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Full Length Research Paper

Acetylcholineterase inhibitory and antioxidant properties of *Rhododendron yedoense* var. *Poukhanense* bark

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The aim of the present study was to evaluate the antiacetylcholinesterase and antioxidant potential of 80% methanolic extract of *Rhododendron yedoense* var. *poukhanense* bark (RYBE). The extract was rich in total phenolic content (160 mg gallic acid equivalent/g of dry extract) and possessed flavonoids (2.2 mg quercetin equivalent/g of dry extract). The acetylcholinesterase (AChE) inhibitory capacity of RYBE was determined against electric eel AChE enzyme. The extract exhibited substantial inhibitory activity with IC_{50} value of 169.01 µg/ml. In addition, the extract revealed its strong antioxidant activity by various *in vitro* assay systems including 1,1-diphenyl-2-picrylhydrazyl, 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid), ferrous ion chelating and nitric oxide scavenging assays. The reducing power of RYBE was also determined by ferric reducing antioxidant power assay. Furthermore, this extract exhibited dose dependent inhibition of lipid peroxidation in rat liver homogenate. In conclusion, due to considerable AChE inhibitory and potent antioxidant nature of RYBE, further *in vivo* studies along with structural elucidation to find the active ingredient(s) will prove useful in searching an effective treatment for Alzheimer's disease.

Key words: Acetylcholinesterase, antioxidant, Ferric (Fe^{3+}) reducing antioxidant power (FRAP), free radical, *Rhododendron yedoense* var. *Poukhanense* bark, total flavonoid content, total phenolic content.

INTRODUCTION

Recently, the number of citizens suffering from neurological disorders has advanced universally, particularly in the developed nations. Within them, neurodegenerative diseases such as Parkinson, Alzheimer, as well as psychiatric ones like anxiety and depression are the most prevalent (López et al., 2009).. Alzheimer's disease (AD) is the most common mental disability called dementia, which is characterized by decline in cognitive function and mental atrophy, because of substantial and progressive loss of neurons from diverse regions of the brain (Suganthy et al., 2010).

Inhibition of AChE, the fundamental enzyme in the breakdown of acetylcholine by terminating nerve impulse transmission at the cholinergic synapses, is considered as a promising strategy for the treatment of AD (Mukherjee et al., 2007). Almost all known synthetic cholinesterase inhibiting drugs used in the treatment of AD reported several drawbacks, such as hepatotoxicity, short duration of biological action, low bioavailability, adverse cholinergic side effects in the periphery and narrow therapeutic windows.

Therefore, in current scenario, to investigate the active and safe natural cholinesterase inhibitors is a prime focus (Hung et al., 2008). Considerable studies have published anticholinesterase efficacy of the plant extracts, among

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them a few herbal extracts proved to act on the central nervous system (Ahmed and Urooj, 2010). Oxidative stress is directly related to neurodegenerative diseases, therefore the antioxidant potentials of various extracts can be helpful to provide neuroprotection. Free radicals are highly reactive molecules derived from oxygen and produced by metabolic processes and several external factors (López et al., 2009). *Rhododendron* species are common garden plants with glossy, evergreen leaves and large, showy flower displays (Carballeira et al., 2008). Toxic honey from many of these species is used in The Black Sea Region as an alternative medicine for the treatment of several disorders (Silici et al., 2010). *Rhododendron yedoense* var. *poukhanense* (*R. yedoense*) family Ericaceae, grows widely on the sunny-side of mountains in all regions of Korea (Jung et al., 2007).

It is evergreen in mild winters, but deciduous in cold winter climates. It has been reported that the flowers of *R. yedoense* possess very good antioxidant activity (Jung et al., 2007), but, other parts of this plant are not evaluated for any of the biological activities. Therefore, in the present study, we evaluated the antiacetylcholinesterase and antioxidant effects of *R. yedoense* bark.

MATERIALS AND METHODS

Plant material

The dried bark of *R. yedoense* (RYB) was obtained from "Korean Collection of Herbal Extracts", a Biotech company in Korea. A voucher specimen is available from the company (Korea Collection of Herbal Extracts, 2000).

Preparation of extracts

The dried bark of *R. yedoense* (100 g, dry weight) was extensively extracted with 80% aqueous methanol at room temperature for 3 days and filtered. The extract was concentrated using rotary vacuum evaporator to give a residue, which was freeze-dried to obtain a dry powder (4.3 g) and stored in a glass bottle at 4˚C until used. For evaluating the extract through various *in vitro* assays, the powder was first dissolved in methanol to obtain RYB methanolic extract (RYBE) with different concentrations depending on the study type.

Chemicals

AChE (type VI-S from electric eel), acetylthiocholine iodide (ATCI), 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium persulfate, ferrozine, ferrous sulfate, potassium ferricyanide, trichloroacetic acid, ferric chloride, sodium nitroprusside, Griess reagent, L-ascorbic acid, Folin-Ciocalteu reagent, gallic acid, citric acid, aluminium chloride and quercetin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Other commercially available reagents and solvents were used as received.

Determination of total phenolic content (TPC)

TPC of the extract was determined using Folin-Ciocalteu assay as described by Sancheti et al. (2010a). TPC was expressed as gallic acid equivalent (GAE) in mg per gram dry extract.

Determination of total flavonoids content (TFC)

TFC of the extract was determined using aluminium chloride colorimetric assay as described by Chang et al. (2002) with minor modifications. In this, 10 µl of extract, 60 µl of methanol, 10 µl aluminium chloride (10% w/v), 10 µl of potassium acetate (1 M) and 120 µl of distilled water were mixed and incubated at room temperature for 30 min. The absorbance was measured at 415 nm. TFC was expressed as quercetin equivalent (QE) in mg per gram dry extract.

Microplate assay for AChE

The enzyme inhibition activity for AChE (from electric eel) was evaluated according to the method previously reported by Ellman et al. (1961) and modified by Sancheti et al. (2010b). RYBE inhibitory potential against AChE was determined using enzyme at a concentration of 0.03 U/ml.

DPPH free radical scavenging assay

The free radical scavenging activity of RYBE at different concentrations was measured as per the method described by Blois (1958) and little modified by Sancheti et al. (2010a). L-ascorbic acid was used as a reference standard.

ABTS radical cation decolorization assay

The ABTS radical cation decolorization assay was carried out using the method reported by Re et al. (1999) and Ling et al. (2009) with slight modifications. $ABTS^{\cdot+}$ was generated by oxidation of ABTS with potassium persulfate.. The ABTS stock solution was prepared by adding 0.0768 g of ABTS salt and 0.0132 g of potassium persulfate in 20 ml of distilled water. Stock solution was kept in dark for 12-16 h (overnight) at room temperature prior to use. The $ABTS^{\bullet+}$ solution was diluted with methanol to an absorbance of 0.700 ± 0.020 at 734 nm. After addition of 200 µl of diluted ABTS solution ($A_{734\ nm}$ = 0.700 ± 0.020) to 10 µl of the sample at various concentrations, the absorbance was read at 734 nm at 30˚C using microplate reader exactly after 6 min after initial mixing. The positive control contained 10 µl of methanol instead of test sample. L-ascorbic acid was served as a reference standard.

Ferrous ion-chelating assay

The ferrous ion chelating potential of the extract at different concentrations was investigated according to the method of Decker and Welch (1990) and Wang et al. (2009) with minor modifications. In this, 2 mM ferrous sulfate ($FeSO_4$) solution and 5 mM ferrozine solution were prepared and diluted 20 times at the time of experiment. 50 µl of diluted $FeSO_4$ and 50 µl of the extract were mixed in a microplate and the reaction was initiated by the addition of 50 µl of diluted ferrozine. The solutions were well mixed and allowed to stand at 25˚C for 10 min. After incubation, the absorbance was measured at 562 nm. Methanol was used as positive control instead of sample. Distilled water was used as blank instead of ferrozine, which was used for error correction. Citric acid

was used as reference standard.

Nitric oxide scavenging assay

The nitric oxide (NO) scavenging activity of the extract was measured according to the method described by Ho et al. (2010). This method consisted addition of 50 µl of sample solution with 50 µl of 10 mM sodium nitroprusside solution into a 96-well flat-bottomed plate and the plate was incubated under light at room temperature for 90 min. Finally, an equal volume of Griess reagent (1% of sulphanilamide and 0.1% of naphthylethylenediamine in 2.5% HPO_3) was added to each well to measure the nitrite content immediately at 546 nm. L-ascorbic acid was evaluated as a reference standard.

Ferric (Fe^{3+}) reducing antioxidant power (FRAP) assay

The reducing power of the RYBE was determined according to the method described by Sancheti et al. (2010b). L-ascorbic acid was taken as a reference standard.

Determination of lipid peroxidation

In this, male Wistar rats were fasted overnight and anaesthetized using diethyl ether. Their livers were quickly removed and cut into small pieces and homogenized in phosphate buffer (50 mM, pH 7.4), to give a 10% w/v liver homogenates. Each homogenate was then centrifuged at 5000g for 15 min at 4°C. The oxidant pair Fe^{2+}/ascorbate was used to induce oxidative stress in the rat liver homogenate (Ardestani and Yazdanparast, 2007; Bahramikia et al., 2009). The reaction mixture was composed of 0.5 ml of each liver homogenate, 0.9 ml phosphate buffer (50 mM, pH 7.4), 0.25 ml $FeSO_4$ (0.01 mM), 0.25 ml ascorbic acid (0.1 mM) and 0.1 ml of different concentrations of RYBE. The reaction mixture was incubated for 30 min at 37°C.

The extent of lipid peroxidation of the rat liver homogenate in the presence and absence of RYBE was evaluated by measuring the product of thiobarbituric acid reactive substances (TBARS) using the Cayman's TBARS assay kit (Seoul, Korea). The MDA (malondialdehyde)-TBA adduct formed by the reaction of MDA and TBA under high temperature and acidic conditions was measured colorimetrically at 532 nm. The amount of TBARS formed was calculated using the MDA standard curve. Quercetin was used as a reference standard.

Statistical analysis

All assays were performed at least three times with triplicate samples. All results are expressed as mean ± SD. IC_{50} values were determined by plotting a percent inhibition versus concentration curve for all the assays (excluding FRAP and lipid peroxidation), in which the concentration of sample required for 50% inhibition was determined and expressed as IC_{50} value.

RESULTS AND DISCUSSION

Total phenolic and flavonoid content

In vitro assays for TPC and TFC revealed that, RYBE contained high amounts of polyphenolic compounds (160 mg gallic acid equivalent/g of dry extract) and considerable amounts of flavonoids (2.2 mg quercetin equivalent/g of dry extract). Most of the antioxidant potential of medicinal plants is due to the redox properties of phenolic compounds, which enable them to act as reducing agents, hydrogen donors and singlet oxygen scavengers (Moyo et al., 2010). In addition, flavonoids act through scavenging or chelation. Therefore, they generally possess good antioxidant potential and their effects on human nutrition and health are considerable. Furthermore, flavonoids and other phenolic compounds have been shown to possess anticholinesterase properties (Orhan et al., 2007; Ji and Zhang, 2008).

AChE inhibitory activity

One of the processes to alleviate the symptoms of AD is to inhibit AChE, which catalyses the hydrolysis of the neurotransmitter, acetylcholine. Several AChE inhibitors (such as tacrine, donepezil, rivastigmine and galanthamine) were used for the treatment of AD, but, maximum of them show hepatotoxicity and gastrointestinal disturbances. Therefore, the search for new AChE inhibitors is of great interest for AD treatment (Sacan and Yanardag, 2010). In this study, RYBE exhibited considerable acetylcholinesterase inhibitory potential against electric eel AChE. The optimal concentration of RYBE required for the 50% inhibition (IC_{50}) against AChE was 169.01 µg/ml. This assay presented dose dependent inhibition (Figure 1).

The results elucidated that, the inhibitory potential of RYBE against AChE was comparatively lower. This is because crude extracts contain non-active components along with the active ones, therefore to isolate the active compound(s) from this plant will help identify the potent natural inhibitor(s) of AChE, in turn helping prevent and/or treat AD.

Antioxidant activities

It is well known that free radicals are one of the causes of several diseases, such as Parkinson's disease, Alzheimer type dementia etc (Sacan and Yanardag, 2010). Also, AD nowadays is associated with a process of inflammation, in which free radicals are one of the causes of an inflammatory process (Hernandez et al., 2010). Therefore, ameliorating oxidative stress through treatment with antioxidants is an effective strategy for reducing neurological complications (Sacan and Yanardag, 2010). Based on this assumption, in the present study, we evaluated a detailed antioxidant potential of RYBE with various antioxidant assay systems. The model of scavenging the stable DPPH radical is a widely used method to evaluate antioxidant activity. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable

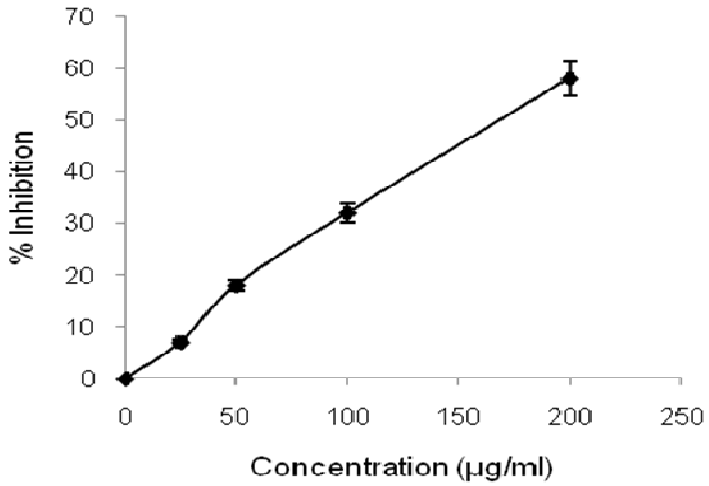

Figure 1. Acetylcholinesterase inhibitory effect of RYBE (25 to 200 µg/ml). Each value is mean of three observations in triplicate. IC_{50} value of RYBE = 169.01 µg/ml.

Table 1. DPPH radical scavenging activity of RYBE

Sample	Concentration (µg/ml)	% Inhibition	IC50 value (µg/ml)
RYBE	14.20	27.8 ± 0.21	31.92
	28.41	45.6 ± 0.82	
	56.82	82.6 ± 0.54	
	113.64	90.3 ± 0.43	
	227.28	95.5 ± 0.87	
L-Ascorbic acid			7.55

diamagnetic molecule (Sacan and Yanardag, 2010). The amount of DPPH reduced could be quantified by measuring a decrease in absorbance at 517 nm. The dose response relationship of DPPH radical scavenging activity of the extract demonstrated that it is capable of scavenging DPPH radical in a concentration-dependent manner (Table 1).

This potent activity was might be due to the presence of phenolic compounds in the RYBE. Conclusively, a strong antiradical activity was undoubtedly confirmed by the DPPH assay; therefore, the extract has been proven able to prevent the initiation of free radical-mediated chain reactions. ABTS assay is used for the determination of the antioxidant capacity of hydrogen donating antioxidants. $ABTS^{\cdot+}$ is a blue chromophore produced by the reaction between ABTS salt and potassium persulfate. Addition of RYBE to this pre-formed radical cation reduced it to ABTS in a concentration dependent manner (Table 2). In the literature, it has been observed that, the ABTS reducing property is directly proportional to the amount of phenolics, therefore, the potent ABTS reduction observed in this assay might be due to the phenolicscompounds present in the extract. The ferrous ion chelating assay for RYBE was estimated using ferrozine. Ferrozine can quantitatively form complex with ferrous ion. However, in the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complex is decreased. Therefore, measurement of the color reduction allows estimation of chelating activity of the coexisting chelator (Ebrahimzadeh et al., 2008). In this assay, RYBE interfered with the formation of ferrous and ferrozine complex, suggesting that it has chelating activity and captures ferrous ion before ferrozine. The results for ferrous ion chelating assay were dose dependent and revealed that RYBE has an effective capacity for ion binding, suggesting that its action as an antioxidant may be related to its ion binding capacity. The data with IC_{50} value is given in Table 3. In the reducing power assay, we investigated the ferric ion-ferrous ion transformation in the presence of RYBE. The investigated extract expressed a significant capacity to reduce Fe^{3+}, and thus, had the ability to donate electrons, which suggested that it may act as a free radical scavenger

Table 2. ABTS radical cation decolorization activity of RYBE.

Sample	Concentration (µg/ml)	% Inhibition	IC_{50} value (µg/ml)
RYBE	7.45	36.23 ± 0.45	12.5
	14.90	57.95 ± 0.63	
	29.80	92.76 ± 0.51	
	59.60	99.09 ± 0.82	
L-Ascorbic acid			6.9

Table 3. Ferrous ion-chelating activity of RYBE.

Sample	Concentration (µg/ml)	% Inhibition	IC_{50} value (µg/ml)
RYBE	52.08	6.05 ± 0.23	1080.1
	104.16	10.92 ± 0.61	
	208.33	28.52 ± 0.52	
	416.66	36.44 ± 0.91	
	833.33	45.83 ± 0.43	
	1666.66	62.98 ± 0.66	
Citric acid			9764

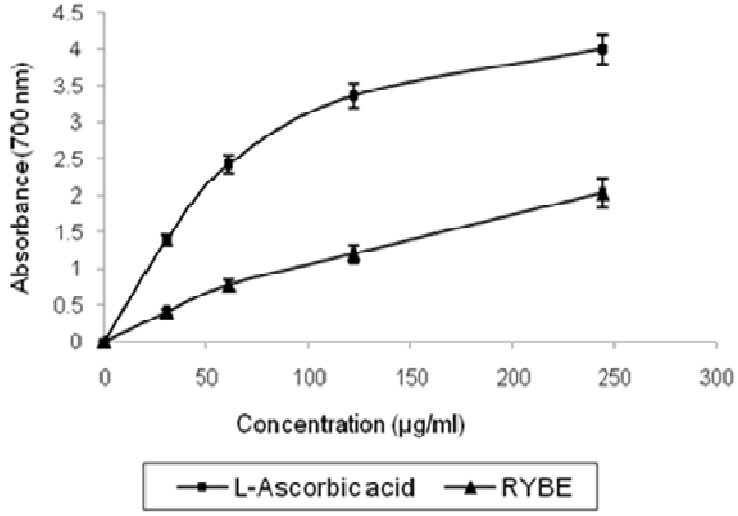

Figure 2. Reducing power of RYBE and L-ascorbic acid (50-250 µg/ml) by FRAP assay.

(Figure 2). However, the reducing power of ascorbic acid was relatively more pronounced than that of RYBE.

Nitric oxide (NO), an essential bioregulatory molecule, is required for several physiological processes like neural signal transmission, immune response, cardiovascular dilation and blood pressure. In this study, RYBE in sodium nitroprusside (SNP) solution decreased levels of nitrite, a stable oxidation product of $NO^{\cdot}$ liberated from SNP in a dose dependent manner and the data along with IC_{50} value is given in Table 4. To determine *in vitro* oxidative stress reducing capacity of RYBE, the lipid peroxidation assay was carried out, in which MDA forms a pink chromogen with TBA that absorbs at 535 nm. Incubation with Fe^{+2}–ascorbate system produced a noteworthy increase in MDA formation in rat liver microsomes (Table 5). RYBE dose dependently inhibited the amount of MDA generated and thus lipid peroxidation in liver microsomes. Therefore, it demonstrated the ability

Table 4. Nitric oxide scavenging activity of RYBE.

Sample	Concentration (µg/ml)	% Inhibition	IC_{50} value (µg/ml)
RYBE	156.25	13.22 ± 0.50	1001.8
	312.50	22.98 ± 0.33	
	625.00	37.56 ± 0.91	
	1250.00	58.67 ± 0.38	
L-ascorbic acid			220.0

Table 5. Lipid peroxidation assay using rat liver homogenate on RYBE.

Sample	Concentration (µg/ml)	MDA equivalents (µmole/liter)
Oxidative stress	--	27.56
RYBE	15.63	23.33
	31.25	19.12
	62.50	18.31
Quercetin	50	19.58

to inhibit the formation of TBA reactive species by scavenging oxygen radicals generated by Fe^{2+}/ascorbate-dependent chemistry in a concentration-dependent manner (Table 5).

In conclusion, the acetylcholinesterase inhibitory potential together with the strong antioxidant activity and ample amounts of phenolic compounds indicate RYBE as an effective treatment in the prevention and/or treatment of neurodegenerative diseases such as AD and Parkinsons's diseases. Further scrutiny regarding structural isolation of active compound(s) and specified *in vivo* tests including toxicity studies to explore the detailed pharmaceutical use of RYBE is warranted.

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