Biomedical & Pharmacology Journal Vol. **1**(1), 115-120 (2008)

Comparative study on the antioxidant activities of extracts from *Piper betle* leaves

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(Received: February 12, 2008; Accepted: April 04, 2008)

ABSTRACT

Betel (*Piper betle*) is widely grown in many parts of the Indian sub-continent and is used in several common household remedies. This study was aimed to evaluate the antioxidant activity of the extracts from leaves of *Piper betle*. The extracts of the betel leaves were prepared in water and organic solvents. The aqueous extracts were enzymatically hydrolyzed by two carbohydrate degrading enzyme (cellulase, diastase) and three protein degrading enzymes (trypsin, neutrase, alcalase) and potential antioxidant activities of the resultant aqueous extracts as well as the organic solvent extracts were evaluated using two different reactive oxygen species (ROS) scavenging assays viz. 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical assay and superoxide anion scavenging assay. The results showed that enzyme extracts as well as polar organic solvent extracts from betel leaves confirmed antioxidant activities comparable to their commercial counterparts such as BHA, BHT and á-tocopherol. Total phenolic content as well as the reducing power of the solvent extracts was found to associate with their antioxidant properties. As the organic solvent in food use may raise issues of safety, such extracts can be explored for potential non-food use. However, the enzyme extracts can be used as a potential water soluble antioxidant source in food industry.

Key words: *Piper betle*, antioxidant activity, enzymatic extract.

INTRODUCTION

Oxidative stress has been implicated in etiology of many human diseases (Halliwell and Gutteridge, 1998). Autooxidation is also a grave concern in the food industry which results in spoilage of various food products (Duthie, 1993). The use of antioxidants can reduce these problems (Lean and Mohamed, 1999). However, continuous use of synthetic antioxidants is reported to pose health hazards, resulting in the search for an alternative non-toxic and inexpensive antioxidant from natural sources (Dinesh *et al*., 2000). Betel (*Piper betle*) is an evergreen Indo-Malayan climbing or trailing shrub that has been cited in medicine as far back as two thousand years ago. Susruta Samhita, an Ayurvedic medico-treatise describes it to possess digestive and stimulant properties (Balasubramanyam *et al*., 1994). According to Hakeem Hashmi, outstanding unani physician, betel leaves have been used from ancient times as an aromatic stimulant and anti flatulent. It is useful in preventing secretion or bleeding and is an aphrodisiac. In India, betel leaves are widely used as masticatory.

Betel leaves are reported to be a potential non-toxic natural antioxidant (Dasgupta and De, 2004, Rathee *et al*., 2006, Arambewela *et al*., 2006). However, these studies have used aqueous extracts, ethanol extracts and essential oil from betel leaves for determination of the antioxidant properties. Therefore, the present study was undertaken whereby antioxidant activities of different solvent extract of betel leaves were evaluated. Also they were enzymatically hydrolyzed to release possible antioxidants present in interstitial tissues

and their potential antioxidant activities were compared. Antioxidant capability was assayed by superoxide radical scavenging activity and free radical scavenging activity, using DPPH, a stable free radical. The effects of enzymatic treatment on their antioxidant activities, total phenolic content and reducing power were also evaluated.

MATERIAL AND METHODS

Materials

Fresh betel leaves were purchased from the main market, Hisar, Haryana, India. They were rinsed carefully in fresh water and stored at -20°C. The frozen samples were lyophilized and homogenized with a grinder before extraction. Trypsin, 1, 1-diphenyl-2-picrylhydrazyl (DPPH), cellulase, Folin-Ciocalteu reagent, a-tocopherol, butylated hydroxyanisole (BHA) and butylated hydroxytoluene BHT were procured from Sigma Chemicals Co., U.S.A. Alcalase (2.4L FG) and Neutrase (0.8L) were procured from Novozymes, Denmark. Pyrogallol was procured from E-Merck, India. Diastase was procured from Loba Chemie, India. Organic solvents and all other chemicals were purchased from CDH, India were of analytical grade.

Preparation of organic solvent extracts from betel leaves

The solvent extracts of pulverized betel leaf were obtained with hexane, chloroform, acetone, methanol, acetone-water-acetic acid (AWA) mix and water, respectively. Sample (0.1g) was extracted with 5 ml solvent under agitation for 15 min at room temperature and clarified by centrifugation at 3000g for 20 min. Clear supernatant was collected and the pellet was re-extracted with same volume of solvent and clarified. Supernatants obtained were collected, combined and filtered through Whatman no 1 filter paper. Concentration of all the extracts was adjusted to 2 mg/ml.

Preparation of enzymatic extracts from betel leaves

To 0.1 g pulverized sample, 10 ml of the appropriate buffer solution (Table 1) was added and then 100 mg of the enzyme was mixed. The enzymatic reactions were performed for 24 h at the optimum temperatures to achieve an optimum degree of hydrolysis. Each sample was clarified by centrifugation at 3000g for 20 min and then filtered through Whatman no. 1 filter paper to remove the unhydrolysed residue. Concentrations of all the extracts were adjusted to 2 mg/ml and used for antioxidant assays. Preparations of commercial antioxidants (a-tocopherol, BHA and BHT at a concentration of 2 mg/ml each) were assayed together as controls.

Radical scavenging activity

Free radical scavenging activities of the extracts were determined by using a stable free radical, DPPH, according to a modified method of Blois, 1958. DPPH was prepared at the concentration of 3 X 10^{-4} M in ethanol. During the assay, 10 µl of the sample was mixed with 190 µl DPPH solution in a 96-well microtiter plate. After incubating in the dark for 30 min, absorbance was recorded at 517nm using ELISA reader. The percentage inhibition was calculated as follows:

$$\% \text{ inhibition} \frac{A_{DPPH(517)nm} - A_{sample\ (517m)}}{A_{DPPH\ (517nm)}} \times 100$$

Superoxide anion scavenging assay

Superoxide anion scavenging activities were determined by measuring the inhibition of the auto-oxidation of pyrogallol using a slightly modified method of Marklund and Marklund, 1974. A sample solution (0.3 ml) and 2. 53 ml of 50 mM Tris-Cl containing 10 mM EDTA (pH 8. 24) were added to freshly prepared 20 µl of 3 mM pyrogallol (dissolved in 10 mM HCl). The inhibition rate of pyrogallol auto-oxidation was measured at 420 nm. Absorbance of each extract was recorded at every 10 s interval for 3 min. The percentage of scavenging was determined as follows:

$$\% \text{ inhibition} \frac{A_{3min(420nm)} - A_{0min\ (420nm)}}{A_{3min(420nm)}} \times 100$$

Determination of total phenolic content

Phenolic contents were determined using a protocol similar to Heo *et al.*, 2005. Each 1 ml of extract, 1 ml 95 % ethanol, 5 ml distilled water, and 0.5 ml of 50 % Folin-Ciocalteu reagent were mixed. The mixtures were allowed to react for 5 min, then 1 ml of 5% Na_2CO_3 was added, and the mixture

was thoroughly mixed and placed in dark for 1h. Absorbance was measured at 725 nm and gallic acid standard curve was obtained for calibration of phenolic content.

Reducing power

The reducing power of extracts was determined according to the method of Oyaizu, 1986 with modifications. Extracts in phosphate buffer (0. 2 M, pH 6.6) were added to 0.5 ml potassium ferricyanide (1%), and the mixture was incubated at 50ºC for 20 min. Trichloroacetic acid solution (10%) was added to the mixture, which was then centrifuged at 600g for 10 min. The supernatant was mixed with distilled water and ferric chloride solution (0.1 %) for 10 min, and the absorbance at 700 nm was measured.

Statistical analysis

Data were analysed using the Sigma Plot 2001 for Windows version 7. 101. Values were expressed as mean ± standard error (SE).

RESULTS

Over the past several decades, plant extracts have been studied as novel sources which possess potential antioxidant activity and medicinal value. In the present study, betel leaves were subjected to various treatments and their effects on antioxidant activities were studied.

Radical scavenging activity

Free radical scavenging ability of various solvent extracts from betel leaves was evaluated as the change of absorbance caused by the reduction of DPPH radical. The percentage scavenging activity of each extract has been shown in Table 2. Significant differences in the activities among organic solvent extracts and aqueous enzymatic extracts were observed with the acetone-water-acetic acid (AWA) mix extract showing maximum activity and hexane fraction showing least inhibition. Interestingly, aqueous extract showed poor free radical scavenging effects, whereas the aqueous extracts prepared by the carbohydrate hydrolysing enzymes indicated strong free radical scavenging effects (Fig. 1A). Proteolytic treatment of aqueous extracts did not have much effect on the radical scavenging activity of the extracts, except for neutrase, where the activity levels reached around 55 %. The methanolic and AWA extracts having around 85 % scavenging activities were comparable to the commercial antioxidants such as BHA, a-tocopherol and superior to BHT. Diastase, cellulase and neutrase treated aqueous extracts were found to be inferior to the commercial antioxidants BHA, a-tocopherol but slightly superior to BHT.

Superoxide anion scavenging assay

Superoxide anion scavenging activity of the extracts was measured using the pyrogallol auto-oxidation system and the results have been expressed as inhibitory rate of the superoxide productivity. As shown in Table 2, all the organic extracts showed relatively poor activity than the commercial antioxidants while the less polar solvent (hexane and chloroform) extracts did not exhibit any activity. Yet again, all the enzymatically treated aqueous extracts showed higher activity than the untreated aqueous extract (Fig. 1B). Proteolytic treatment with trypsin showed higher superoxide anion scavenging activity than the commercial antioxidants while neutrase and alcalase treatment indicated activity that were relatively comparable to

Table 1: Optimal hydrolyzation conditions of particular enzymes

Enzyme	Optimum conditions		Buffer used	Enzyme characteristics
	pH	Temperature		
Diastase	6	50ºC	0.2 M phosphate buffer	A heat stable α-amylase
Cellulase	4.5	50ºC	0.1 N acetate buffer	Cellulose hydrolysing
Alcalase	8	50ºC	0.2 M phosphate buffer	An endo protease
Neutrase	7	50ºC	0.2 M phosphate buffer	An endo protease
Trypsin	8	50ºC	0.2 M phosphate buffer	Protease

Table 2: Antioxidant activities, total phenolic contents and reducing power of the extracts obtained from betel (*Piper betle*) leaves

Solvent extracts	% Inhibition		Total phenolics	Reducing
	DPPH	O_2^-	(mg/ml)	power (OD_{700})
Hexane	18.9750 ± 4.3156	-	0.3110 ± 0.011	0.7546 ± 0.0941
Chloroform	38.6950 ± 4.5650	-	0.3224 ± 0.0102	1.5434 ± 0.0932
Acetone	47.8250 ± 0.9200	27.7436 ± 0.5821	0.8705 ± 0.1053	2.5507 ± 0.2931
Methanol	84.8900 ± 0.0500	13.5116 ± 0.2302	1.5640 ± 0.0530	3.8737 ± 0.0872
AWA	86.7600 ± 0.0700	27.1944 ± 0.3428	1.7010 ± 0.0340	3.4051 ± 0.1732
Aqueous Untreated	15.7650 ± 0.6350	19.8208 ± 0.1900	0.1238 ± 0.031	0.9587±0.0527
Aqueous + Diastase	76.3600 ± 0.3600	24.2775 ± 0.6985	0.7380 ± 0.0410	2.7334 ± 0.1036
Aqueous + Cellulase	48.1800 ± 0.9200	25.8325 ± 0.0425	1.0886 ± 0.0393	3.3982 ± 0.1996
Aqueous + Trypsin	11.5800 ± 0.5400	41.0521 ± 0.7297	0.8628 ± 0.1034	2.4824 ± 0.1289
Aqueous + Neutrase	55.5400 ± 0.7600	35.9856 ± 1.0023	0.9372 ± 0.0732	3.5529 ± 0.0267
Aqueous + Alcalase	10.1000 ± 0.2100	26.3300 ± 0.4546	0.7600 ± 0.009	3.3781 ± 0.3303
BHA	86.7050 ± 0.3750	26.3744 ± 0.3465	-	2.8627 ± 0.0924
BHT	40.0400 ± 2.5900	31.8892 ± 0.1984	-	2.4882 ± 0.0179
a-tocopherol	88.2950 ± 0.1050	37.7772 ± 0.7268	-	3.2312 ± 0.0455

Values are mean ± S.E. of four replicates

commercial antioxidants (Fig 2). Superoxide anion scavenging activity of carbohydrate hydrolysing enzyme extracts were comparable to the commercial antioxidant BHA but were inferior to -tocopherol and BHT.

Total phenolic content and reducing power

The total phenolic content of the extracts was measured and the results were expressed in mg/ml as shown in Table 2. The total phenolic content of the extracts was found to associate with the radical scavenging activity of the extracts. Phenolic content increased with the increasing polarity of the organic solvents. In case of aqueous extract, the total phenolic content was very low, which increased significantly on enzymatic treatment. With regard to the reducing power, the increased absorbance (Fe^{+3}-Fe^{+2} transformations)

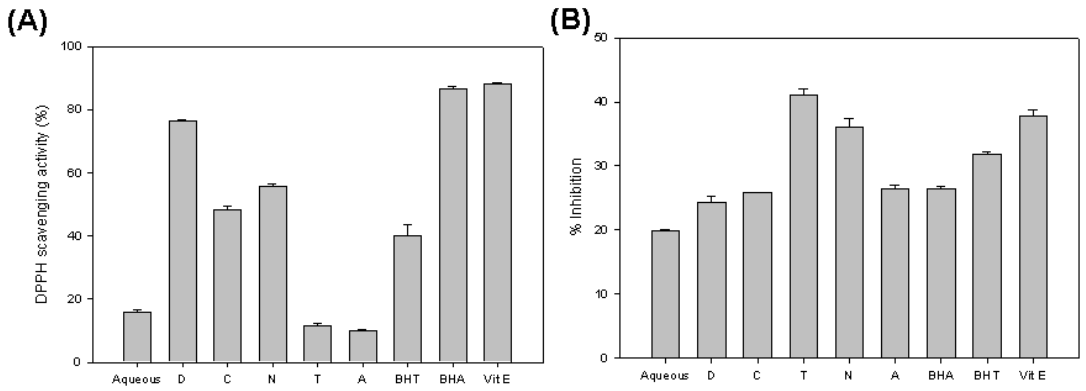

Fig. 1: (A) and (B) represent DPPH free radical scavenging activity and superoxide anion radical scavenging activity of *Piper betle* L. aqueous extract after treatment with enzymes (D-diastase, C-cellulase, T-trypsin, N- neutrase, A-alcalase)

of the reaction mix in presence of all the extracts as well as commercial antioxidants were measured spectrophotometrically at 700 nm. Higher absorbance of the reaction mixture indicated greater reducing power. The results in Table 2 indicate greater reducing power in the extracts that possessed higher antioxidant activity with the methanol extract showing highest reducing power.

DISCUSSION

The DPPH free radical scavenging activity of a compound indicates its hydrogen-donating tendency (Blois, 1958). A high correlation between DPPH radical scavenging activities and total polyphenolics has been reported by many researchers (Heo *et al*., 2005). Similarly, antioxidant activity of plant extracts is also correlated with their reducing powers (Pin-Der-Duh, 1998).

The aqueous extract of betel leaves was not found to possess good antioxidant activity compared to other solvent extracts. However, the antioxidant potential of organic solvents was found to increase with increasing polarity. Interestingly, antioxidant activity of aqueous extracts was found to increase appreciably on enzymatic treatment with neutrase and carbohydrate degrading enzymes (cellulase and diastase) suggesting degradation of proteins and carbohydrates in the tissue resulting in liberation of moieties responsible for the antioxidant activities, which may otherwise be unavailable. The increase in the total phenolic content of the aqueous extracts after enzymatic treatment might also contribute to higher antioxidant activities as it is well known that plant phenolics, in general, are highly effective free radical scavengers and antioxidants (Rathee *et al*., 2006). However, trypsin and alcalase digestion of betel leaves did not increase the antioxidant activity significantly, although they contained as much phenolic compounds as other enzymatic digests. It is thought that presence of other materials, such as small molecular weight polysaccharides, pigments, proteins or peptides, may influence the activity (Heo *et al*., 2005).

Gulcin *et al*., 2004 have reported association between reducing power and antioxidant potential of compounds. As seen in case of other analysis, reducing power of aqueous extract was seen to increase with enzymatic treatment. Proteolytic as well as carbohydrate hydrolyzing enzymes might be degrading the complex plant material thereby leading to the formation and exposure of other materials, which may influence the antioxidant activity.

Extraction with polar solvents like methanol, AWA and acetone resulted in better extraction of antioxidant compounds, with chloroform and hexane being least desirable as solvent systems. These polar solvent extracts could be used as promising antioxidants in lipid-based systems. Also, with regard to the antioxidant activities, betel leaves treated with enzymes, particularly carbohydrases, showed better scavenging effects on DPPH, superoxide and also higher reducing power and phenolic content as compared to their aqueous extract. Hence, these enzymatic extracts from betel leaves could be used as a potential water-soluble antioxidant source in the food industry. However, further studies with regard to the isolation and characterization of compounds responsible for antioxidant activity need to performed.

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