

Study on Antioxidant Activity and Constituents of *Radix Paeonia veitchii*

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Abstract: The crude extract, fractions and subfractions derived from RPV were evaluated for the DPPH radical scavenging ability, the inhibition ability of bleaching β -carotene and the reducing power. The EAF (ethyl acetate-soluble fraction) and EAF6 (a subfraction derived from EAF) were the most valuable fraction and subfraction, respectively. Furthermore, bioactivity-guided chromatographic fractionation revealed that four pure compounds greatly contributed to the antioxidant activities. Qualitative and quantitative analyses of the major antioxidant constituents in the extract were systematically conducted by NMR, Mass spectra and RP-HPLC. The results demonstrated that gallic acid, (+)catechin, galloyl-paeoniflorin and galloyl-oxypaeoniflorin were the major antioxidative constituents in RPV. These compounds showed stronger activity than the BHT in four tested concentration levels. The results from this study indicate that RPV extract as well as isolated compounds are promising antioxidants which can be used as food additives.

Key words *radix Paeonia veitchii*; antioxidant activity; antioxidative constituents; structure determination; quantification

赤芍抗氧化活性及其成分研究

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摘 要: 利用 DPPH 法、 β -胡萝卜素法和还原力法评估赤芍提取物的抗氧化活性。结果显示乙酸乙酯提取物具有最高的抗氧化活性。利用硅胶, RP-18 和 Sephadex LH-20 对其进行活性追踪分离, 得到四种纯的抗氧化活性成分。利用 1D、2DNMR 和 ESI-MS 对其结构进行鉴定。结果显示为没食子酸、儿茶精、没食子酸芍药甙和没食子酸氧化芍药甙。其抗氧化活性均大于同浓度的人工合成抗氧化剂 BHT。实验证明赤芍及其提取物是一种高效价廉的天然的抗氧化剂, 可用作食品添加剂。

关键词: 赤芍; 抗氧化活性; 抗氧化成分; 结构测定; 定量

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Oxidative stress or excessive production of reactive oxygen species (ROS) is being implicated in many diseases such as cancer, atherosclerosis, degenerative diseases, ageing, diabetes, and so on^[1]. In addition, ROS are considered to induce lipid peroxidation causing the deterioration of foods^[2]. Antioxidants that can neutralize free radicals may be used to protect the human body from diseases and retard lipid rancidity in foods. In recent years, there has been increasing interests in finding novel antioxidants, especially those of plant origin. Natural antioxidants are presumed to be safe since they occur in plant foods, and are more desirable than their synthetic counterparts. Several research studies have demonstrated that herbal plants contain various potential antioxidants, and these antioxidative components may help the human body to reduce oxidative damages and prevent lipid peroxidation in foods. We screened and evaluated various medicinal plant species for their potential antioxidant activities. Among them, the crude extracts of radix *Paeonia veitchii* (RPV) (Chinese name: Chuanchishao, family: *Paeoniaceae*) exhibited the strongest antioxidant activity. RPV is a well-known Chinese folk medicine with the functions of cleansing heat, cooling blood, and invigorating blood circulation, etc. and used as an anticoagulant, antiinflammatory, analgesic and sedative agent. It is also frequently used as a remedy for female genital diseases^[3]. In traditional Chinese medicinal system, RPV is also used very frequently as substitution to the radix *Paeonia lactiflora* Pall. There are many reports for the various chemical constituents and pharmacological actions of *P. lactiflora*. In contrast, only several reports have been published on the chemical constituents^[4]. Literature survey revealed that there is no scientific data has been documented or investigated on antioxidative property and antioxidant constituents of RPV. Therefore, in this report, further works on the antioxidative evaluation of the extract and its fractions as well as isolation, identification, and quantification of the potent antioxidative constituents are performed.

1 Materials and Methods

1.1 Plant material

Radix *Paeonia veitchii* Lynch (RPV) was purchased and identified (voucher specimen ID: QD04301) from the local herbal drugstore at Qingdao, Shandong Province of China, in March, 2006.

1.2 Chemicals

1, 1-Diphenyl-2-picrylhydrazyl (DPPH), β -carotene, and

butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. (St. Louis, MO). Linoleic acid, Tween-40 (polyoxyethylene sorbitan monopalmitate) and ammonium molybdate were purchased from Shanghai Chemical Reagents Co. (Shanghai, China). All other organic chemicals used in the experiments were of analytical grade.

1.3 Extraction and isolation of antioxidant components from RPV

Dried sample (1.0 kg) was ground into small pieces by a Waring blender and extracted with methanol-chloroform (1:1, V/V) in an ultrasonicator for 1 h. The extraction was repeated twice. The extract was decanted, filtered under vacuum. The resulting extractive solution, which was designated as CE, was evaporated to dryness under reduced pressure in a rotary flash evaporator (Büchi Labortechnik AG, Switzerland) dissolved in a small amount of water, and then partitioned by liquid-liquid partitions sequentially in three different solvents, petroleum ether, ethyl acetate, and *n*-butanol, to afford four fractions including petroleum ether-soluble fraction (PEF), ethyl acetate-soluble fraction (EAF) and *n*-butanol-soluble fraction (BF) and aqueous residue (AR), respectively. The resulting four fractions, which were designated as PEF, EAF, BF, and AR, respectively, were evaporated to dryness and stored in the dark at 4 °C until the experiment was carried out.

An antioxidant assay-guided fractionation was conducted. The EAF fraction was further purified because it showed the highest activity in the subsequent antioxidant assays. It was applied to a column (2 × 20 cm) packed with silica gel (200~300 mesh, Qingdao Mar. Chem. Ind. Co. Ltd., Qingdao, China) and eluted with methanol/chloroform (gradient elution performed by changing from 0/100 to 100/0). The elutes were monitored according to thin-layer chromatography (TLC) analysis, which was performed on precoated 0.2 mm thick silica gel 60 GF254 plates (Qingdao Mar. Chem. Ind. Co. Ltd.). Spots were detected by spraying with anisaldehyde-H₂SO₄ reagent, followed by heating, to afford eight subfractions, EAF1-EAF8. One of the subfractions, EAF6, which revealed the highest activity in the subsequent antioxidant assay, was further purified by repeated column chromatography over silica gel, RP-18 (40~75 μ m, Fuji Silysia Chemical Ltd) and Sephadex LH-20 (18~110 μ m, Pharmacia), to afford four compounds. Solvent in each subfraction was evaporated under reduced pressure in a rotary flash evaporator and further removed under a purified nitrogen stream to afford four pure compounds (1~4, Fig. 1).

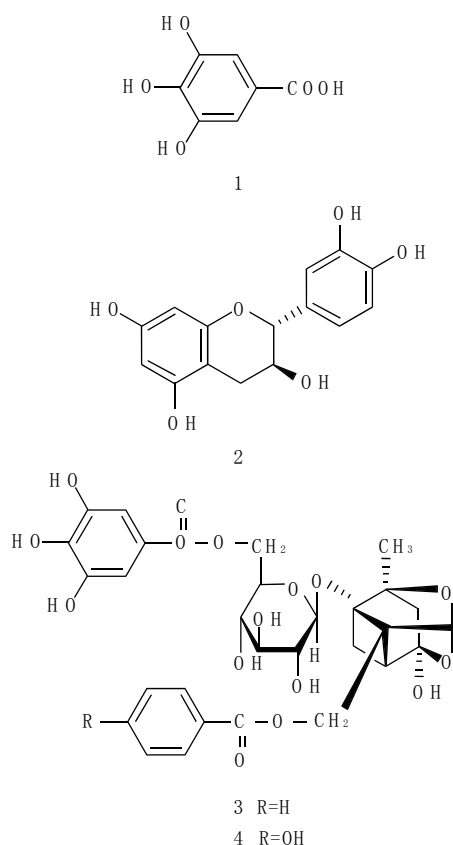


Fig.1 Chemical structures of compounds 1~4 isolated from RPV

1.4 Structure determination and quantification of antioxidant compounds

The structures of all isolated antioxidative compounds were elucidated using spectroscopic analyses. NMR spectra were recorded with Bruker Avance 500 MHz FT-NMR spectrometer, at 500 MHz (^1H) and 125 MHz (^{13}C), respectively. ^1H - ^1H COSY, DEPT, HMQC, and HMBC spectra were obtained with the usual pulse sequences. MS data were collected with a VG Autospet-3000 mass spectrometer.

Quantification of the antioxidative compounds was performed by high performance liquid chromatography (HPLC) using a Dionex HPLC system equipped with a P680 pump, and a photodiode array UVD340U detector. A $5\mu\text{m}$ C-18 column ($250\text{ mm} \times 8\text{ mm}$, Merck, Darmstadt, Germany) was employed in this study with solvent system containing H_2O and MeOH. The gradient linear elution was conducted, with the flow rate 1.0 ml/min . The detector wavelength was set at 280 nm . The antioxidant compounds in the CE, which were identified by comparing retention time of specific peaks with those of the isolated pure compounds as standards in this study under the same chromatographic conditions, were obtained at retention time of 9.247, 12.135, 25.786 and 27.525 min for compounds 1~4, respectively. The individual peak

area corresponding to the isolated pure compound in the HPLC profile of the CE was determined at the observed maximal absorbance of OD_{280} . The standard calibration curves (peak area vs weight) of the isolated pure compounds were obtained. Quantification of the antioxidant compounds in the CE was then performed by HPLC analysis. The peak area of the antioxidant compound in the chromatogram of the CE (with known loading concentration) was then defined, and its content in the extract was calculated on the basis of the quantity calibrated from the standard calibration curve.

1.5 Determination of DPPH radical scavenging activity

The DPPH radical scavenging activity of the extract, fractions, subfractions, and the isolated pure compounds derived from RPV, as well as positive control BHT was measured using the method of Brand-Williams^[5], with slightly modified as follows: a 2.0 ml of methanol solution of DPPH ($2.0 \times 10^{-4}\text{ mol/L}$) was mixed with equivalent aliquot of different concentrations of samples ($0.4, 2, 10$, and $50\text{ }\mu\text{g/ml}$) and after standing in dark for 30 min , and absorbance at 517 nm was measured against methanol. Controls containing methanol instead of the sample and blank containing methanol instead of DPPH solution were also measured, respectively. Positive control BHT was used for comparative purpose. The inhibition of the DPPH radical by the samples was calculated according to the following formula:

$$\text{DPPH scavenging activity (\%)} = [1 - (\text{Abs. of sample} - \text{Abs. of blank}) / \text{Abs. of control}] \times 100$$

1.6 Antioxidant activity assay with the β -carotene-linoleate model system

The antioxidant activity was evaluated according to following procedures previously described by Jayaprakasha^[6]. Generally, a 0.2 mg sample of β -carotene in 0.2 ml of chloroform, 20 mg of linoleic acid, and 200 mg of Tween-40 were mixed. Chloroform was removed at $40\text{ }^\circ\text{C}$ under vacuum and the resulting mixture was diluted with 10 ml of water and mixed well. This emulsion was added 40 ml of oxygenated water. Four-milliliter aliquots of the emulsion were pipetted into different test tubes containing 0.2 ml of different concentrations of solvent fractions and subfractions. Controls containing 0.2 ml of solvent and 4 ml of the above emulsion were prepared. The tubes were placed at $50\text{ }^\circ\text{C}$ in a water bath and the absorbance at 470 nm was taken at zero time ($t=0$). The measurement of absorbance was continued at intervals of 30 min until the color of β -carotene disappeared in the control tubes ($t=180\text{ min}$). A mixture was prepared as above without β -carotene as the blank. BHT was used for comparative purpose. All determinations were carried out in

triplicate. The antioxidant activity (AA) of the samples was evaluated in terms of bleaching of the β -carotene, using the following formula:

$$AA(\%) = [1 - (A_0 - A_t) / (A_0^0 - A_t^0)] \times 100$$

Where A_0 and A_0^0 are the absorbance values measured at zero time of the incubation for the test sample and the control, respectively, and A_t and A_t^0 are the absorbance values measured in the test sample and the control, respectively, after incubation for 180 min.

1.7 Determination of reducing power by phosphomolybdenum complex method

Reducing power was evaluated by the method of Prieto^[7]. Briefly, a 0.1 ml sample solution of different concentration (0.4, 2, 10 and 50 $\mu\text{g/ml}$) was combined with reagent solution (0.6 mol/L sulfuric acid, 28 mmol/L sodium phosphate, and 4 mmol/L ammonium molybdate). In the case of the blank, 0.1 ml of solvent was used in place of sample. The tubes were capped and incubated in a boiling water bath at 95 $^{\circ}\text{C}$ for 90 min. After the samples had cooled to room temperature, the absorbance of each aqueous solution was measured at 695 nm against a blank. The reducing power of the sample was evaluated by means of the absorbance value of the reaction mixture.

1.8 Statistical analysis

All determinations were conducted in triplicate and all results were calculated as mean \pm standard deviation (SD) in this study.

2 Results and Discussion

2.1 DPPH radical-scavenging activity of the crude extract, fractions, and subfractions derived from RPV

The antioxidant activity of RPV extract was first assayed as its capacity to scavenge free radicals of DPPH, which has been widely used to evaluate the antioxidant activity of natural products from plant and microbial sources. Although radical scavenging activity should not be considered as being synonymous with antioxidant activity, it is a fact that almost all of the powerful natural antioxidants such as tocopherol, carnosol, and ascorbic acid are also strong scavengers of the DPPH radical. Thus, good activity in this test is also an indication of the presence of possible antioxidants.

The result of DPPH free radical scavenging assay was listed in Table 1. Among the four fractions isolated by different solvent partitions, the EAF exhibited the highest scavenging activity (82.89%) to DPPH radical at a concentration of 50 $\mu\text{g/ml}$, which was 4.8 times higher than that of the crude methanol-chloroform extract (MCE) itself (17.25%) and significantly higher than that of the synthetic antioxidants, BHT (65.60%), followed by the BF (56.26%). In addition, EAF and BF showed concentration-dependent manner on scavenging DPPH free radical in the tested concentration range. In contrast, PEF and AR did not show significant scavenging activity to DPPH. Among eight subfractions (EAF1-EAF8) isolated from EAF, it was found that EAF6 exhibited the strongest scavenging activity on DPPH (94.06%) and had higher scavenging activity than EAF itself (82.89%) at a concentration of 50 $\mu\text{g/ml}$, which suggested that the com-

Table 1 DPPH radical-scavenging activities of extracts derived from RPV

Extracts	Scavenging activity ($\bar{X} \pm \text{SD}$, %) ^a			
	0.4 $\mu\text{g/ml}$	2 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$
Crude extract	2.80 \pm 0.44	2.77 \pm 0.01	3.55 \pm 0.17	17.25 \pm 1.69
Petroleum ether-soluble fraction	8.02 \pm 0.96	9.60 \pm 0.09	10.96 \pm 0.34	10.28 \pm 0.78
Ethyl acetate-soluble fraction	9.73 \pm 0.72	26.37 \pm 0.33	58.40 \pm 0.51	82.89 \pm 0.19
<i>n</i> -Butanol-soluble fraction	4.44 \pm 1.71	7.05 \pm 1.10	34.34 \pm 0.21	56.26 \pm 1.02
Aqueous residue	2.04 \pm 0.54	1.80 \pm 0.66	3.98 \pm 0.18	5.87 \pm 0.26
EAF1	6.61 \pm 0.79	6.01 \pm 0.91	7.00 \pm 0.67	18.33 \pm 0.18
EAF2	5.24 \pm 0.00	3.64 \pm 0.30	3.65 \pm 0.45	13.99 \pm 1.45
EAF3	10.99 \pm 2.55	10.63 \pm 0.84	11.63 \pm 0.36	11.89 \pm 0.36
EAF4	5.91 \pm 0.08	6.15 \pm 1.51	8.66 \pm 0.26	16.11 \pm 0.32
EAF5	3.50 \pm 0.28	6.17 \pm 0.93	8.36 \pm 0.90	22.26 \pm 0.14
EAF6	3.09 \pm 0.29	19.30 \pm 0.36	68.60 \pm 0.17	94.06 \pm 1.77
EAF7	6.81 \pm 1.02	8.65 \pm 1.07	16.20 \pm 0.03	69.40 \pm 0.23
EAF8	5.30 \pm 0.02	13.13 \pm 0.52	47.80 \pm 0.53	64.25 \pm 0.29
Gallic acid (1)	20.59 \pm 0.40	52.42 \pm 0.02	80.39 \pm 0.21	96.18 \pm 0.24
(+)-Catechin (2)	7.53 \pm 0.39	17.43 \pm 0.11	42.37 \pm 0.20	75.17 \pm 1.09
Galloyl-paeoniflorin (3)	5.16 \pm 0.76	9.42 \pm 0.22	27.37 \pm 0.22	71.90 \pm 0.21
Galloyl-oxypaeoniflorin (4)	3.16 \pm 0.26	9.02 \pm 0.12	26.37 \pm 0.10	71.20 \pm 0.23
BHT	2.86 \pm 0.14	8.93 \pm 0.03	25.64 \pm 0.46	65.60 \pm 0.22

Note: ^a Each value is the mean \pm SD of triplicate measurements.

Table 2 Inhibition of extracts derived from RPV in β -carotene-linoleate model system

Extracts	Inhibition($\bar{X} \pm \text{SD}$, %) ^a			
	0.4 $\mu\text{g/ml}$	2 $\mu\text{g/ml}$	10 $\mu\text{g/ml}$	50 $\mu\text{g/ml}$
Crude extract	2.32 \pm 0.21	5.62 \pm 0.14	8.22 \pm 0.15	5.78 \pm 0.17
Petroleum ether-soluble fraction	6.34 \pm 0.02	9.80 \pm 0.11	13.31 \pm 0.04	10.31 \pm 0.07
Ethyl acetate-soluble fraction	3.48 \pm 0.07	12.34 \pm 0.21	28.82 \pm 0.08	45.62 \pm 0.05
<i>n</i> -Butanol-soluble fraction	2.26 \pm 0.03	3.58 \pm 0.08	10.94 \pm 0.05	19.38 \pm 0.04
Aqueous residue	0.62 \pm 0.08	3.23 \pm 0.12	1.54 \pm 0.02	0.63 \pm 0.07
EAF1	7.06 \pm 0.07	15.68 \pm 0.05	7.76 \pm 0.01	26.26 \pm 0.03
EAF2	3.79 \pm 0.06	9.65 \pm 0.07	6.15 \pm 0.13	6.56 \pm 0.04
EAF3	1.49 \pm 0.14	4.12 \pm 0.11	4.05 \pm 0.05	4.10 \pm 0.01
EAF4	4.01 \pm 0.05	5.86 \pm 0.08	3.72 \pm 0.03	3.86 \pm 0.01
EAF5	2.50 \pm 0.20	9.96 \pm 0.68	12.50 \pm 1.20	29.96 \pm 0.68
EAF6	7.06 \pm 0.07	15.68 \pm 0.05	37.76 \pm 0.01	56.26 \pm 0.03
EAF7	3.65 \pm 0.61	10.25 \pm 0.16	23.66 \pm 0.60	30.25 \pm 0.16
EAF8	5.47 \pm 0.82	7.24 \pm 0.37	15.47 \pm 0.86	21.24 \pm 0.87
Gallic acid (1)	8.57 \pm 0.73	20.34 \pm 1.00	44.15 \pm 0.21	66.21 \pm 0.58
(+)-Catechin (2)	7.53 \pm 0.39	17.43 \pm 0.11	42.37 \pm 0.20	63.17 \pm 1.09
Galloyl-paeoniflorin (3)	6.16 \pm 0.24	9.42 \pm 0.22	23.33 \pm 0.22	61.90 \pm 0.21
Galloyl-oxypaeoniflorin (4)	4.14 \pm 0.26	8.02 \pm 0.11	20.17 \pm 0.10	61.20 \pm 0.23
BHT	1.65 \pm 0.14	12.17 \pm 0.10	26.50 \pm 0.09	53.51 \pm 0.12

Note: ^a Each value is the mean \pm SD of triplicate measurements.

pounds with high DPPH scavenging activity were enriched to the subfraction EAF6 level.

2.2 Antioxidant assay with the β -carotene-linoleate model system

Table 2 shows the antioxidant activities of extract, fractions, and subfractions derived from RPV, as well as BHT determined by the β -carotene-linoleate model system. Among the crude extract and four fractions, the EAF fraction again exhibited the highest antioxidant activity and significantly stronger than those of MCE itself and other fractions in all of four tested concentration levels. EAF exhibited activity in a concentration-dependent manner. In addition, the EAF showed higher antioxidant activity than the positive control BHT at lower concentrations (0.4, 2, and 10 $\mu\text{g/ml}$). At the lowest concentration (0.4 $\mu\text{g/ml}$) tested, the EAF exhibited stronger activity, which was 2.1 times higher than that of BHT. The other fractions did not show significantly protective effects in β -carotene-linoleate model system. Table 2 also shows the antioxidant effects of subfractions EAF1-EAF8 in β -carotene-linoleate model system. It was found that EAF6 was more effective than the other subfractions in all tested concentrations and higher than those of BHT in all of four tested concentration levels. EAF6 also showed a dose-dependent manner.

2.3 Reducing power by the formation of phosphomolybdenum complex (PMC)

The PMC method is based on the reduction of Mo(VI) to Mo(V) by the antioxidant compounds and the formation of

a green Mo(V) complex with a maximal absorption at 695 nm. Generally, the stronger reducing power of the sample related to the higher absorbance value of the reaction mixture. As showed in Table 3, it was observed that EAF and BF had a significant higher reducing power than MCE and the other two fractions, but the effect was more profound in EAF. In addition, the reducing powers of EAF and BF were significant stronger than that of BHT. In comparison of the reducing powers among EAF and its subfractions, EAF6 exhibited the highest reducing power which was higher than that of the EAF itself.

2.4 Identification of antioxidative constituents from RPV

The most active subfraction EAF6 was purified by and repeated column chromatography over a silica gel, RP-18, and Sephadex LH-20, and four pure antioxidant compounds were obtained (1~4). Their structures were determined by interpretation of the 1D and 2D NMR, as well as mass spectral data (Fig. 1).

Compound 1 was obtained as a white powder. Pseudo molecular ion peaks at m/z 171 $[\text{M}+\text{H}]^+$ in ESI-MS along with the analysis of ^1H -, ^{13}C -NMR, and DEPT spectra showed its molecular formula to be $\text{C}_7\text{H}_6\text{O}_5$. The ^1H and ^{13}C NMR were consistent with previously published data^[8]. Compound 1 was determined to be gallic acid (1).

Compound 2 was obtained as a white powder. Pseudo molecular ion peaks at m/z 291 $[\text{M}+\text{H}]^+$ in ESI-MS along with the analysis of ^1H -, ^{13}C -NMR, and DEPT spectra showed its molecular formula to be $\text{C}_{15}\text{H}_{14}\text{O}_6$. Based on the NMR spectral

Table 3 Reducing power of the extracts derived from RPV

Extracts	Absorbance ($\bar{X} \pm SD$) ^a			
	0.4 μ g/ml	2 μ g/ml	10 μ g/ml	50 μ g/ml
Crude extract	0.022 \pm 0.007	0.034 \pm 0.004	0.049 \pm 0.006	0.084 \pm 0.015
Petroleum ether-soluble fraction	0.038 \pm 0.001	0.096 \pm 0.005	0.069 \pm 0.002	0.056 \pm 0.004
Ethyl acetate-soluble fraction	0.047 \pm 0.001	0.070 \pm 0.002	0.123 \pm 0.001	0.288 \pm 0.001
<i>n</i> -Butanol-soluble fraction	0.040 \pm 0.006	0.051 \pm 0.003	0.069 \pm 0.007	0.122 \pm 0.001
Aqueous residue	0.004 \pm 0.005	0.015 \pm 0.004	0.025 \pm 0.033	0.053 \pm 0.030
EAF1	nm	nm	0.003 \pm 0.000	0.002 \pm 0.001
EAF2	nm	nm	0.048 \pm 0.001	0.049 \pm 0.001
EAF3	nm	nm	0.001 \pm 0.001	0.013 \pm 0.002
EAF4	nm	nm	0.004 \pm 0.002	0.030 \pm 0.004
EAF5	nm	nm	0.018 \pm 0.004	0.053 \pm 0.012
EAF6	0.085 \pm 0.001	0.084 \pm 0.002	0.166 \pm 0.001	0.345 \pm 0.005
EAF7	0.008 \pm 0.001	0.012 \pm 0.002	0.021 \pm 0.001	0.139 \pm 0.004
EAF8	0.004 \pm 0.001	0.025 \pm 0.002	0.016 \pm 0.006	0.104 \pm 0.049
Gallic acid(1)	0.105 \pm 0.001	0.145 \pm 0.003	0.206 \pm 0.001	0.495 \pm 0.006
(+)-Catechin(2)	0.055 \pm 0.001	0.095 \pm 0.002	0.126 \pm 0.002	0.365 \pm 0.034
Galloyl-paeoniflorin(3)	0.033 \pm 0.001	0.062 \pm 0.002	0.107 \pm 0.002	0.145 \pm 0.003
Galloyl-oxypaeoniflorin(4)	0.036 \pm 0.011	0.066 \pm 0.037	0.089 \pm 0.084	0.135 \pm 0.030
BHT	0.007 \pm 0.001	0.033 \pm 0.001	0.059 \pm 0.018	0.100 \pm 0.052

Note: nm, not measured.

^a The reducing power of the sample was evaluated by means of the absorbance value of the reaction mixture. Each value is the mean \pm SD of triplicate measurements.

evidences as well as by a comparison of the data in the literature report^[9], compound 2 was determined to be (+) catechin(2). The identification was further supported by RP18-HPLC comparison (retention time and UV spectrum) with authentic compound(Sigma).

Compound 3 was obtained as a white powder. Pseudo molecular ion peaks at m/z 633 $[M+H]^+$ in ESI-MS along with the analysis of 1H -, ^{13}C -NMR, and DEPT spectra showed its molecular formula to be $C_{30}H_{32}O_{15}$. Based on the NMR spectral evidences as well as by a comparison of the data in the literature report^[10], compound 3 was determined to be galloyl-paeoniflorin(3). The identification was further supported by RP18-HPLC comparison (retention time and UV spectrum) with authentic compound(Sigma).

Compound 4 was obtained as a white powder. Pseudo molecular ion peaks at m/z 649 $[M+H]^+$ in ESI-MS along with the analysis of 1H -, ^{13}C -NMR, and DEPT spectra showed its molecular formula to be $C_{30}H_{32}O_{16}$. A comparison of the 1H - and ^{13}C -NMR spectral data between 3 and 4 revealed that the structures of the two compounds were very similar, except for an additional hydroxyl substitution for 4. Based on the analysis of the 1H -, ^{13}C -NMR, HMQC, and HMBC spectra data as well as by a comparison of the data in the literature report^[11], compound 4 was determined to be galloyl-oxypaeoniflorin(4).

2.5 Quantification of major antioxidants from RPV

The major antioxidative compounds in the crude extract

of RPV, which were identified by comparing retention time of peaks with those of the isolated pure compounds in this study under the same chromatographic conditions, were obtained at retention time of 9.247 min(1), 12.135 min(2), 25.786 min(3) and 27.525 min(4). The peak area of each compound in the chromatogram of the CE (with known loading concentration) was then defined, and its content in the extract was calculated on the basis of the quantity calibrated from the standard calibration curve ($R^2 > 0.99$). According to HPLC analysis, each gram of whole dried plant tissue of RPV contains 12.00 mg of gallic acid (1), 6.52 mg of (+)catechin(2), 2.85 mg of galloyl-paeoniflorin(3), and 2.12 mg of galloyl-oxypaeoniflorin(4).

2.6 Antioxidative activity of isolated constituents from RPV

The antioxidative activities of the four major antioxidative compounds (1~4) were also evaluated for the DPPH radical scavenging ability, the inhibition ability of bleaching β -carotene, and the reducing power. The results of DPPH free radical scavenging assay were listed in Table 1. Four isolated compounds showed significant scavenging activity to the DPPH radical in the following order: Gallic acid(1) > (+)catechin(2) > galloyl-paeoniflorin(3) ~ galloyl-oxypaeoniflorin(4). The compound 1 showed the highest free radical scavenging effect and significantly stronger than the positive control BHT in all of four tested concentration levels and exhibited activity in a concentration-dependent

manner. Its scavenging rate to the DPPH radical arrived to 80.39% at a concentration of 10 µg/ml and 96.18% at a concentration of 50 µg/ml. Following the compound 2, 3 and 4 also showed higher antioxidant activity than the positive control BHT in all of four tested concentration levels. They also showed a dose-dependent manner.

It is known that the antioxidant activity of phenolic compounds is correlated to their chemical structure. In general, the free radical scavenging effect of phenolics depends mainly on the number and position of hydrogen-donating hydroxyl groups on the aromatic ring of the phenolic molecules. The results of the present work suggested that the galloyl group or ortho-dihydroxy structure in the compound 1, 2, 3 and 4 seemed to play an important role in the antioxidative function. In addition, the differences among the compound 1, 2 and 3, 4 in the free radical scavenging effect were as that 3 and 4 had the glycosidic substituent, which, probably, reduced the activity for steric hindrance.

The antioxidative activities of the compounds (1~4) were also assayed by using the β -carotene-linoleate model system (Table 2). It can be seen from Table 2 that compounds (1~4) showed stronger activity than the BHT in four tested concentration levels. The order was gallic acid(1) > (+) catechin(2) > galloyl-paeoniflorin(3) ~ galloyl-oxypaeoniflorin(4), which was the same as that of scavenging activity to the DPPH radical. Generally, the tested samples showed significantly higher activity in DPPH assay than in β -carotene assay.

The reducing powers of the compounds (1~4) were summarized in Table 3. The compound 1 showed the highest reducing power and significantly stronger than the BHT in all of four tested concentration levels. The compound 2, 3 and 4 showed also higher antioxidant activity than the positive control BHT in all of four tested concentration levels.

2.7 Comparative analysis among different antioxidant screening methods

Statistical analyses indicated that, at a concentration of 50 µg/ml, there was a strong correlation between the DPPH radical-scavenging activity and reducing activity in isolated compounds ($R^2=0.9366$), followed by that between the DPPH radical-scavenging activity and the antioxidant activity in the β -carotene assay ($R^2=0.8866$). The correlation coefficient between the reducing power and the β -carotene assay was also positive and significant but relatively lower than the others ($R^2=0.8405$). Such results indicated that these three

methods seemed to depend on a similar mechanism, most likely, the propensity to donate hydrogen.

3 Conclusion

RPV is a widespread herbal plant and is easy and unexpensive to be obtained. The results from this study demonstrate that the compounds (1~4) could be mainly responsible for the antioxidative effect of an ethyl acetate soluble fraction of RPV, and might be suitable for further development as potential leading natural antioxidant. Active phenolic constituents obtained from RPV may be an alternative to the more toxic synthetic antioxidants as additives in food industry. The results of the present work also suggested that, apart from the use in traditional medicinal system, RPV may be used to act as a prophylactic agent to prevent oxidative related diseases, and, regular consumption of this medicinal plant may improve antioxidant status and therefore possibly reduce the risks of chronic diseases that associated with oxidative stress. This is the first report so far on the comprehensive investigation for the antioxidant capacity and antioxidant constituents of RPV.

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