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Antioxidant activity and nitrite scavenging ability of each fractions from *Phyllostachys bambusoides* ethanolic extract

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SUMMARY

This study was conducted to investigate the efficacy of antioxidative activity and nitrite scavenging ability of each fractions from *Phyllostachys bambusoides* S. et Z. (*P. bambusoides*) trunk ethanolic extract using reverse-phase flash chromatography. Among the each fractions, fraction 3 (H_2O : MeOH = 1:1) showed high DPPH free radical scavenging activity (81.33%) at 80 μg/mL concentrations and strongly inhibited autooxidation of pyrogallol by superoxide dismutase-like activity (45.8%) at 0.46 mg/mL concentrations compared with different fractions. The fraction 3 was also increased to 76.62% cell viability against hydrogen peroxide-mediated cytotoxicity. Nitrite scavenging ability was the most remarkable under pH 1.2 condition among various pH regions examined and effectively exhibited to 65.6% by treatment of the fraction 3 with a concentration of 0.2 mg/ml. In general, nitrite scavenging ability decreased with higher pH condition. These results suggest that fraction 3 from *P. bambusoides* ethanolic extract can be used for bioacitve and functional materials.

Key words: *Phyllostachys bambusoides*; Antioxidative activity; Nitrite scavenging ability

INTRODUCTION

There is now increasing evidence to suggest that many age-related human diseases such as heart disease, cancer, inflammation, arthritis, immune system impairment and brain dysfunction are the result of cellular damage by free radicals. Antioxidants could play an important role in preventing such disease (Gao *et al.*, 1999; Carr and Frei, 2000; Perry *et al.*, 2000). Several cancer chemopreventive agents exhibit antioxidant activity through their ability to scavenging oxygen radicals, including singlet oxygen peroxyl radicals, superoxide, and hydroxyl radicals (Wei and Frenkel, 1993; Ito *et al.*, 1999). High nitrate and amine-rich food intake has been shown to result in an increased risk of endogenous formation of carcinogenic N-nitroso compounds (NOCs) (Kolb *et al.*, 1997; Vermeer *et al.*, 1998). Generally, about 80% of gastric nitrite in the normal acidic stomach arises from the reduction of ingested or endogenous nitrate (Mirvish, 1995) and the remaining 20% of gastric nitrite arises from ingested nitrite in nitrite-preserved meat and fish and other foods (National Academy of Sciences, 1981; Mirvish, 1983). Nitrite

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can subsequently react in the stomach with secondary and tertiary amine present in food to form carcinogenic NOCs (Mirvish, 1975; Hotchkiss, 1989). Recent studies suggest that diet containing phenolic compounds inhibits the formation of nitroso compounds (Cooney and Ross, 1987; Ho, 1992). Moreover, Choi *et al.* (2002) reported that consumption of Korean green tea and Maesil extracts with nitrate and amine-rich diet resulted in inhibition of endogenous nitrosamine formation in food.

There are ~280 species of bamboo within 10 genera, among which *Phyllostachys* and *Sasa albo* are well-known as edible shoots in Asia. The leaves of bamboo have been used in Asian countries as a food wrapping material to prevent food deterioration since ancient time. The leaves have been also utilized clinically in the treatment of hypertension, arteriosclerosis, cardiovascular disease, and certain forms of cancer (Shibata *et al.*, 1975). However, there have been no detailed studies carried out to date for pharmacological effect in bamboo culm.

Interestingly, we have recently reported efficacy of antioxidant activity and nitrite scavenging ability of ethanol extract of *Phyllostachys bambusoides* S. et Z (Lim *et al.*, 2004). In detail, the electron donating ability of the ethanol extract was shown at RC_{50}, 116.75 μg/ml. After addition of 0.92 mg/ml ethanol extract, autooxidation of pyrogallol was inhibited to 44% by superoxide dismutase-like activity. In the antioxidative activity of ethanol extract against linoleic acid during incubation time of 4 and 6 day at 40°C, TBA values decreased 74.76% and 58.48% by the addition of 50 mg/ml, respectively. Nitrite scavenging ability showed the most remarkable effect under pH 1.2 among various pH region examined and exhibited to 43% at the addition level of 0.2 mg/ml.

Therefore, this study was aimed at determining the antioxidant activity and nitrite scavenging ability of each fraction from bamboo (*Phyllstachys bambusoides*) culm ethanolic extract using reverse-phase flash chromatography.

MATERIALS AND METHODS

Materials

The trunks of *Phyllostachys bambusoides* S. et Z., obtained from Tae-sung food corporation and dried in the shade. Octadecyl-functionalized silica gel (C18), 1,1-diphenyl-2-picrylhydrzyl (DPPH), pyrogallol, sodium nitrite, sulfanilic acid, α-naphtylamine and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (st. Louis. MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were supplied from Gibco. Co. Monkey kidney vero cells were obtained from Korean cell line bank (KCLB). Solvents and the other chemicals were used reagent grade.

Ethanol extract

The dried and chopped trunks of *P. bambusoides* (780.0 g) were extracted with ethanol (3 × 4 L) at room temperature for 24 hours. The combined ethanol extracts were filtered, and the solvent was evaporated in vacuo. This afforded a brown gum (35.59 g, 4.6%).

Reverse-phase flash chromatography

The ethanol extract (10.0 g) was coated onto C18 (20.0 g) and packed onto a C18 column (80.0 g). This column was developed in a stepwise manner with H_2O, H_2O : MeOH mixtures, MeOH, MeOH: $CHCl_3$ mixtures, $CHCl_3$ and hexane. Details of eluent volume and fraction mass are given in Table 1 (Blunt *et al.*, 1987). Each fraction was used for assay of below.

DPPH free radical scavenging activity

Assay for DPPH free radical scavenging potential is based on the scavenging activity of stable DPPH free radicals (Chen *et al.*, 1999). Briefly, 0.6 mM DPPH solution was added to test sample dissolved in methanol. After incubation at room temperature for 30 min, absorbance was then measured at 517 nm by UV-Vis spectrophotometer and percent

inhibition was calculated.

Superoxide dismutase (SOD)-like activity

The measurement of SOD-like activity was carried out according to the method of Marklund and Marklund (Marklund and Marklund, 1974). The reaction mixture consist of 50 mM Tris-HCl containing 10 mM EDTA (pH 8.5), 7.2 mM pyrogallol and test sample was incubated at 25°C for 10 min. After the reaction was stopped by 1 N HCl, absorbance was then measured at 420 nm by UV-Vis spectrophotometer. SOD-like activity was calculated as below.

SOD-like activity (%) = 100
- [(sample OD/control OD) × 100]

Cell culture and drug treatment

Vero cells were cultured in DMEM supplemented with 10% FBS. Stock culture of exponentially grown cells were trypsinized, and plated (1×10^5 cells per well) into 24 well plates and incubated in a humidified atmosphere of 5% CO_2 at 37°C for 24 hours. Fractions 3 and 4 were then added to the wells with concentrations of 10, 20, 30, 45 and 90 μg/ml and were incubated for 10 min before the addition of hydrogen peroxide (final concentration 1.6 mM). After hydrogen peroxide treatment, the cells were incubated for 24 hours and then checked up cell viability.

Determination of cell viability

Cell viability could be quantified using MTT, which yields a blue formazan product in living cells, but not in dead cells or their lytic debris (Mossmann, 1983). The stock solution of MTT (2 mg/ml) was added at the end of incubation to a final concentration of 0.2 mg/ml and then incubated at 37°C for 4 hours. The resultant formazan product was resolved with DMSO and detected by a UV-VIS spectrophotometer at 570 nm. Cell viability was calculated as below.

Cell viability (%) = 100 [(sample OD/control without hydrogen peroxide OD) × 100]

Nitrite scavenging ability

Nitrite scavenging ability was measured according to the method of Kato *et al.* (1987). The sample added 1 mM $NaNO_2$ was adjusted to pH 1.2, 3.0 and 6.0 with 0.1 M HCl (pH 1.2) and 0.2 M citrite buffer (pH 3.0 and 6.0). Reaction solution was filled up to 10 ml with distilled water and was incubated at 37°C for 1 hr. 2% acetic acid and Griess reagent (1% sulfanilic acid:1% naphthylamine in 30% acetic acid) was added to the reaction solution. After the resulting mixture was incubated at room temperature for 15 min, absorbance was measured at 520 nm by UV-VIS spectrophotometer. The nitrite scavenging ability was calculated as below.

Nitrite scavenging ability (%) = 100 - [(sample OD/control OD) × 100]

RESULTS AND DISCUSSION

The dried and chopped *P. bambusoides* were extracted thrice with ethanol for 24 hours at room temperature. The ethanol extract from *P. bambusoides* exhibited to have two major components (R_f = 0.655, 0.845) as performing TLC fingerprint. In order to fractionate the resultant ethanol extracts, it was applied over reverse-phase flash chromatography using the solvents of H_2O, H_2O : MeOH mixtures, MeOH, MeOH : $CHCl_3$ mixtures, $CHCl_3$ and hexane (Table 1). All fractions were subjected to comparison of antioxidant activity including DPPH free radical scavenging activity, superoxide-like activity and protection effect against hydrogen peroxide-mediated cytotoxicity as well as nitrite scavenging ability.

A preliminary antioxidant activity assay revealed that the ethanol extract from *P. bambusoides* exhibited DPPH free radical scavenging activity (RC_{50} = 116.67 μg/ml, 35.54% at 80 μg/ml) and SOD-like activity of 30.41% by treatment of 0.46 mg/ml (Table 2) (Lim *et al.*, 2004). As shown in Fig. 1, DPPH free radical scavenging activity of each fractions was observed to have an order of effectiveness of Fr. 4 (H_2O:MeOH = 1:3, 81.51%) >

Table 1. C_{18} Reverse-phase flash chromatography of the ethanol extract from *P. bambusoides*

Fraction No.	Eluent	Volume (ml)	Mass (mg)
1	H_2O	90	6,260
2	H_2O : MeOH (3 : 1)	90	1,330
3	H_2O : MeOH (1 : 1)	90	760
4	H_2O : MeOH (1 : 3)	90	596
5	H_2O : MeOH (1 : 9)	90	173
6	MeOH	90	102
7	MeOH : $CHCl_3$ (3 : 1)	90	175
8	MeOH : $CHCl_3$ (1 : 1)	90	260
9	$CHCl_3$	90	267
10	Hexane	90	6.3

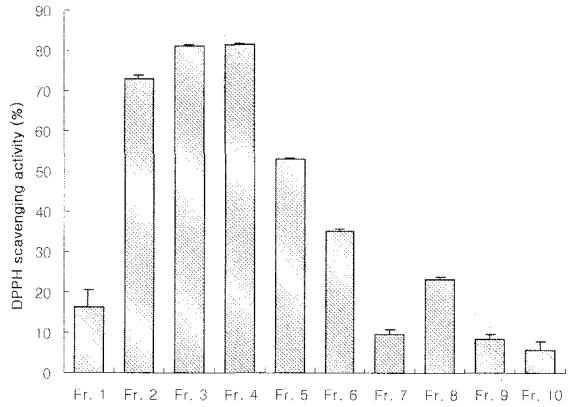

Fig. 1. DPPH scavenging activity of each fraction from *P. bambusoides* ethanolic extract. RC_{50} values of ascorbic acid and BHA as positive control showed 2.53 μg/ml and 5.62 μg/ml, respectively. Experiments were carried out at 80 μg/ml concentrations of samples as described under Materials and Methods. The values represent the mean ± standard deviations for triplicate experiments.

Fr. 3 (H_2O : MeOH = 1 : 1, 81.33%) > Fr. 2 > Fr. 5 > Fr. 6 > Fr. 8 > Fr. 1 > Fr. 7 > Fr. 9 > Fr. 10 at a concentration of 80 mg/ml. The free racical scavenging activity of fractions depends on the

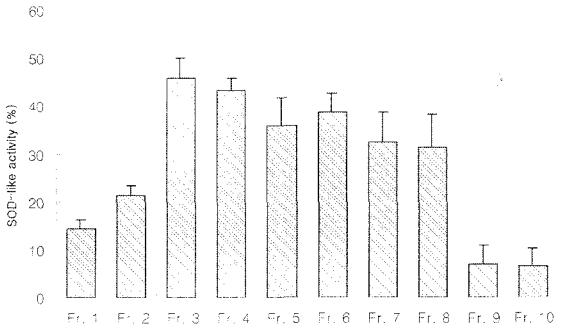

Fig. 2. SOD-like activity of each fraction from *P. bambusoides* ethanolic extract. Experiments were carried out at 0.46 mg/ml concentrations of samples as described under Materials and Methods. The values represent the mean ± standard deviations for triplicate experiments.

availability of phenolic hydrogens and on the possibility for stabilization of the resulting phenoxyl radicals formed by hydrogen donation (Silva *et al.*, 2000). The percent of superoxide dismutase represent as inhibition rate of each fractions against autooxidation of pyrogallol. The SOD-like activity of each fractions showed 45.8 - 6.6% at a concentration of 0.46 mg/ml and Fr. 3 (H_2O : MeOH = 1 : 1) exhibited the highest SOD-like activity compared with different fractions (45.8%, Fig. 2). In order to investigate the effect of Frs. 3 and 4 on cytotoxicity by hydrogen peroxide was performed MTT assay. The Fr. 3 and 4 have no cytotoxicity as cell viability of Frs. 3 and 4 exhibited more than 97.9% and 99.1% by treatment of 100 mg/ml concentrations. The results are shown in Fig. 3. Fr. 3 offered effective protection against hydrogen peroxide-mediated cytotoxicity. The strongest protection exhibited by treatment of 20 mg/ml concentrations which was increased to

Table 2. Comparison between ethanol extract and fraction 3 of ethanolic extract from *P. bambusoides* on antioxidative activity and nitrite scavenging ability

Parameters	Ethanol extract	Fraction 3
DPPH free radical scavenging activity	35.54% at 80 μg/ml	81.33% at 80 μg/ml
SOD-like activity	30.41% at 0.46 mg/ml	45.8% at 0.46 mg/ml
Nitrite scavenging ability	43.02% at 0.2 mg/ml	65.6% at 0.2 mg/ml

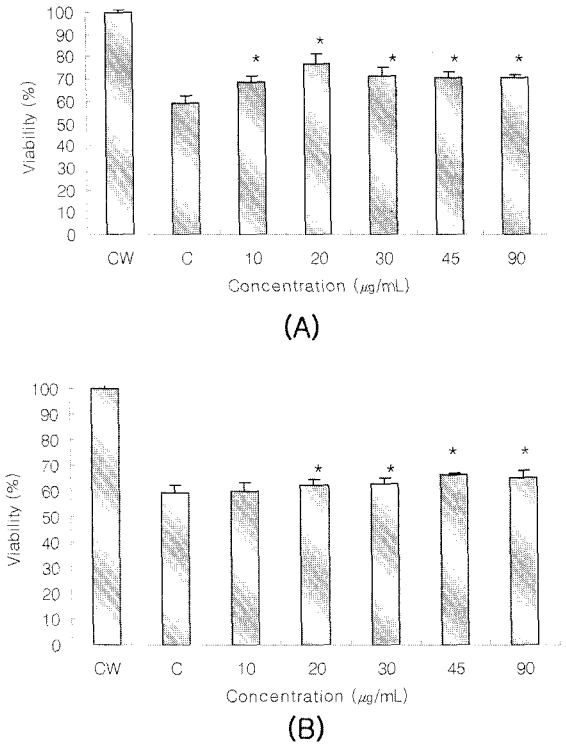

Fig. 3. The effect of the fraction 3 (A) and 4 (B) on hydrogen peroxide-mediated cytotoxicity. CW; Control without hydrogen peroxide. C; Control *Significantly different from the control values ($P < 0.05$). The values represent the mean ± standard deviation for triplicate experiments.

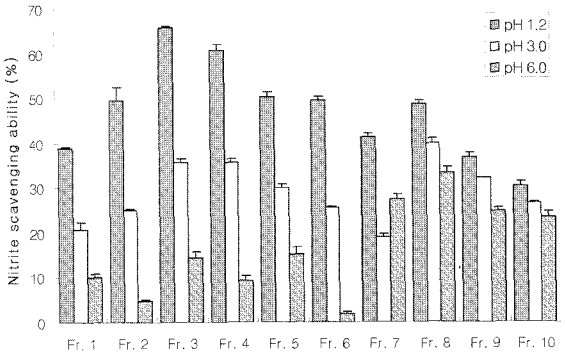

Fig. 4. Nitrite scavenging ability of each fraction from *P. bambusoides* ethanolic extract. Experiments were carried out at 0.2 mg/ml concentrations of samples as described under Materials and Methods. The values represent the mean ± standard deviations for triplicate experiments.

76.62% cell viability compared with control (59.29 %). However, this fraction was shown to similar cell viability at other concentrations. Fr. 4 showed only subtle effect. Subsequently, Fr. 3 (H_2O : MeOH = 1 : 1) was considered as superior active fraction, which have protective effect against hydrogen peroxide-mediated cytotoxicity as well as DPPH free radical scavenging activity and SOD-like activity. Hu *et al.* (2000) have recently reported that bamboo *Phyllostachys nigra* leaf extract (BLE) was shown to exhibit free radical scavenging in different model systems. They investigated the antioxidant behavior of BLE *in vitro* and found the presence of polyphenolic compound, such as caffeic acid and chlorogenic acid and luteolin 7-glucoside responsible for the antioxidant activity of BLE (Hu *et al.*, 2000). Kweon *et al.* (2001) have also reported about the antioxidant effect of the novel chlorogenic acid derivatives from bamboo (*Phyllostachys edulis*). Therefore, the Fr. 3 (H_2O : MeOH = 1 : 1), the most active fraction studied in this work seem to include similar active compounds or functional structure with bamboo leaf (BLE). Nitrite-derived nitroso-compounds (NOC) are formed in the stomach from a component of nitrite-preserved meat and fish. Nitrite scavenging ability of each fractions was examined under various pH 1.2, 3.0 and 6.0 conditions. Preliminarily, we referred that nitrite scavenging ability showed to 43.02% under pH 1.2 condition by treatment of the ethanol extract from *P. bambusoides* at a concentration of 0.2 mg/ml (Table 2) (Lim *et al.*, 2004). As result, nitrite scavenging ability of each fractions was the highest at pH 1.2 and decreased with higher pH condition (Fig. 4). Especially, Fr. 3 and 4 exhibited the most effective nitrite scavenging ability (65.6%, 60.8%) against sodium nitrite under pH 1.2 condition compared with different fractions at a concentration of 0.2 mg/ml (Fig. 4). Inhibition of NOC formation by vegetable and fruit juices was shown to be due to both ascorbic acid and the phenolic compounds (Helser and Hotchkiss, 1994; Hicks *et al.*, 1982). Tea is also useful for

demonstrating the role of polyphenols in inhibiting nitrosation (Stich, 1992; Xu *et al.*, 1993), because green and black teas are rich in polyphenols, e.g. epigallocatechin, and contain little ascorbic acid (Yang and Wang, 1993). Hence, we considered that Fr. 3 showing strong nitrite scavenging ability may be included polyphenolic compound.

In conclusion, these results proved that fraction 3 of the ethanol extract from *P. bambusoides* may be an antioxidative agent against oxidative stress and can inhibit formation of carcinogenic nitroso-compound. The separation of the main bioactive components from the fraction 3 of *P. bambusoides* ethanolic extract need to be studied further and the results will be discussed elsewhere.

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