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ARTICLE

Evaluation of antioxidant potential of ethyl acetate fraction of *Rosmarinus officinalis* L. and its major components

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Abstract The solvent fractions of rosemary methanol extract were obtained by successive extraction with *n*-hexane, chloroform, ethyl acetate, butanol, and water. The ethyl acetate fraction (EAF) contained a remarkable amount of polyphenol and flavonoid as well as high levels of alkyl and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical scavenging activity. The activity guided fractionation and repeated chromatographic separations over silica gel, RP C18, and Sephadex LH-20 led to isolation of six compounds from the EAF. ^{1}H NMR, ^{13}C NMR, 2D NMR, MS, and IR spectroscopies determined the compounds to be caffeic acid (**1**), rosmarinic acid (**2**), rosmarinic acid methyl ester (**3**), luteolin (**4**), apigenin (**5**), and hispidulin (**6**), and high-performance liquid chromatography quantification was used to determine concentrations in EAF. Among the six isolated compounds, rosmarinic acid methyl ester showed the highest scavenging activities against di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium, alkyl and ABTS radicals. The EAF mixture, but not individual isolated compounds, shielded dermal fibroblast cells from H_2O_2-induced cytotoxicity at concentrations that encompass the SC_{50} of alkyl and ABTS radical. Therefore, our findings suggested for the first time that antioxidant capacity of the EAF mixture result in a synergistic effect on the antioxidant action.

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Introduction

The free radical theory of aging proposes the cumulative damage by reactive oxygen species (ROS) on biomolecules and is one of the predominant internal factors of aging (Fu et al. 2015). In healthy cells, equilibrium exists between the production of these highly reactive species and the defense systems, either enzymatic or non-enzymatic. When this equilibrium is disrupted, oxidative damage due to free radical accumulation, defined as oxidative stress, occurs and consequently many diseases, e.g., cancer, cardiovascular problems, and diabetes, are promoted (Mata et al. 2007). ROS, generated from various sources, targets human skin by oxidative stress (Qin et al. 2014) and plays an important role in skin aging.

Strategies have been developed for measuring the antioxidant activity by scavenging free radicals generated in aqueous phases. Detection methods for antioxidant activity measurement vary and are based on fluorescence inhibition, oxygen uptake, and absorbance. For example, di(phenyl)-(2,4,6-trinitrophenyl) iminoazanium (DPPH) assay has been widely used to test the ability of compounds. DPPH is a stable free radical that decreases with exposure to proton radical scavengers (Moon et al. 2013). Alkyl radicals are primary intermediates in many hydrocarbon reactions, and the alkyl radicals generated by the process of the radical chain reaction further induce lipid peroxides and attack other components in the organism (Nakao et al. 1998). Direct production of the blue/green 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic) acid $(ABTS)^{+}$ chromosphere is brought about by reacting ABTS with potassium persulfate; antioxidant-induced

scavenging of the $ABTS^+$ reduces the radical to ABTS and decreases absorbance at 734 nm (Guedes et al. 2013). No single antioxidant test method can determine the real antioxidant ability of a sample as methods differ in reaction strategies and sensitivities to test conditions (Karaçelik et al. 2015). Electron spin resonance (ESR) spectrometry is an analytical technique that specifically detects the free radicals involved in autoxidation and related processes (Antolovich et al. 2002) and is more sensitive and accurate than UV–visible spectrometry.

Natural antioxidants have a wide range of biochemical activities, including inhibition of ROS generation and scavenging of free radicals (Finkel and Holbrook 2000). These natural antioxidants protect against health problems that may arise from the use of synthetic antioxidants such as butylated hydroxyanisole (BHA), which may have toxic effects (Aruoma et al. 1992). Therefore, suitable alternatives from plants require exploration, such as the widely cultivated herb of Mediterranean origin Rosemary (*Rosmarinus officinalis* Lamiaceae) (Mata et al. 2007). Rosemary could prevent acute liver damage by improving the structural integrity of the hepatocyte through the scavenging activity of the free radicals induced by CCl_4 (Sotelo-Félix et al. 2002). Also rosemary extracts decreased the generation of UVB-induced intracellular ROS and was also capable of preventing UV-induced DNA damage, which may contribute to reduction of the risk for further skin disorders (Pérez-Sánchez et al. 2014). Several phytochemicals have been isolated and reported from rosemary, and the seasonal and locational variation has been known to govern active components in *R. officinalis* (Celiktas et al. 2007; Borrás-Linares et al. 2014). *R. officinalis* cultivated in subtropical Jeju island has α-pinene (40.96 %) and 1,8 cineole (21.3 %) as major components in the essential oil (Jeon et al. 2013). However, thus far, no studies have examined the antioxidant efficacy and phytochemicals present in rosemary extracts produced in Jeju. Additionally, the antioxidant activity of rosemary using ESR measurement has not been reported yet.

The present study was carried out to measure the total phenolic content of various fractions of rosemary extract produced in Jeju; assess their antioxidant capacity against several free radicals; and evaluate the protective effects of EAF and the individual isolated compounds from EAF on H_2O_2-induced DNA damage in human dermal fibroblast cells.

Materials and methods

Instrumental analysis

NMR spectra were recorded on a JEOL, JNM-ECX400 FT-NMR spectrometer (Japan Electronic Optics Laboratory Co. Ltd., Japan). IR spectra were obtained with IFS-66/S spectrometer (Shimadzu Corporation, Japan). The FAB/MS spectra were recorded on a JEOL JMS-700 mass spectrometer (JEOL). Optical rotation was measured on a JASCO P-1030 automatic digital polarimeter (JASCO Co., Japan).

Plant material

R. officinalis sprig was purchased from Urban Farmers Co., Korea. The voucher specimen (No. 2402/014) was deposited in the herbarium of the Subtropical Research Institute of Jeju National University, Jeju, Korea.

Reagents

High-performance liquid chromatography (HPLC) grade H_2O and acetonitrile were obtained from Burdick & Jackson Co. (USA). Silica gel (0.063–0.2 mm), (Merck, Germany), octadecyl silica gel (ODS, Lichroprep RP-18, Merck), and Sephadex LH-20 (25–100 μm), (GE Healthcare, Sweden) for gel filtration chromatography were used for column chromatography. Thin-layer chromatography (TLC) was performed on a Kiesel gel 60 F254 plate (silica gel 60, 70–230 mesh, Merck) and DC-Fertigplatten RP-18 F254S (Merck). TLC plates developed in 10 % H_2SO_4 were visualized using a Spectroline ENF 260C/F UV lamp (USA). All other reagents used for isolation and analysis were of analytical grade.

Determination of total polyphenol and flavonoid levels

Total polyphenol levels were determined using Folin–Ciocalteu phenol reagent method with minor modifications (Cheung et al. 2003). Absorbance at 725 nm was recorded using a Tecan Sunrise microplate reader (Sunrise, Tecan, Austria). Results were displayed as mg gallic acid equivalents (GAE/g of dried sample). Flavonoid contents were measured using a colorimetric assay developed previously (Zhishen et al. 1999). Absorbance was measured at 510 nm against a blank of Dimethyl Sulfoxide (DMSO), and flavonoid contents were expressed as mg rutin equivalents (RE/g of dried sample). All analyses were performed in at least triplicate.

DPPH radical scavenging activity

The free scavenging activity of fractions was measured by DPPH (Sigma-Aldrich, USA) using Nanjo et al. (1996) method. We detected using JES-FA electron spin resonance spectrometer (JEOL). The radical scavenging activities were calculated by

$$\text{Scavenging activity} = (A_o - A_x)/A_o \times 100\%,$$

where A_0 and A_x are signal intensities of samples and only solvent, respectively.

Alkyl radical scavenging activity

Alkyl radicals were generated by 2,2′-Azobis (2-amidinopropane) hydrochloride (AAPH). The different samples diluted in DMSO and reaction mixture containing 40 mM AAPH and 40 mM 4-POBN were incubated at 37 °C for 30 min in a water bath (Hiramoto et al. 1993). The spin of sample was recorded on ESR. The alkyl radical scavenging activities were calculated according to same formula of DPPH radical scavenging activity.

ABTS radical scavenging activity

Determination of the antioxidant capacity was carried out using previous protocols (Gião et al. 2007); briefly, ABTS was dissolved in distilled water and added to a 7 mM concentration. ABTS radical cation ($ABTS^+$) was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 16 h before use. The stock solution was diluted with water to obtain an absorbance of 0.700 ± 0.005 at 734 nm, measured with a UV 1800 spectrophotometer (Shimadzu). A 100 μL of sample in DMSO was added to 900 μL of this diluted solution, and the absorbance at 734 nm was determined after 2 min of initial mixing. The antioxidant solution reduced the radical cation to ABTS, which reduced the color. The extent of decolorization was calculated as the percentage reduction in absorbance.

Cytotoxicity assay

The ability of the extracts to protect cultured cells from H_2O_2-induced cell death was evaluated by MTT colorimetric assay cell viability (Hansen et al. 1989). Fresh human foreskin specimens were obtained from 8 donors aged from 6 to 12 years, who received a routine circumcision procedure of Jeju National University Hospital, Korea. The primarily cultured cell from the baby foreskin was prepared by Professor Moonjae Cho from Department of Biochemistry, School of Medicine, Jeju National University. The established human dermal fibroblasts were cultured at a density of 5×10^4 cells/well in 96-well plates for 1 day and pre-treated with samples. After 1-h incubation, 200 μM H_2O_2 solutions were added to the wells and re-incubated for 4 h. MTT reagent (5 mg/mL) was added to each well after H_2O_2 solutions were removed, and then the plate was incubated at 37 °C for additional 4 h. The media was removed and dissolved in 150 μL DMSO. Absorbance was detected at 570 nm using microplate reader.

Extraction and isolation

The shade-dried sprig *Rosmarinus officinalis* (8 kg) was extracted with 80 % aqueous methanol (MeOH, 80 L × 3) for 24 h, giving a concentrated extract (2 kg). The concentrated extract was dissolved in water (12 L) and successively extracted with *n*-hexane (12 L × 3), $CHCl_3$ (12 L × 3), EtOAc (12 L × 3), and *n*-BuOH (10.8 L × 3), yielding a concentrated extract of *n*-hexane (HF, 140.5 g), $CHCl_3$ (CF, 508 g), EtOAc (EAF, 145 g), *n*-BuOH (BF, 480 g), and H_2O (WF, 726.5 g) fractions. The concentrated EtOAc fraction (EAF, 145 g) was applied to silica gel (SiO_2) column (15 × 12 cm) chromatography (c.c.) and eluted with $CHCl_3$–MeOH (18:1, 4 L → 12:1, 16 L, → 4:1, 6 L). Each eluent fraction was monitored by TLC, with 14 fractions (EAF-1 to EAF-14) obtained. Fraction EAF-7 [31.3 g, elution volume/total volume (Ve/Vt) 0.46–0.67] was subjected to SiO_2 c.c. (ϕ 12 × 12 cm) with elution of $CHCl_3$–MeOH (12:1, 19.4 L, to yield fractions EAF-7–1 to EAF-7–18. EAF–7–4 [155.8 mg, Ve/Vt 0.16–0.17] subjected to Sephadex LH-20 c.c. (ϕ 2 × 55 cm) with elution MeOH–H_2O (2:1, 360 mL) yielded 17 fractions (EAF-7–4–1 to EAF-7–17) with isolation of compound **6** [EAF-7–4–15, 10.8 mg Ve/Vt 0.68–0.73, TLC (RP-18 F_{254}s) R_f 0.45, acetone–H_2O (1:1)] and compound **5** [EAF-7–4–17, 11.8 mg Ve/Vt 0.85–1.0, TLC (RP-18 F_{254}s) R_f 0.42 acetone–H_2O (1:1)]. Fraction EAF-7–7 [190.7 mg, Ve/Vt 0.25–0.26] underwent to Sephadex LH-20 c.c. (ϕ 2 × 55 cm) with elution MeOH–H_2O (1:1, 2400 mL) to yield fractions EAF-7–1–1 to EAF-7–1–14 with isolation of compound **3** [EAF-7–8–6, 72.2 mg Ve/Vt 0.59–0.65, TLC (RP-18 F_{254}s) R_f 0.33, acetone–H_2O (1:1)] and compound **4** [EAF-7–8–12, 4.9 mg Ve/Vt 0.92–1, TLC (RP-18 F_{254}s) R_f 0.20, acetone-H_2O (1:1)]. Further, fraction EAF-7–9 [2.57 g, Ve/Vt 0.29–0.30] was subjected to Sephadex LH-20 c.c. (ϕ 2 × 50 cm) with elution MeOH-H_2O (2:1, 4000 mL) yielded 17 fractions (EAF-7–9–1 to EAF-7–9–17). Further EAF-7–9–8 [135.2 mg, Ve/Vt 0.19–0.24] subjected to ODS c.c. (ϕ 2 × 5 cm) with elution MeOH–H_2O (2:3, 1100 mL) yielded 10 fractions with isolation of compound **1** [EAF-7–9–8–5, 52 mg Ve/Vt 0.15–0.40, TLC (RP-18 F_{254}s) R_f 0.63, MeOH–H_2O (3:2)]. Further EAF-7–13 [12.5 g, Ve/Vt 0.48–0.60] subjected to ODS c.c. (ϕ 4 × 4 cm) with elution acetone (1:3, 1100 mL) yielded 8 fractions with isolation of compound **2** [EAF-7–13–2, 58 mg Ve/Vt 0.10–0.13 TLC (RP-18 F_{254}s) R_f 0.60, MeOH-H_2O (3:2)].

Compound 1

Whitish amorphous powder; IR (CaF_2, λ, cm^{-1}) 3425, 1612; FAB-MS at *m/z* 179 $[M–H]^{-1}$; ^1H NMR (400 MHz, CD_3OD, δ) 7.49 (1H, d, $J = 16.0$ Hz, H-7), 6.99 (1H, d, $J = 2.0$ Hz, H-2), 6.88 (1H, dd, $J = 8.0$, 2.0 Hz, H-6), 6.73 (1H, d, $J = 8.0$ Hz, H-5), 6.17 (1H, d, $J = 16.0$ Hz, H-8). ^{13}C NMR (100 MHz, CD_3OD, δ) 171.14 (C-9), 149.40 (C-4), 147.20 (C-3), 146.87 (C-7), 127.75 (C-1), 123.34 (C-6), 116.81 (C-5), 115.62 (C-8), 114.65 (C-2).

Compound 2

Light brown oil; $[\alpha]_D^{23}$ + 41.2° (*c* 0.1, MeOH); IR (CaF_2, λ, cm^{-1}) 3427, 1616; FAB-MS at *m/z* 359 $[M-H]^{-1}$; ^1H NMR (400 MHz, CD_3OD, δ) 7.55 (1H, d, $J = 15.8$ Hz, H-7), 7.04 (1H, br s., H-2), 6.94 (1H, br d., $J = 7.5$ Hz, H-6), 6.78 (1H, d, $J = 8.5$ Hz, H-5), 6.76 (1H, s, H-2′), 6.71 (1H, d, $J = 8.0$ Hz, H-5′), 6.62 (1H, br. d, $J = 8.0$ Hz, H-6′), 6.27 (1H, d, $J = 15.8$ Hz, H-8), 5.19 (1H, dd, $J = 8.0$, 4.25 Hz, H-8′), 3.10 (1H, dd, $J = 14.3$, 4.0 Hz, H-7′a), 3.01(1H, dd, $J = 14.3$, 8.5 Hz, H-7′b). ^{13}C NMR (100 MHz, CD_3OD, δ) 174.20 (C-9), 168.60 (C-9), 149.87 (C-4), 147.82 (C-7), 146.84 (C-3), 146.19 (C-3′), 145.31 (C-4′), 129.39 (C-1′), 127.73 (C-1), 123.30 (C-6), 121.94 (C-6′), 117.68 (C-2′), 116.61 (C-5), 116.40 (C-5′), 115.32 (C-2), 114.50 (C-8), 74.78 (C-8′), 37.99 (C-7′).

Compound 3

Brown oil; $[\alpha]_D^{23}$ + 35.5° (*c* 0.1, MeOH); IR (CaF_2, λ, cm^{-1}) 3442, 1667; FAB-MS at *m/z* 373 $[M–H]^{-1}$; ^1H NMR (400 MHz, CD_3OD, δ) 7.56 (1H, d, $J = 16.0$ Hz, H-7), 7.05 (1H, d, $J = 2$ Hz, H-2), 6.95 (1H, dd, $J = 8.0$, 2.0 Hz, H-6), 6.78 (1H, d, $J = 8.0$ Hz, H-5), 6.72 (1H, d, $J = 2.0$ Hz, H-2′), 6.70 (1H, d, $J = 8.0$ Hz, H-5′), 6.57 (1H, dd, $J = 8.0$, 2.0 Hz, H-6′), 6.27 (1H, d, $J = 16.0$ Hz, H-8), 5.19 (1H, dd, $J = 7.5$, 5.5 Hz, H-8′), 3.68 (3H, s, $COO\underline{C}H_3$), 3.04(2H, m, H-7′). ^{13}C NMR (100 MHz, CD_3OD, δ) 172.33 (C-9′), 168.48 (C-9), 149.90 (C-4), 148.09 (C-7), 146.90 (C-3), 146.28 (C-3′), 145.46 (C-4′), 128.87 (C-1′), 127.68 (C-1), 123.37 (C-6), 121.94 (C-6′), 117.66 (C-2′), 116.63 (C-5), 116.44 (C-5′), 115.36 (C-2), 114.24 (C-8), 74.78 (C-8′), 52.83 ($COO\underline{C}H_3$), 37.98 (C-7′).

Compound 4

Yellow powder; IR (CaF_2, λ, cm^{-1}) 3430, 1640, 1553; FAB-MS at *m/z* 285 $[M–H]^{-1}$; ^1H NMR (400 MHz, CD_3OD, δ) 7.37 (1H, br. d, $J = 7.8$ Hz, H-6′), 7.36 (1H, br. s, H-2′), 6.89 (1H, d, $J = 7.8$ Hz, H-5′), 6.50 (1H, s, H-3), 6.40 (1H, s, H-8), 6.19 (1H, s, H-6). ^{13}C NMR (100 MHz, CD_3OD, δ) 182.76 (C-4), 165.23 (C-7), 162.03 (C-2), 160.00 (C-5), 157.96 (C-9), 150.42 (C-4′), 146.15 (C-3′), 122.80 (C-1′), 119.29 (C-6′), 116.05 (C-5′), 113.15 (C-2′), 104.03 (C-10), 102.76 (C-3), 99.30 (C-6), 94.15 (C-8).

Compound 5

Yellow powder; IR (CaF_2, λ, cm^{-1}) 3427, 1629, 1580; FAB-MS at *m/z* 269 $[M-H]^{-1}$; ^1H NMR (400 MHz, pyridine d_5, δ) 7.88 (1H, d, $J = 8.0$ Hz, H-2′,6′), 7.20 (1H, d, $J = 8.0$ Hz, H-3′, 5′), 6.85 (1H, s, H-3), 6.82 (1H, s, H-8), 6.73 (1H, s, H-6). ^{13}C NMR (100 MHz, pyridine d_5, δ) 182.54 (C-4), 166.37 (C-7), 164.34 (C-2), 162.89 (C-5), 162.50 (C-4′), 158.27 (C-9), 128.69 (C-2′, 6′), 122.03 (C-1′), 116.66 (C-1′), 104.75 (C-3′,5′), 103.63 (C-3), 98.66 (C-6), 94.68 (C-8).

Compound 6

Yellow powder; IR (CaF_2, λ, cm^{-1}) 3427, 1668, 1580; FAB-MS at *m/z* 299 $[M–H]^{-1}$; ^1H NMR (400 MHz, pyridine d_5, δ) 7.91 (1H, d, $J = 8.0$ Hz, H-2′,6′), 7.21 (1H, d, $J = 8.0$ Hz, H-3′, 5′), 6.89 (1H, s, H-8), 6.88 (1H, s, H-3), 3.95 (3H, H-6-OCH_3). ^{13}C NMR (100 MHz, pyridine d_5, δ) 183.30 (C-4), 164.75 (C-2), 162.91 (C-4′), 159.26 (C-7), 154.31 (C-5), 153.93 (C-9), 132.82 (C-6), 129.06 (C-2′,6′), 122.50 (C-1′), 117.04 (C-3′,5′), 105.39 (C-3), 95.41 (C-8), 60.49 (C-3-CH_3).

HPLC analysis

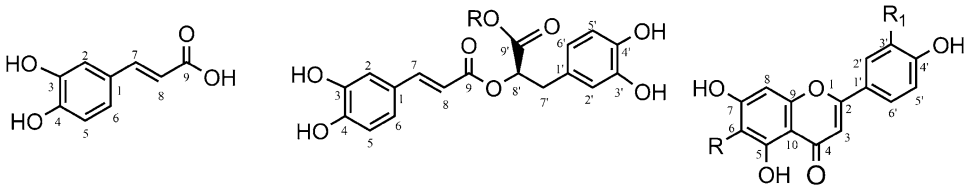
HPLC analysis was done in UFLC LC20 A (Shimadzu Corporation), equipped with a Shim-Pack GIS ODS column (4.6 × 250 mm; 5 μm; Shimadzu). The concentrated EtOAc fraction (25 mg/mL) and compounds (1 mg/mL) were prepared and sieved through a 0.2-μm syringe filter for HPLC analysis. The injection volume for HPLC analysis was 20 μL, and the flow was 1 ml/min and column temperature at 40 °C with measurement at 280 nm. The mobile phase comprised water (solvent A) and acetonitrile (solvent B). For the gradient elution, solvent B was 15 % at start, increased to 43 % over 40 min, then to 100 % in 43–46 min, and finally to 15 % in 50–55 min. HPLC calibration curves of standard solutions at four concentrations were prepared in H_2O (0.4, 0.2, 0.05, and 0.01 mg/mL) for compounds (**1**–**3**) and three concentrations (0.2, 0.1, and 0.05 mg/mL) for compounds (**4**–**6**). The compounds were detected within the retention time of 38 min.

Table 1 Total polyphenol, flavonoid content, and radical scavenging activities of rosemary solvent fractions

Fractions	Total polyphenol content (mg GAE/g)	Total flavonoid content (mg RE/g)	DPPH radical SC_{50} (μg/ml)	Alkyl radical SC_{50} (μg/ml)	ABTS radical SC_{50} (μg/ml)
ME	21.6 ± 14.6[a]	23.1 ± 7.4	33.8 ± 4.5	47.9 ± 11.4	>100
HF	16.2 ± 9.9	11.5 ± 5.7	61.0 ± 3.4	49.2 ± 11.5	97.0 ± 1.3
CF	14.4 ± 8.6	7.8 ± 4.1	94.7 ± 1.0	42.1 ± 7.0	>100
EAF	65.3 ± 22.4	93.0 ± 10.6	>100	17.1 ± 4.1	33.5 ± 4.2
BF	43.1 ± 14.7	61.6 ± 6.2	79.2 ± 4.9	19.2 ± 1.0	56.5 ± 3.7
WF	11.6 ± 8.3	12.5 ± 2.5	46.6 ± 12.8	63.9 ± 11.6	>100

ME aqueous methanol extract, *HF* *n*-hexane fractions, *CF* chloroform fractions, *EAF* ethyl acetate fraction, *BF* n-butanol fractions, *WF* distilled water fraction, *GAE* Gallic acid equivalents, *RE* Rutin equivalents, *SC_{50}* concentration of 50 % radical scavenging activity

[a] Values are mean ± SD ($n = 3$)



1

2: R=OH
3: R=OCH_3

4: R=H, R_1=OH
5: R=R_1=H
6: R=OCH_3, R_1=H

Fig. 1 Structures of isolated compounds **1**–**6** from sprig of *R. officinalis* L

Results and discussion

Extraction

The shade-dried *Rosmarinus officinalis* (8 kg) was extracted with 80 % aqueous methanol for 24 h and gave a concentrated extract (2 kg). Successively extracted with *n*-hexane, $CHCl_3$, EtOAc, *n*-BuOH, and water, the concentrated extracts yielded 140.5, 508, 145, 480, and 726.5 g of material, respectively.

Total polyphenols and flavonoids

Natural phenolic compounds from plants have been reported to exhibit antioxidant activity due to the ability to scavenge free radicals (Moon et al. 2013). A causative relationship has been demonstrated between total phenolic contents and antioxidant activity (Jayaprakasha and Patil 2007). The present study shows polyphenol and flavonoid contents increased according to polarity of solvent used in the fractionation process in the following order: EtOAc fraction (EAF) > *n*-BuOH fraction (BF) > 80 % MeOH extract (ME) > H_2O fraction (WF) > *n*-hexane fraction (HF) > $CHCl_3$ fraction. The EAF contained the highest polyphenol and flavonoid contents with value of 65.3 mg GAE/g and 93.0 mg RE/g. The total phenol and flavonoid contents of ME obtained in our study were similar to that of other study (Chen et al. 2015). However, there are no reports on the total phenol and flavonoid contents of solvent fractions.

Radicals scavenging activities of solvent fractions

The free radical scavenging activities of the various solvent fractions were determined by ESR spectrometry, which is more sensitive and accurate than UV–visible spectrometry and yet has not been utilized for measuring the free radical scavenging activity of rosemary (Yang and Mu 2013). As shown in Table 1, the ME displayed the highest scavenging activity of the DPPH radical and moderate alkyl radical scavenging activity but not much ABTS radical scavenging activity. The DPPH radical scavenging activity of ME obtained in our study exhibited lower SC_{50} value (33.2 μM) compared to that (54.0 μM) of the previous reports (Erkan et al. 2008). The EAF, which contained the highest amount of phenolic compounds, showed the greatest scavenging activities of both alkyl and ABTS radicals but very marginal scavenging activity of DPPH radicals. In a previous study, antioxidant capacity for fruits, vegetables, and beverages detected by ABTS assay was significantly higher than by DPPH assay (Floegel et al. 2011). Similarly, our results show both EAF and BF exhibit

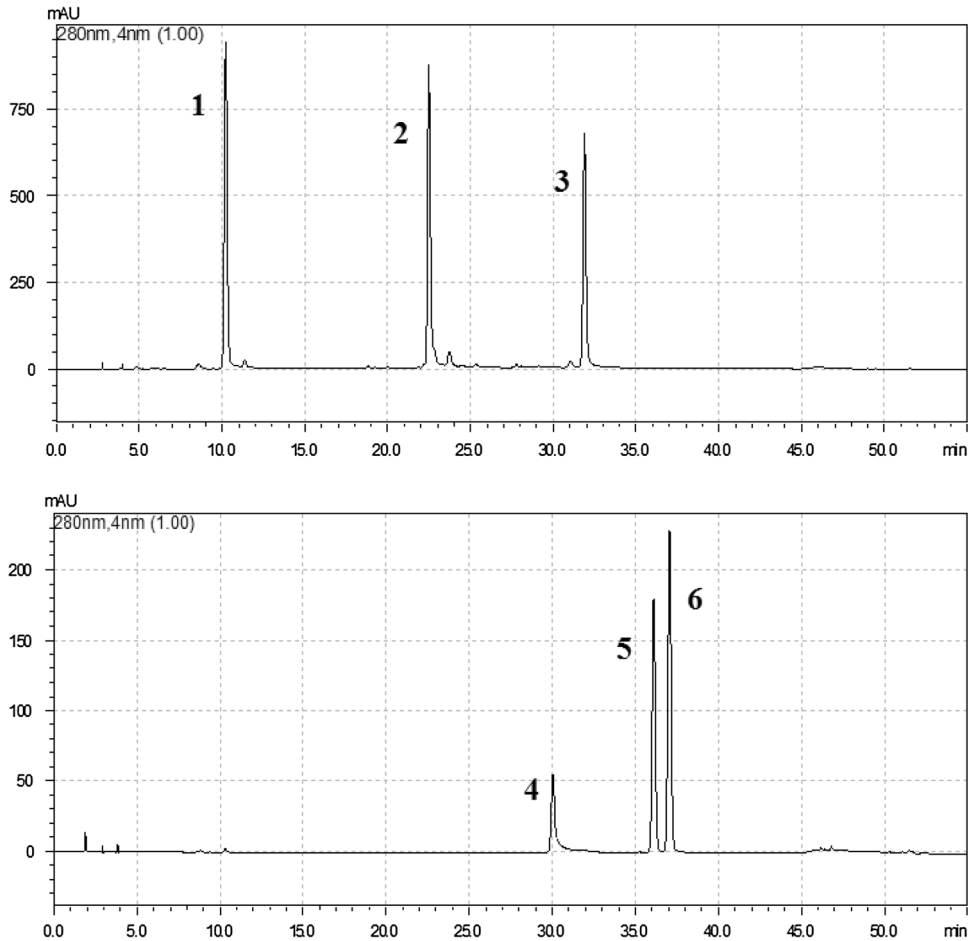


Fig. 2 HPLC chromatograms of isolated compounds. Measured at 280 nm

Table 2 Radical scavenging activities of isolated compounds

Compound	DPPH radical SC_{50} (μM)	Alkyl radical SC_{50} (μM)	ABTS radical SC_{50} (μM)
1	12.1 ± 2.1[a]	27.2 ± 4.1	81.8 ± 0.7
2	4.0 ± 3.1	63.2 ± 12.4	56.7 ± 0.9
3	3.0 ± 2.9	46.8 ± 13.0	37.7 ± 0.9
4	5.7 ± 3.1	32.2 ± 4.8	>100
5	>20	94.3 ± 6.9	>100
6	>20	45.0 ± 7.5	93.3 ± 2.1

SC_{50} Concentration of 50 % radical scavenging activity

[a] Values are mean ± SD ($n = 3$)

greater radical scavenging activities of ABTS than of DPPH. ABTS assay is applicable to both hydrophilic and lipophilic antioxidant systems, whereas DPPH assay is applicable to hydrophobic systems (Kim et al. 2002). EAF and BF compounds have superior correlation with ABTS assay compared to DPPH assay. The SC_{50} values of each fraction obtained in our study could not be compared to those of previous reports since no reports about the antioxidant activities on the various solvent fraction of rosemary methanol extract were found. However, it had been reported previously that both ethyl acetate and butanol fractions of guava (*Psidium cattleianum*) leaves showed potential as a rich source of DPPH, hydroxyl, and alkyl radical scavengers among the tested various solvent fractions (Moon et al. 2013).

Isolation of antioxidant phytochemical by activity guided fractionation and repeated chromatography

The EAF, rich in polyphenol and flavonoid compounds and exhibiting superior radical scavenging activity of alkyl and ABTS radicals, was selected for isolation of the bona fide

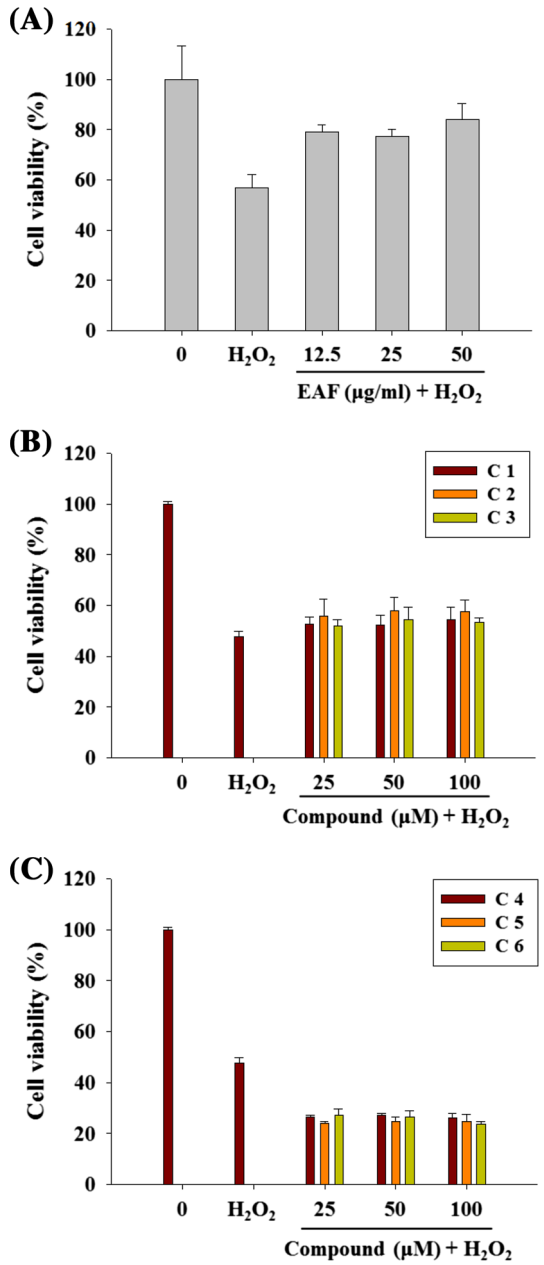


Fig. 3 Protective effect of EAF and isolated compounds. (**A**) Dermal fibroblast cell culture was treated for 1 h with 12.5–50 μg/ml of EAF and then incubated with 200 μM of H_2O_2 for 4 h. (**B**),(**C**) Cell treated with 25–100 μM for 1 h with isolated compounds. Cell viability was detected using MTT assay

antioxidant component from rosemary. The activity guided fractionation of EAF lead to isolation of antioxidant components of *R. officinalis* grown in Jeju. Spectroscopic analysis and comparison with literature values was used to identify the structures of isolated compounds as caffeic acid (**1**) (Jeong et al. 2011), rosmarinic acid (**2**) (Lecomte et al. 2010), rosmarinic acid methyl ester (**3**) (Lecomte et al. 2010), luteolin (**4**), apigenin (**5**), and hispidulin (**6**) (Wawer and Zielinska 2001) (Fig. 1). HPLC quantification showed compounds **1** at 0.09, **2** at 3.9, **3** at 1.89, **4** at 0.012, **5** at 0.008 mg/g, and **6** at 0.031 mg/mg in EAF (Fig. 2).

Radicals scavenging activities of isolated compounds

Next, we investigated the effects of isolated compounds on DPPH, alkyl, and ABTS radical scavenging activity. Each compounds exhibited characteristic antioxidant activities depending on the types of radicals (Table 2). Compounds **3**, **2**, and **4** showed similar SC_{50} values (3.0, 4.0, and 5.7 μM) of DPPH assay and compounds **1**, **4**, and **6**, respectively, suitable for alkyl radical scavenging, whereas compounds **3** and **2** have good activity in the ABTS assay. ABTS assay results of the isolated compounds showed similar SC_{50} values compared to the previous reports (Chen et al. 2007; Begum and Prasad 2012). However, SC_{50} value of DPPH assay was lower than in previous studies (Lin et al. 2014; Zhou et al. 2014), possibly due to the high sensitivity of ESR measurement for DPPH assay. The phytochemicals isolated from rosemary were present in low quantities in the EAF. For example, 25 μg/mL of EAF showed 80 % alkyl radical scavenging activity, while compound **2** showed 80 % alkyl radical scavenging activity at 100 μM. Actually 25 μg/mL of EAF contained 0.0975 μg of compound **2** and can be converted to molar concentration 2.71×10^{-1} μM. The present results suggest the antioxidant effects of EAF, rich in polyphenol compounds, were more significant than the individual components present in EAF with given molar concentration.

Protective Effects of EAF and isolated compounds on H_2O_2-induced cytotoxicity

Human skin is a primary target of oxidative stress from ROS. The role of isolated compounds in the protection of the dermal fibroblast cells from H_2O_2-induced oxidative stress were evaluated using the MTT assay. Exposure of cells to 200 μM H_2O_2 for 4 h increased cell death by 50 %. Cell viability was nearly 84 % by pre-treatment with EAF constituents (Fig. 3A) but not by pre-treatment with single isolated compounds. These results suggest that pre-incubation of cells with EAF isolated compounds for 1 h prior to inducing the oxidative stress could rescue the cytotoxicity induced by H_2O_2 (Fig. 3B, C).

In summary, rosemary EAF is rich in polyphenol compounds and a more effective antioxidant than single isolated phytochemicals, indicating remarkable potential use for human health. This is the first report showing that a rosemary extract exhibits protective effects on H_2O_2-induced cytotoxicity in human fibroblast cells. More extensive studies are needed to develop new antioxidant agents universal to different types of radicals.

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