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Full Length Research Paper

Comparison of biological activities of *Ribes fasciculatum* according to regional differences

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Ribes fasciculatum **has been traditionally used as febrifuge, diuretic and antidote against urushiol in Korea, although its biological functions have not been well studied. In this study, we determined the anti-oxidant activities, anti-microbial activities and α-glucosidase inhibitory effects of methanol extract and the fractions of** ***R. fasciculatum*** **stem from Chuncheon and Jeju in South Korea. The highest anti-oxidant activity obtained by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay and reducing power assay was found in the EtOAc fraction from Jeju-MeOH extract, which contained the highest level of total phenolic compounds compared to the other fractions. In addition, EtOAc fraction from Chuncheon-MeOH extract exhibited higher inhibitory activities against α-glucosidase (IC_{50} = 4.38 ug/ml) compared to the IC_{50} of the EtOAc fraction from Jeju-MeOH extract. Chuncheon-MeOH extract showed antimicrobial activities against** ***Staphylococus aureus*****,** ***Escherichia coli*** **and** ***Pichia jadinii*****. Although extracts obtained from two regions contained different biological activities, these** ***in vitro*** **studies indicate that this plant can likely be a potential medicinal plant resource for developing effective dietary health supplements.**

Key words: Anti-oxidant activity, anti-microbial activity, alpha glucosidase inhibitory effect, *Ribes fasciculatum*.

INTRODUCTION

Ribes fasciculatum is a deciduous shrub of the Saxifrage family, which consists of about 150 species worldwide and is mainly distributed across northeast Asia. This shrubby tree grows up to 1.5 m high and has glabrous leaves, 10 to 15 cm across, with 3 to 5 lobes and a finely toothed margin. Flowers of *R. fasciculatum* appear in the spring with the leaves and the fruits remain on the branches during the whole winter (Ahn, 1998; Park, 2004). In traditional Korean medicine, roots of *R. fasciculatum* are used for treating menstrual irregularities and cramps, the fruit as a febrifuge and diuretic and the leaf and stem as an antidote against urushiol (Ahn, 1998; Park, 2004; Park et al., 2006). In a pharmaceutical study, it was shown that threo-(7*S*,8*R*)-1-(4-hydroxyphenyl)-2-[4-(*E*)-propenylphenoxy]-propan-1-ol, catechin and (±)-gallocatechin, octadecanyl 3-(4-hydroxy-3-methoxy-phenyl)-acrylate ester from MeOH extract of *R. fasciculatum* stem and twigs contain inhibitory activity against nuclear factor of activated T cells (NFAT) transcription factor (Dat et al., 2005). Although this finding indicates that extract of *R. fasciculatum* has potential as a crude drug for the treatment of immunological and inflammatory diseases, study of the biological activity of *R. fasciculatum* has been limited. In addition, its pharmacological functions have not been introduced in Western countries, whereas its extracts have been used for dietary health supplements due to its numerous pharmacological functions in east Asia.

Therefore, we analyzed the biological activities of *R. fasciculatum* crude extract and fractions. In order to better understand the biological activities of *R. fasciculatum*, we analyzed its anti-microbial and

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Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; **Pnpg,** 4-Nitrophenyl-α-$_D$-glucopyranoside; **ROS,** reactive oxygen species.

anti-diabetic activities and determined the relationship between the amounts of phenols, flavonoids and anti-oxidants. Furthermore, careful analysis of electron donation ability and reducing power was performed. This study presents basic data on *R. fasciculatum*, which could be useful in food companies.

MATERIALS AND METHODS

Chemicals

α-tocopherol, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 4-Nitrophenyl-α-$_D$-glucopyranoside (*p*NPG) and α-glucosidase (E.C. 3.2.1.20) were obtained from Sigma Chemical Co. (St. Louis, MO). Acarbose was obtained from Bayer Schering Pharma. All reagents were of analytical grade or better.

Plant materials and extraction

R. fasciculatum was obtained from Hangkuk tojong yasaeng sanyacho yeonguso (Busan, South Korea) and served from Gangwon provincial arboretum (Chuncheon, South Korea). The voucher specimen (KNUA-O-2) was established botanically and deposited in the Department of Applied Plant Sciences, Kangwon National University. Dried *R. fasciculatum* stem (1 kg) was extracted three times with methanol at a 10:1 solvent-sample ratio for 24 h at room temperature. The solution was filtered, evaporated under reduced pressure and lyophilized. The combined methanolic extract was partitioned with organic solvents to yield *n*-hexane, ethyl-acetate (EtOAc), *n*-butanol (BuOH; water saturated), and aqueous fractions. All processes were carried out in triplicate.

Electron donation ability (EDA) assay

The EDA of sample was determined by the method of Kim et al. (2010). This assay is based on the capacity of a substance to scavenge stable DPPH free radicals. The EDA of *R. fasciculatum* was measured as follows; the reaction mixture contained 1 ml of 0.15 mM DPPH-methanol solution, 3.98 ml of methanol, and 20 µl of different concentration samples or α-tocopherol, BHT and methanol (control). The mixture was allowed to react for 30 min at room temperature and the absorbance values were measured at 517 nm using a spectrophotometer (V-530, Jasco Co., Tokyo, Japan). The experiment was conducted in triplicate. The EDA was expressed as the reduction rate of absorbance in accordance with the following equation:

EDA (%) = [1-(absorbance value of sample/absorbance value of control)] × 100.

Determination of reducing power activity

The reducing power of samples was determined via the method of Oyaizu (1986), with some modifications. Reducing power activity is based on the reduction of (Fe^{3+}) ferricyanide in stoichiometric excess relative to the antioxidants (14). Samples with different concentrations were mixed with 0.5 ml of 0.2 M sodium phosphate buffer (pH 6.6) and 0.5 ml of 1% potassium ferricyanide (w/v). The mixture was incubated for 20 min at 50°C. After incubation, 2 ml of 10% TCA (w/v) was added to the mixture, followed by 10 min of centrifugation at 650 rpm. The upper layer (0.5 ml) was mixed with 0.5 ml of deionized water and 0.1 ml of 0.1% ferric chloride (w/v) and the absorbance of the resultant solution was measured at 700 nm. α-Tocopherol and BHT were used as reference compounds.

Total phenol and flavonoid analysis

The total phenolic content was determined using Folin-Ciocalteu reagent in accordance with the method described by Singleton and Rossi (1965). In brief, 0.1 ml of sample and 50 µl of 2 N Folin-Ciocalteu reagent were added to a 5 ml volumetric flask. The solutions were mixed and allowed to stand for 3 to 5 min at room temperature. Next, 0.3 ml of a 20% sodium carbonate solution (w/v) was added. The solutions were mixed and kept aside for 15 min. Finally, 1 ml of distilled water was added. The blue color was measured against a reagent blank at 725 nm. The total phenolic content was expressed in milligrams of gallic acid equivalents (GAE) per gram of samples.

The total flavonoid content of the extracts was determined via the colorimetric method as described by Park et al. (1997). An aliquot of 0.2 ml was added to test tubes containing 0.1 ml of 10% aluminum nitrate (w/v), 0.1 ml of 1 M potassium acetate and 4.6 ml of 80% ethanol. After 40 min at room temperature, the absorbance was determined at a wavelength of 415 nm. The total flavonoid contents of the sample were determined by comparison with the optical density values of different concentrations of a standard flavonoid compound, quercetin. This analysis for each sample was analyzed in triplicate and a calibration curve of quercetin was plotted by plotting the absorbance vs. the concentration of quercetin.

Inhibition of α-glucosidase

α-Glucosidase (50 µl, 0.5 U/ml) and 0.2 M potassium phosphate buffer (pH 6.8, 50 µl) was mixed with test sample (50 µl, 10, 50 and 100 ppm). After incubation at 37°C for 15 min, 3 mM *p*NPG (100 µl) was added. The reaction was incubated again at 37°C for 10 min and then stopped by 0.1 M Na_2CO_3 (750 µl). The absorption of 4-nitrophenol was measured at 405 nm. The reaction mixture without sample was used as a control and the mixture without substrate was used as a blank. The experiment was performed in triplicate. The percent inhibition of α-glucosidase was calculated as follows:

Inhibition rate (%) = {1-(Abs sample-Abs blank)/ Abs control} ×100

Where Abs sample is the absorbance of the experimental sample, Abs blank is the absorbance of the blank and Abs control is the absorbance of the control.

Anti-microbial activity test

The Gram-positive bacteria *Bacillus subtilis* (KTCT 1021) and *Staphylococcus aureus* (KTCT 1916), the Gram-negative bacteria *Escherichia coli* (KTCT 1924) and the yeast *Candida albicans* (KTCT 7965) and *Pichia jadinii* (KTCT 7293) were obtained from the Korean collection for type cultures (KCTC), Korea. The microorganisms were grown in liquid media (micrococcus, nutrient, and YM media) at a suitable temperature (25, 26, 30 or 37°C).

Extract, fractions and isolated compounds were screened for anti-microbial activity using a serial two-fold dilution assay. Inocula were prepared from 12 h broth cultures of the bacterial and yeast

strains, then diluted 100-fold with nutrient broth (Kobayashi et al., 1993). Compound samples were serially diluted with sterile nutrient broth medium to obtain the desired concentrations (7.8, 15.6, 31.3, 62.5, 125, 250, 500 and 1,000 µg/ml). Briefly, 96-well plates were prepared by dispensing 180 µl of the diluted inocula into the first row of wells and 100 µl into the remaining wells. Aliquots of 20 µl of the stock solution of each sample (prepared at a concentration of 10 mg/ml) were added to the first row and 100 µl was serially diluted into the remaining wells. The final volume in each well was 100 µl. The contents of each well were gently mixed using a micropipette for 20 s, followed by incubation at the appropriate temperature for 24 h. The minimum inhibitory concentration (MIC) of each sample was defined as the lowest concentration that inhibited microorganism growth. Microorganism growth was evaluated visually based on the degree of turbidity for bacteria and yeast. A control experiment was performed in parallel to examine the effects of the solvent itself (without sample) and standard drugs (tetracycline and ketoconazole) on the growth of the six test organisms.

Statistical analysis

All data were expressed as mean value ± standard deviation (SD) of the number of experiments (n = 3). Significant differences for multiple comparisons were determined using one-way analysis of variance (ANOVA). Duncan's multiple range tests was used to assess the significant differences using SPSS statistical analysis. Differences at $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Anti-oxidant properties of *R. fasciculatum*

Since several epidemiological studies have suggested the importance of high consumption of fruits and vegetables as a counterpart to oxidative stress, many plants have been identified as having potential anti-oxidant activities (Katalinic et al., 2006). Scientific evidences strongly suggest that anti-oxidants from plants reduce the risk of chronic diseases like cancer (Schlesier et al., 2002), indicating that free radical scavenger activities of plants are the main factors responsible for the observed efficacy in reducing chronic diseases.

To investigate the biological activities of *R. fasciculatum*, we initially analyzed the free radical scavenger activities of *R. fasciculatum* extracts using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method. Dried *R. fasciculatum* stem (1 kg) obtained from two regions, Jeju and Chuncheon in South Korea, were extracted with MeOH and these extracts were incubated with DPPH-MeOH solution. As shown in Figure 2, Jeju extract showed stronger anti-oxidant activity compared to those of Chuncheon at 1 mg/ml (55 and 19%, respectively). At a concentration of 1 mg/ml, anti-oxidant activities of solvent fractionated Jeju-MeOH extract were determined to be in following order: EtOAc fraction>BuoH> hexane fraction> aqueos fraction (95, 61, 23 and 22% respectively). Especially, EtOAc fraction of Jeju-MeOH extract showed stronger anti-oxidant activity (95% at 1 mg/ml) compared to BHT (24%) and similar activity compared to that of BHA (93%) or α-tocopherol (96%).

To further characterize the anti-oxidant properties of the extracts, we tested the anti-oxidant capacities of different fractions using ferric-reducing ability power (FRAP) assay. When 1, 5 and 10 mg/ml Jeju-MeOH extract were incubated with potassium ferricyanide, the reducing ability ranged from 0.4 to 1.9 OD_{700}, whereas Chuncheon-MeOH extract displayed less differences in its reducing power values, which ranged from 0.2 to 1.1 OD_{700} (Figure 3). In addition, EtOAc fraction (OD_{700} = 2.6) of Jeju-MeOH extract exhibited stronger activity than BHT (OD_{700} = 2.4), although α-tocopherol and BHA showed higher activity compared to that of the EtOAc fraction.

Phenolic compounds including polyphenols possess ideal structural chemistry for free radical-scavenging activities (Rice-Evans et al., 1997). Accumulated experimental evidences by *in vitro* tests demonstrated that these compounds have more effective anti-oxidant efficacy than vitamin C and E (Rice-Evans et al., 1996; Cai et al., 2004), indicating that phenolic compounds have a major contribution to anti-oxidant activity. Therefore, we suppose that the diversity of anti-oxidant activity between the Jeju and Chuncheon samples may have contributed to the difference in total phenolic content in the extracts. Consequently, the relationship between anti-oxidant activity and total phenolics was investigated. The total phenolic contents of Chuncheon and Jeju were 0.74 and 1.01 mg GAE/g, respectively. The EtOAc fraction had higher total phenolic content in each sample (Chuncheon, 1.20 and Jeju, 4.54). The results were highly consistent with the EDA and reducing power activity results and demonstrated that the EtOAc fraction had higher total flavonoid content equal to 0.15 and 0.58 mg of QE/g, respectively. Total flavonoid levels were determined to be in following order: EtOAc fraction > hexane fraction > methanolic extract > BuOH fraction > aqueous fraction (Table 1). These findings, together with the anti-oxidant results, suggest that phenolic compounds act as major anti-oxidants in *R. fasciculatum*.

Although *R. fasciculatum* is widely cultivated from the far-southern area (Jeju) to mid-north area (Chuncheon) (Figure 1), the different anti-oxidant activities and phenolic compounds of the *R. fasciculatum* samples obtained from Chuncheon and Jeju were most likely due to regional and climate differences due to temperature, soil composition, etc. In fact, the differences in root yield and contents of active compounds between *Bulpeurum falcatum* L cultivated in two regions, Chuncheon and Jeju, have been reported (Kim et al., 2005). In addition, climate differences cause a variety of anti-oxidant

Figure 1. Map of South Korea showing collection sites of *Ribes fasciculatum* var. for various biological activity analyses.

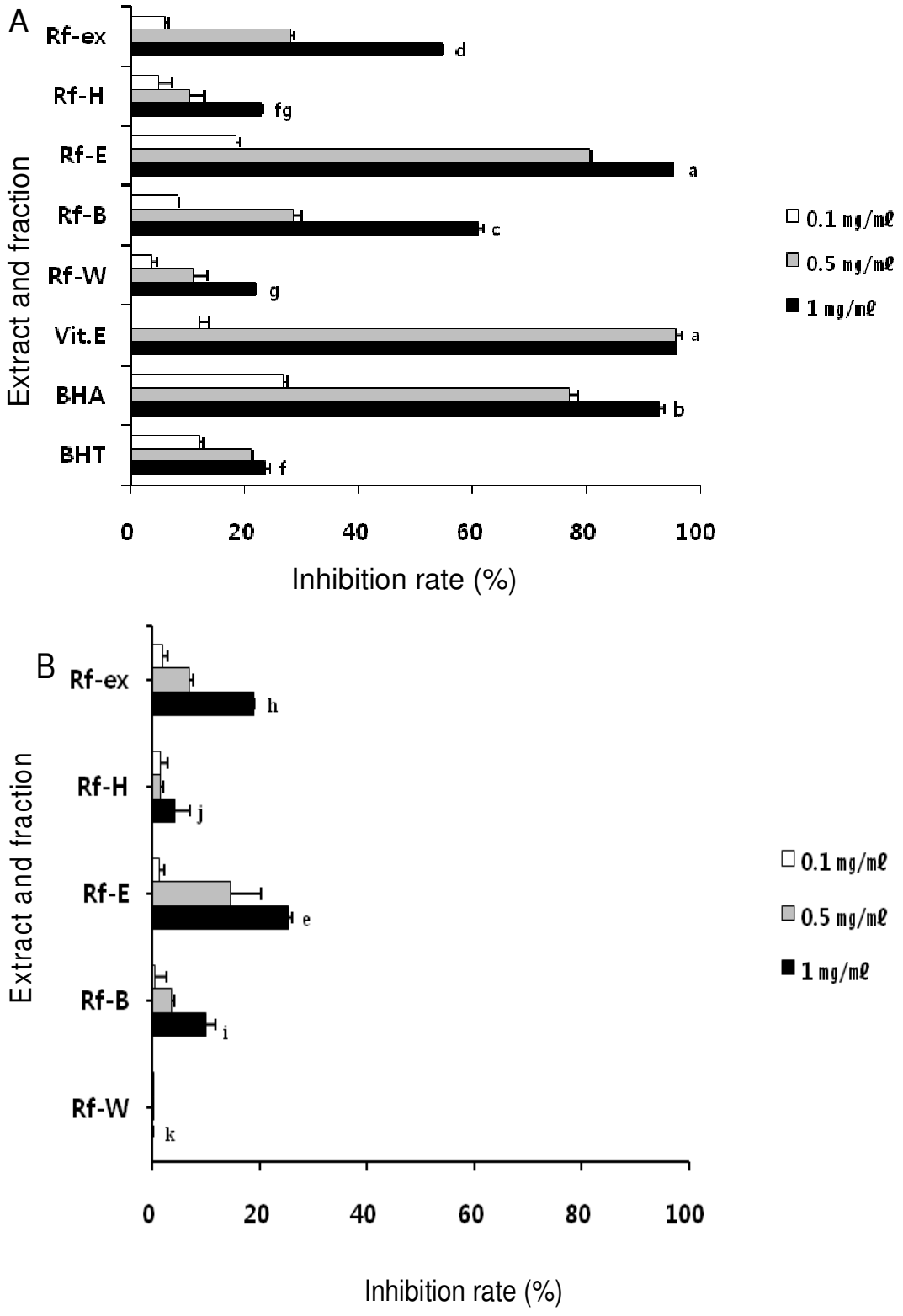

Figure 2. Electron donationg ability of extracts and fractions from *Ribes fascisulatum* var. in Jeju (A) and Chuncheon (B) areas. Rf-ex., methanolic extract; Rf-H., n-Hexane fraction; Rf-E., EtOA fraction; Rf-B., BuOH fraction; Rf-W., aqueous fraction; Vit. E, α-tocopherol; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene.

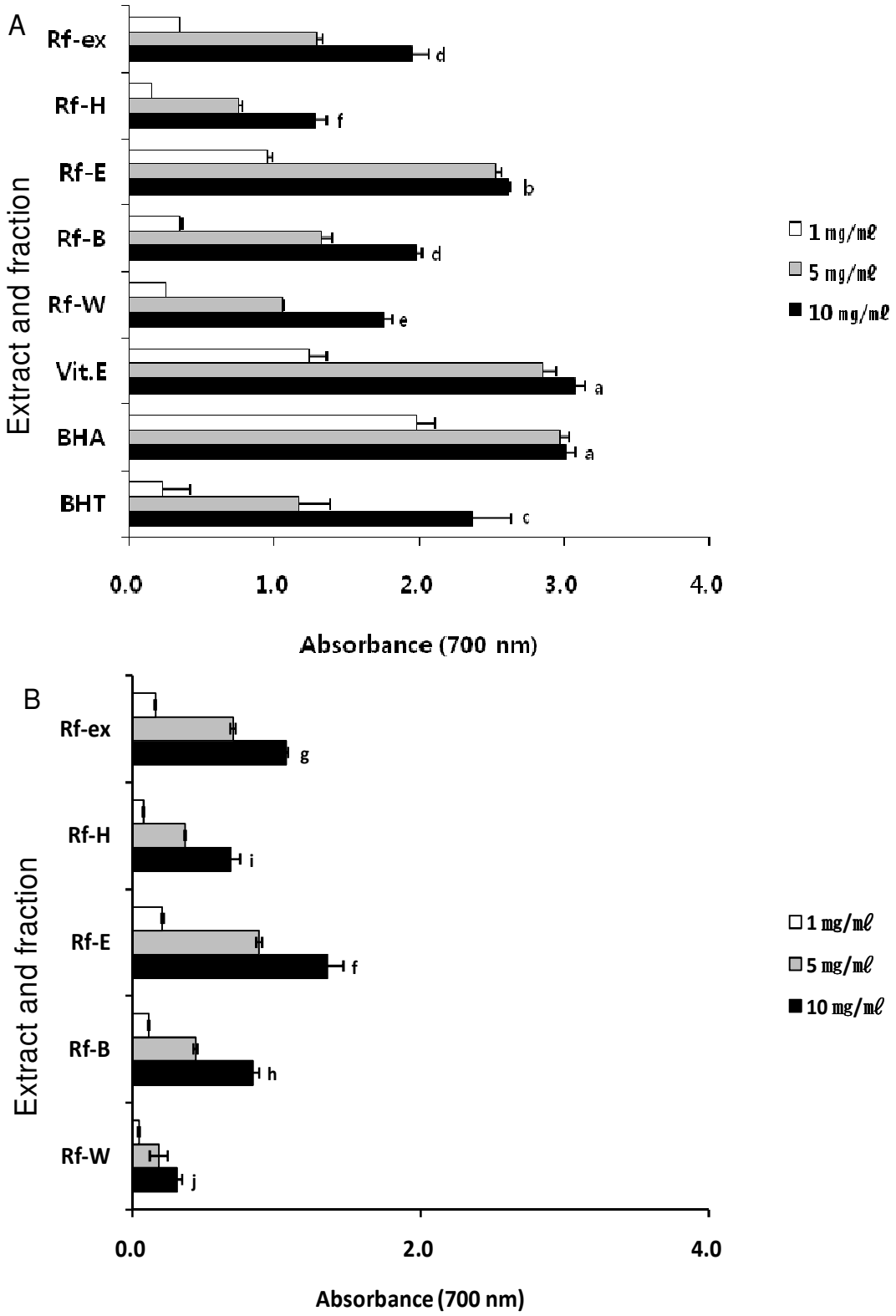

Figure 3. Reducing power of extracts and fractions from *Ribes fascisulatum* var. in Jeju (A) and Chuncheon(B) areas. Rf-ex., methanolic extract; Rf-H., n-Hexane fraction; Rf-E., EtOAc fraction; Rf-B., BuOH fraction; Rf-W., aqueous fraction; Vit. E, α-tocopherol; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene.

Table 1. Total phenolic content and total flavonoid content of the extracts and fractions from *Ribes fascisulatum* var. in Jeju and Chuncheon areas.

Cultivating area	Extract and fractions	TPC[1)] (mg GAE/g)	TFC[2)] (mg QE/g)
Jeju	Rf-ex[3)]	1.01 b[4)]	0.11[cd]
	Rf-H	0.25[ef]	0.30[b]
	Rf-E	4.54[a]	0.58 [a]
	Rf-B	1.18[b]	0.03[de]
	Rf-W	0.37[e]	0.01[e]
Chuncheon	Rf-ex	0.74[c]	0.11[cd]
	Rf-H	0.26[ef]	0.12[c]
	Rf-E	1.20[b]	0.15[c]
	Rf-B	0.53[d]	0.03[c]
	Rf-W	0.19[f]	0.01[e]

[1)] Total phenol content analyzed as gallic acid equivalent (GAE) mg/g of extract, values are the average of triplicates. [2)] Total flavonoid content analyzed as quercetin equivalent (QE) mg/g of extract, values are the average of triplicates. [3)] Rf-ex, methanolic extract; Rf-H, n-hexane fraction; Rf-E, EtOAc fraction; Rf-B, BuOH fraction; Rf-W, aqueous fraction. [4)] Mean separation within.

Table 2. α-Glucosidase inhibition activities of extracts and fractions from *Ribes fascisulatum* var. in Jeju and Chuncheon areas.

Extract and fractions	IC_{50}[1)](μg/ml)	
	Jeju	Chuncheon
Rf-ex[2)]	7.18[a3)]	5.51[a]
Rf-H	20.26[a]	10.06[a]
Rf-E	6.33[a]	4.38[a]
Rf-B	14.22[a]	65.5[b]
Rf-W	9.42[a]	5362.13[c]
Acarbose	0.0069 ± 0.0	

[1)] Amount required for 50% reduction of α-glucosidase. [2)] Rf-ex, methanolic extract; Rf-H, n-hexane fraction; Rf-E, EtOAc fraction; Rf-B, BuOH fraction; Rf-W, aqueous fraction. [3)] Mean separation within columns by Duncan's multiple range test, $P<0.05$.

capacities and phenolic level in wines (Mitic et al., 2010).

Inhibitory effects of *R. fasciculatum* on α-glucosidase activity

The potential of polyphenolic compounds, which have the ability to bind with carbohydrate hydrolyzing enzymes, have long been recognized as a good source of anti-diabetic agents (McDougall and Stewart, 2005). In fact, many plant polyphenols show inhibitory effects on α-glucosidase and α-amylase activities, which are targets of current drugs for the treatment of type 2 diabetes (McDougall and Stewart, 2005; Onal et al., 2005; Mai et al., 2007; Kim et al., 2011). The presence of different levels of total phenolics in the Chuncheon- and Jeju-MeOH extracts might indicate that both extracts contained different levels of anti-diabetic activities. To support this supposition, the extracts and fractions of *R. fasciculatum* were tested for α-glucosidase inhibitory activity (Table 2). The EtOAc fractions from the Chuncheon- and Jeju-MeOH extracts showed higher α-glucosidase inhibitory effects than the other samples (IC_{50} = 5.51 to 5362.13 μg/ml). Although Chuncheon-MeOH extracts and its fractions contained low levels of phenolic contents compared to those of the Jeju-MeOH extracts and fractions, the methanolic extract, hexane fraction and EtOAc fraction of Chuncheon-MeOH extracts exhibited stronger α-glucosidase inhibitory effects than those of Jeju. This result suggests that phenolic compounds did not act as a major inhibitor of α-glucosidase in *R. fasciculatum*, indicating that *R. fasciculatum* might be

Table 3. Antimicrobial activity of extracts and fractions from *Ribes fascisulatum* var. in Jeju and Chuncheon areas.

Cultivating area	Extract and fractions	MIC[1] (μg/ml)						
		Bacteria strain					Yeast strain	
		B. s.[2](+)	S. a.[2](+)	E. c.[2](-)	S. t.[2](-)	K. p.[2](-)	C. a.[2]	P. j.[2]
Jeju	Rf-ex[3]	1000	>1000	>1000	>1000	>1000	250	500
	Rf-H	500	>1000	1000	>1000	>1000	250	500
	Rf-E	500	>1000	1000	1000	1000	250	500
	Rf-B	>1000	>1000	>1000	1000	>1000	250	500
	Rf-W	>1000	>1000	>1000	>1000	>1000	500	1000
Chuncheon	Rf-ex	500	500	125	>1000	>1000	500	250
	Rf-H	500	125	63	>1000	>1000	500	250
	Rf-E	500	500	125	>1000	>1000	500	250
	Rf-B	>1000	>1000	>1000	>1000	1000	1000	500
	Rf-W	>1000	>1000	>1000	>1000	>1000	>1000	>1000
Ketoconazole							250	250
Tetracycline		8	8	8	8	8		

[1] MIC values against bacteria and yeast were determined by the serial two-fold dilution method. The growth of the bacteria and yeast were evaluated based on the degree of turbidity of the culture using the naked eye. [2] S. a.: *Staphylococus aureus* KCTC 1916, B. s.: *Bacillus subtilis* KCTC 1021, K. p.: *Klebsiella pneumonia* KCTC 2208, E. c.: *Escherichia coli* KCTC 1924, S. t.: *Salmonella typhimurium* KCTC 1925, P. j.: *Pichia jadinii* KCTC 7293, C. a.: *Candida albicans* KCTC 7965. [3] Rf-ex, methanolic extract; Rf-H, n-hexane fraction; Rf-E, EtOAc fraction; Rf-B, BuOH fraction; Rf-W, aqueous fraction.

good source for developing non-phenolic anti-diabetic agents.

Anti-microbial activities of *R. fasciculatum*

We also examined the anti-microbial properties of the *R. fasciculatum* extracts and fractions against micro-organisms involved in food poisoning or infections disease (Grimoud et al., 2003; Hauge, 1955; Pothakamurya et al., 1995; Tsolis et al., 1999) by determining the minimal inhibitory concentration (MIC) (Table 3). Hexane fraction of Chuncheon-MeOH extracts showed the highest anti-microbial activity against the Gram-negative bacterium *E. coli* (MIC = 63 μg/ml), followed by the Gram-positive bacterium *S. aureus* (MIC = 125 μg/ml). In yeast strain, Jeju-MeOH extracts and its fractions displayed higher anti-fungal activities against *C. albicans* compared to the Chuncheon-MeOH extracts, whereas Chuncheon-MeOH extracts and its fractions showed higher anti-fungal activities than those of Jeju-MeOH extracts against *P. jadinii*. In addition, Jeju samples against *C. albicans* and Chuncheon samples against *P. jadinii* showed similar activities compared to the anti-fungal drug ketoconazole (MIC = 250 μg/ml).

Conclusion

Over the last two decades, numerous natural products from traditional medicinal plants have been introduced and their potential as therapeutic agents demonstrated. In this study, we have introduced and determined the biological activities of *R. fasciculatum*. The extract of *R. fasciculatum* stem exhibited anti-oxidant and anti-microbial activities as well as inhibitory effects on α-glucosidase activity. Interestingly, samples obtained from two regions, Chuncheon and Jeju in South Korea, showed different biological activities, most likely due to regional and climate differences. Although, the identification of active compounds from *R. fasciculatum* is required, our results further support the view that *R. fasciculatum* is a promising source of dietary health supplements due to its pharmacological functions. In addition, identification of the α-glucosidase inhibitor in *R. fasciculatum* is absolutely necessary and significant.

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