute alcoholic hyperlipidemia in rats. *Saengyak Hakhoechi*, 33(3): 238-244.

De Gregorio, A., Arena, N. and Giuffrida, D., 2010. Lipoxygenase Activity in Prickly Pear Fruit (Opuntia Ficus Indica [L.] Mill. Cactaceae). *J Food Biochem*, 34(2): 439-450.

Dixit, N., Baboota, S., Kohli, K., Ahmad, S. and Ali, J., 2007. Silymarin: A review of pharmacological aspects and bioavailability enhancement approaches. *Indian J Pharmacol*, 39(4): 172-179.

Dok-Go, H. et al., 2003. Neuroprotective effects of antioxidative flavonoids, quercetin, (+)-dihydroquercetin and quercetin 3-methyl ether, isolated from Opuntia ficus-indica var. saboten. *Brain research*, 965(1-2): 130-6.

Dufour, J.F. and Clavien, P.A., 2010. Hepatocytes, Signaling Pathways in Liver Diseases. *Springer*, pp. 3-24.

Ennouri, M. et al., 2006. Evaluation of some biological parameters of Opuntia ficus indica. 2. Influence of seed supplemented diet on rats. *Bioresource Technol*, 97(16): 2136-2140.

Galati, E.M. et al., 2003. Chemical characterization and biological effects of Sicilian Opuntia ficus indica (L.) mill. Fruit juice: antioxidant and antiulcerogenic activity. *Journal of agricultural and food chemistry*, 51(17): 4903-8.

Galati, E.M. et al., 2005. Opuntia ficus indica (L.) Mill. fruit juice protects liver from carbon tetrachloride-induced injury. *Phytother Res*, 19(9): 796-800.

Galati, E.M. et al., 2007. Opuntia ficus indica (L.) Mill. mucilages show cytoprotective effect on gastric mucosa in rat. *Phytother Res*, 21(4): 344-6.

Galati, E.M., Monforte, M.T., Tripodo, M.M., d'Aquino, A. and Mondello, M.R., 2001. Antiulcer activity of Opuntia ficus indica (L.) Mill. (Cactaceae): ultrastructural study. *J Ethnopharmacol*, 76(1): 1-9.

Galati, E.M., Pergolizzi, S., Miceli, N., Monforte, M.T. and Tripodo, M.M., 2002a. Study on the increment of the production of gastric mucus in rats treated with Opuntia ficus indica (L.) Mill. cladodes. *J Ethnopharmacol*, 83(3): 229-233.

Galati, E.M., Tripodo, M.M., Trovato, A., Miceli, N. and Monforte, M.T., 2002b. Biological effect of Opuntia ficus indica (L.) Mill. (Cactaceae) waste matter - Note I: diuretic activity. *J Ethnopharmacol*, 79(1): 17-21.

Gentile, C., Tesoriere, L., Allegra, M., Livrea, M.A. and D'Alessio, P., 2004. Antioxidant betalains from cactus pear (Opuntia ficus-indica) inhibit endothelial ICAM-1 expression. *Annals of the New York Academy of Sciences*, 1028: 481-6.

Gramenzi, A. et al., 2006. Review article: alcoholic liver disease--pathophysiological aspects and risk factors. *Alimentary pharmacology & therapeutics*, 24(8): 1151-61.

Griffith, M.P., 2004. The origins of an important cactus crop, Opuntia ficus-indica (Cactaceae): New molecular evidence. *Am J Bot*, 91(11): 1915-1921.

Hashimoto, K. et al., 1992. Two glycosides from roots of Asiasarum sieboldi. *Phytochemistry*, 31(7): 2477-2480.

Holtzman, J.L., Margolis, S. and Rothman, V., 1972. Metabolism of Drugs by Isolated Hepatocytes. *Biochem Pharmacol*, 21(4): 581-585.

Hwang, J.S., Kim, E.J., Ko, S.Y. and Min, D.J., 2011. Cosmetic composition used for lips and having lip-moisturizing effect, *Repub. Korean Kongkae Taeho Kongbo*, Korea.

Javier, A.A.F. et al., 2003. Hypoglycemic activity of two polysaccharides isolated from Opuntia ficus-indica and O. streptacantha. *Proceedings of the Western Pharmacology Society*, 46: 139-142.

Jeon, Y.E. et al., 2011. Inhibitory Activity of Aromadendrin from Prickly Pear (Opuntia ficus-indica) Root on Aldose Reductase and the Formation of Advanced Glycation End Products. *Food Sci Biotechnol*, 20(5): 1283-1288.

Jin, C.B., J., K.H., Ryu, J.H., Lee, Y.S. and Kim, J.M., 2012. Medicinal composition containing opuntia ficus-indica extract for improving learning and memory abilities, *Repub. Korean Kongkae Taeho Kongbo*, Korea.

Jin, C.B., Kim, H.J., Lee, Y.S. and Jung, S.Y., 2009. Extracts of Opuntia ficus-indica var. saboten seeds or compounds isolated therefrom for treating liver disorders. Korea Institute of Science and Technology, *S. Korea* . pp. 21pp.

Jones, C.A., Moore, B.P., Cohen, G.M., Fry, J.R. and Bridges, J.W., 1978. Studies on Metabolism and Excretion of Benzo(a)Pyrene in Isolated Adult Rat Hepatocytes. *Biochem Pharmacol*, 27(5): 693-702.

Jun, H.G. and Yoon, U.C., 2002. Extract composition having good antimicrobial activity extracted from Opuntia, Repub. *Korean Kongkae Taeho Kongbo*, Korea.

Kamiya, K., Tanaka, Y., Endang, H., Umar, M. and Satake, T., 2004. Chemical constituents of Morinda citrifolia fruits inhibit copper-induced low-density lipoprotein oxidation. *Journal of agricultural and food chemistry*, 52(19): 5843-8.

Kang, I.J. and Lim, S.S., 2011. Opuntia ficus-indica root extracts for treating diabetes and diabetic complication, *Repub. Korean Kongkae Taeho Kongbo*, Korea.

Kim, I.H., 2008. 알코올간질환의 병태생리. *대한간학회지*, 14: 7-21.

Kim, J.H. et al., 2006. Opuntia ficus-indica attenuates neuronal injury in in vitro and in vivo models of cerebral ischemia. *J Ethnopharmacol*, 104(1-2): 257-62.

Kim, J.M. et al., 2010. The n-butanolic extract of Opuntia ficus-indica var. saboten enhances long-term memory in the passive avoidance task in mice. *Prog Neuro-Psychoph*, 34(6): 1011-1017.

Kim, K.I. and Oh, S.H., 2002. An immunological activity enhancer with Opuntia ficus-indica fruit extract as effective component, *Repub. Korean Kongkae Taeho Kongbo*, Korea.

Kim, N.C., Graf, T.N., Sparacino, C.M., Wani, M.C. and Wall, M.E., 2003. Complete isolation and characterization of silybins and isosilybins from milk thistle (Silybum marianum). *Org Biomol Chem*, 1(10): 1684-1689.

Kim, S.H. et al., 2012. Prickly Pear Cactus (Opuntia ficus indica var. saboten) Protects Against Stress-Induced Acute Gastric Lesions in Rats. *Journal of medicinal food*, 15(11): 968-73.

Kleinman, H.K., McGoodwin, E.B., Rennard, S.I. and Martin, G.R., 1979. Preparation of collagen substrates for cell attachment: effect of collagen concentration and phosphate buffer. *Analytical biochemistry*, 94(2): 308-12.

Lee, E.H. et al., 2003a. Constituents of the stems and fruits of Opuntia ficus-indica var. saboten. *Archives of pharmacal research*, 26(12): 1018-1023.

Lee, J.C., Kim, H.R., Kim, J. and Jang, Y.S., 2002. Antioxidant property of an ethanol extract of the stem of Opuntia ficus-indica var. saboten. *Journal of agricultural and food chemistry*, 50(22): 6490-6.

Lee, M.H. et al., 2006. Inhibition of nitric oxide synthase expression in activated microglia and peroxynitrite scavenging activity by Opuntia ficus indica var. saboten. *Phytother Res*, 20(9): 742-747.

Lee, Y.S. et al., 2003b. Use of an Opuntia ficus-indica extract and compounds isolated therefrom for protecting nerve cells, *PCT Int. Appl*.

Lim, K.T., 2010. Inhibitory effect of glycoprotein isolated from Opuntia ficus-indica var. saboten MAKINO on activities of allergy-mediators in compound 48/80-stimulated mast cells. *Cellular immunology*, 264(1): 78-85.

Lin, C.F., Ni, C.L., Huang, Y.L., Sheu, S.J. and Chen, C.C., 2007. Lignans and anthraquinones from the fruits of Morinda citrifolia. *Nat Prod Res*, 21(13): 1199-204.

Liu, S. et al., 2010. Quercetin protects against ethanol-induced oxidative damage in rat primary hepatocytes. *Toxicology in vitro*, 24(2): 516-22.

Morán-Ramos, S. et al., 2012. Opuntia ficus indica (Nopal) Attenuates Hepatic Steatosis and Oxidative Stress in Obese Zucker (fa/fa) Rats. *Journal of Nutrition*, 142(11): 1956-1963.

Mosmann, T., 1983. Rapid Colorimetric Assay for Cellular Growth and Survival - Application to Proliferation and Cyto-Toxicity Assays. *J Immunol Methods*, 65(1-2): 55-63.

Ncibi, S., Ben Othman, M., Akacha, A., Krifi, M.N. and Zourgui, L., 2008. Opuntia ficus indica extract protects against chlorpyrifos-induced damage on mice liver. *Food and Chemical Toxicology*, 46(2): 797-802.

Olszewska, M.A. and Roj, J.M., 2011. Phenolic constituents of the inflorescences of Sorbus torminalis (L.) Crantz. *Phytochemistry Letters*, 4(2): 151-157.

Park, E.H. and Chun, M.J., 2001. Wound healing activity of Opuntia ficus-indica. *Fitoterapia*, 72(2): 165-167.

Park, E.H., Hwang, S.E. and Kahng, J.H., 1998. Anti-inflammatory activity of opuntia ficus-indica. *Yakhak Hoechi*, 42(6): 621-626.

Shi, Z.-H. et al., 2012. Metabolism-based synthesis, biologic evaluation and SARs analysis of O-methylated analogs of quercetin as thrombin inhibitors. *European Journal of Medicinal Chemistry*, 54(0): 210-222.

Stewart, S., Jones, D. and Day, C.P., 2001. Alcoholic liver disease: new insights into mechanisms and preventative strategies. *Trends Mol Med*, 7(9): 408-413.

Sultan, A., Bahang, Aisa, H.A. and Eshbakova, K.A., 2008. Flavonoids from *Dracocephalum moldavica*. *Chemistry of Natural Compounds*, 44(3): 366-367.

Tilg, H. and Day, C.P., 2007. Management strategies in alcoholic liver disease. Nature clinical practice. *Gastroenterology & hepatology*, 4(1): 24-34.

Tome, S. and Lucey, M.R., 2004. Review article: current management of alcoholic liver disease. *Alimentary pharmacology & therapeutics*, 19(7): 707-14.

Trombetta, D. et al., 2006. Effect of polysaccharides from Opuntia ficus-indica (L.) cladodes on the healing of dermal wounds in the rat. *Phytomedicine*, 13(5): 352-8.

Waibel, R., Benirschke, G., Benirschke, M. and Achenbach, H., 2003. Sesquineolignans and other constituents from the seeds of Joannesia princeps. *Phytochemistry*, 62(5): 805-811.

Wang, X.W., Mao, Y., Wang, N.L. and Yao, X.S., 2008. A New Phloroglucinol Diglycoside Derivative from Hypericum japonicum Thunb. *Molecules*, 13(11): 2796-2803.

Wang, Y., Hamburger, M., Gueho, J. and Hostettmann, K., 1989. Antimicrobial flavonoids from Psiadia trinervia and their methylated and acetylated derivatives. *Phytochemistry*, 28(9): 2323-2327.

Wiese, J., McPherson, S., Odden, M.C. and Shlipak, M.G., 2004. Effect of Opuntia ficus indica on symptoms of the alcohol hangover. *Archives of internal medicine*, 164(12): 1334-40.

Zakhari, S., 2006. Overview: How Is Alcohol Metabolized by the Body? *Alcohol Research & Health*, 29: 245-254.

Zorgui, L., Ayed-Boussema, I., Ayed, Y., Bacha, H. and Hassen, W., 2009. The antigenotoxic activities of cactus (Opuntia ficus-indica) cladodes against the mycotoxin zearalenone in Balb/c mice: prevention of micronuclei, chromosome aberrations and DNA fragmentation. *Food and chemical toxicology*, 47(3): 662-7.

국 문 초 록

천연물로부터 새로운 알코올성 간질환 치료제로 개발될 수 있는 후보물질을 도출하기 위하여 에탄올로 독성을 유발시킨 흰쥐의 일차배양 간세포(rat primary hepatocyte)를 검색계로 사용하여 간세포 보호 활성을 검색하던 중 백년초 종자의 80% 에탄올 추출물이 유의성 있는 보호 활성을 나타냄을 확인하였다.

백년초 (*Opuntia ficus-indica* (L.) Mill.)는 선인장과 (Cactaceae)에 속하는 다년생 초본으로 전통적으로 당뇨, 고혈압, 천식, 이질의 치료, 소염, 해독, 자양강장의 목적으로 사용되어 왔으며, 최근 들어 antioxidant, antiulcer, neuroprotective, hepatoprotective 등의 효과가 있다고 보고되고 있으나 아직까지 에탄올로 유도한 간손상에 대한 *in vitro* 보호 활성과 그에 관련된 성분연구는 체계적으로 이루어진 바 없다. 이에 본 연구에서는 백년초 종자로부터 활성 지향적 분리기법을 통해 간세포 보호 활성 성분을 분리하고자 하였다.

백년초 종자의 총 80% 에탄올 추출물을 물에 현탁한 다음 *n*-hexane, $CHCl_3$, EtOAc 및 *n*-BuOH 로 용매의 극성에 따라 순차적으로 분획하고, 각 분획물의 에탄올 독성에 대한 간세포 보호 활성을 측정하였다. 백년초 종자 분획물 중에서 EtOAc 분획물이 유의성 있는 보호활성을 나타내어 각종 column chromatography 기법을 이용하여 16 종의 화합물을 분리하였다. 분리한 화합물들은 각종 이화학적 성상 및 분광학적 데이터를 종합하여 그 구조를 각각 (+)-aromadendrin (**1**), (+)-taxifolin (**2**), kaempferol (**3**), kaempferol 3-methyl ether (**4**), quercetin (**5**), quercetin 3-methyl ether (**6**), isorhamnetin (**7**), isorhamnetin 3-*O*-β-D-glucopyranoside (**8**), narcissin (**9**), (7*S*,8*S*)-isoamericanin A (**10**), (7*R*,8*R*)-isoamericanoic acid A (**11**), (7*R*,8*R*)-9'-*O*-methylisoamericanol A (**12**), 3,3'-bisdemethyl pinoresinol (**13**), (7*S*,8*S*)-isoprincepin (**14**), (7*S*,8*S*)-princepin (**15**), 1-*O*-feruloyl-β-D-glucopyranoside (**16**)로 동정하였다. 화합물 **1-9** 는 flavonoid 계열, 화합물 **10-15** 는 lignan 계열, 화합물 **16** 는 phenolic 계열 물질이다. 이들 화합물 중 화합물 **11** 과 **16** 은 이 과 식물에서 처음으로 분리, 보고되는 물질이다.

분리한 총 16 종의 화합물에 대하여 에탄올로 독성을 유도한 흰쥐의 일차배양 간세포를 이용하여 MTT assay 를 이용하여 세포 생존율을 측정하였다. 이 중 화합물 **13-15** 의 구조 내 furofuran ring 을 가지는 lignan 계열의 화합물이 유의성 있는 보호활성을 나타내었다. 또한 flavonoid 계열 화합물의 경우, B ring 의 3',4'-dihydroxylation, C ring 의 3 번 위치의 methoxy 기 치환이나 당의 결합 여부가 활성에 영향을 미치는 것으로 나타났다.

주요어 : 백년초 (*Opuntia ficus-indica* (L.) Mill.), 알코올성 간염, flavonoids, lignans, 일차배양 간세포 (rat primary hepatocyte), ethanol

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