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The Butanol Fraction of Bitter Melon (*Momordica charantia*) Scavenges Free Radicals and Attenuates Oxidative Stress

Hyun Young Kim[1], Seung Mi Sin[2], Sanghyun Lee[3], Kye Man Cho[1], and Eun Ju Cho[2]

[1]Department of Food Science, Gyeongnam National University of Science and Technology, Gyeongnam 660-758, Korea
[2]Department of Food Science and Nutrition, Pusan National University, Busan 609-735, Korea
[3]Department of Integrative Plant Science, Chung-Ang University, Gyeonggi 456-756, Korea

ABSTRACT: To investigate radical scavenging effects and protective activities of bitter melon (*Momordica charantia*) against oxidative stress, *in vitro* and a cellular system using LLC-PK_1 renal epithelial cells were used in this study. The butanol (BuOH) fraction of bitter melon scavenged 63.4% and 87.1% of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals at concentrations of 250 and 500 μg/mL, respectively. In addition, the BuOH fraction of bitter melon effectively scavenged hydroxyl radicals (·OH). At all concentrations tested, the scavenging activity of the BuOH fraction was more potent than that of the positive control, ascorbic acid. Furthermore, under the LLC-PK_1 cellular model, the cells showed a decline in viability and an increase in lipid peroxidation through oxidative stress induced by pyrogallol, a generator of superoxide anion (O_2^-). However, the BuOH fraction of bitter melon significantly and dose-dependently inhibited cytotoxicity. In addition, 3-morpholinosydnonimine (SIN-1), a generator of peroxynitrite ($ONOO^-$) formed by simultaneous releases of nitric oxide and O_2^-, caused cytotoxicity in the LLC-PK_1 cells while the BuOH fraction of bitter melon ameliorated oxidative damage induced by $ONOO^-$. These results indicate that BuOH fraction of bitter melon has protective activities against oxidative damage induced by free radicals.

Keywords: bitter melon, LLC-PK_1 cell, oxidative stress, superoxide anion, peroxynitrite

INTRODUCTION

Oxidative stress, defined as an imbalance between the production of reactive oxygen species (ROS) and antioxidant defense, is associated with a number of pathological conditions, such as inflammation, carcinogenesis, aging, atherosclerosis, and reperfusion injury (1). Overproduction of ROS as well as reactive nitrogen species (RNS) mediates damage to cell structures, nucleic acids, lipids, and proteins (2). The harmful biological effects of ROS and RNS are termed oxidative and nitrosative stress, respectively. Free radical-mediated oxidative and nitrosative stress leads to pathological conditions and has been implicated in a variety of degenerative diseases as well as in the aging process (3-6). Therefore, antioxidants that prevent free radical damage have attracted much attention, and there has been a great deal of effort to identify safe and effective therapeutic agents for oxidative stress-related diseases. Compelling evidence indicates that increased consumption of dietary antioxidants or vegetables with antioxidant properties may improve quality of life by delaying the onset and reducing the risk of degenerative diseases (7-9).

Bitter melon (*Momordica charantia*) is an indigenous medicinal and vegetable plant found in the tropical and subtropical regions of the world and is also commonly known as bitter gourd (10). Several biological effects of bitter melon have been reported such as hypoglycemic effects, anti-rheumatic, anti-inflammatory, antiseptic and anti-diabetic remedies (11,12). In addition, bitter melon also has been reported to have other medicinal properties such as anti-carcinogenic, hypocholesterolemic, anti-viral, anti-cytotoxic, hypoglycemic and anti-mutagenic properties and dissipate melancholia (13). In addition, the main constituents of bitter melon which are responsible for these biological and medicinal effects are triterpenes, proteins, steroids, alkaloids, inorganic compounds, lipids, and phenolic compounds (14). We previously reported the protective effects of the methanol extract and their four fractions from bitter melon against oxidative stress and found the butanol (BuOH) fraction has the strongest activity against oxidative stress (15).

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Correspondence to: Eun Ju Cho, Tel: +82-51-510-2837, E-mail: ejcho@pusan.ac.kr

Therefore, we investigated the radical scavenging effect of the active BuOH fraction from bitter melon on 1,1-diphenyl-2-picrylhydrazyl (DPPH) and hydroxyl (·OH) radicals by *in vitro* scavenging assays, and evaluated the protective activity against oxidative damage in a cellular system.

MATERIALS AND METHODS

Materials

M. charantia, bitter melon, was obtained from the Farming Cooperation Hamyang (Hamyang, Korea). The air-dried powdered fruit of bitter melon was extracted with methanol under reflux. The resultant extract was combined and concentrated under reduced pressure to afford the residue. The methanol extract was suspended in water and then fractionated successively with equal volumes of *n*-hexane, dichloromethane, ethyl acetate, and BuOH.

Chemicals

3-Morpholinosydnonimine (SIN-1), pyrogallol, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H* tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

DPPH radical scavenging activity

In a microwell plate, 100 μL of sample (control: 100 μL of 50% ethanol) was added to an ethanolic solution of DPPH (60 mM) according to the method described by Hatano et al. (16). For the assay, phosphate-buffered saline (PBS) was used for sample solution and three sample concentrations (100, 250 and 500 μg/mL) were prepared. After being mixed gently and left for 30 min at room temperature, the DPPH radical was measured at 540 nm using a microplate reader (model SPECTRAmax 340PC, Molecular Devices, Sunnyvale, CA, USA). Ascorbic acid was used as the DPPH-scavenging positive control compound.

$$\text{DPPH radical scavenging activity (\%)} = (Abs_c - Abs_s)/Abs_c \times 100$$

(Abs_c, Absorbance of control; Abs_s, Absorbance of sample)

·OH radical scavenging activity

The reaction mixture contained 0.45 mL of 0.2 M sodium phosphate buffer (pH 7.0), 0.15 mL of 10 mM 2-deoxyribose, 0.15 mL of 10 mM $FeSO_4$-EDTA, 0.15 mL of 10 mM H_2O_2, 0.525 mL of H_2O, and 0.075 mL of sample solution. Three sample concentrations (10, 25 and 50 μg/mL) were prepared for BuOH fraction. The reaction was initiated with the addition of H_2O_2. After incubation at 37°C for 4 hr, the reaction was stopped by adding 0.75 mL of 2.8% trichloroacetic acid and 0.75 mL of 1.0% 2-tribarbituric acid in 50 mL of NaOH. The solution was boiled for 10 min and then cooled in water at room temperature. The absorbance of the solution was measured at 520 nm. ·OH radical scavenging activity was evaluated as the inhibition rate of 2-deoxyribose oxidation by ·OH (17). Ascorbic acid was used as an ·OH radical scavenging positive control compound.

Cell culture

LLC-PK_1 porcine renal epithelial cells were provided by ATCC (Manassas, VA, USA). Delbecco's modified Eagle's medium/nutrient mixture F-12 (DMEM/F-12) and fetal bovine serum (FBS) were purchased from Hyclone (Grand Island, NY, USA) and Gibco (Cleveland, OH, USA), respectively. Commercially available LLC-PK_1 renal tubular epithelial cells were maintained in a culture flask containing 5% FBS-supplemented DMEM/F-12 medium (pH 7.2) at 37°C in a humidified atmosphere of 5% CO_2 in air. All subsequent procedures were carried out under these conditions. The cells were sub-cultured weekly with 0.05% trypsin-EDTA in PBS.

Radical generation

After confluence had been reached, the cells were plated into 96-well plates at 10^4 cells/mL and allowed to adhere for 2 hr. Next, 0.25 mM of pyrogallol, and 1.0 mM of SIN-1 were treated to generate superoxide (O_2^-), and peroxynitrite ($ONOO^-$), respectively. After 24 hr of incubation, the BuOH fraction was treated in the test wells at various concentrations for 24 hr.

MTT cytotoxicity assay

Cell viability was assessed using the MTT colorimetric assay. MTT solution (1 mg/mL) was added to each 96-well culture plate and incubated for 4 hr at 37°C, and then the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 100 mL of dimethyl sulfoxide (DMSO) and the absorbance of each well was read at 540 nm using a microplate reader.

Statistical analysis

Significance was verified by performing Duncan's multiple range tests using SAS software (version 6.0, SAS Institute, Cary, NC, USA).

RESULTS AND DISCUSSION

Biologically active compounds found in vegetables may play roles in reducing the risk of degenerative diseases caused by oxidative stress (18). However, studies examining the protective activity of bitter melon and its active compounds under free radical-induced oxidative stress

are few in number. Therefore, in the present study, before finding active compounds from bitter melon, we investigated the ability of the BuOH fraction, the active fraction of bitter melon, against free radical-induced oxidative stress in an *in vitro* and cellular system.

DPPH is a stable free radical that is widely used to test for the ability of compounds or plant extracts to act as free radical scavengers or hydrogen donors. Antioxidants react with the DPPH radical directly and restore it by transferring electrons or hydrogen. Moreover, ·OH causes injury to surrounding organs and plays a vital role in some clinical disorders. Therefore, removal of ·OH is the most effective defense against diseases (19). ·OH is an extremely reactive and short-lived species that can attack biological molecules such as DNA, proteins, and lipids. The reactivity of ·OH has been shown to be related to several human diseases such as neurodegenerative diseases and diabetes. Therefore, radical scavenging activity has received much attention (1,20,21).

The DPPH and ·OH radical scavenging effects of the BuOH fraction from bitter melon are shown in Fig. 1 and 2. Treatment with the BuOH fraction increased the DPPH radical scavenging activity in a dose-dependent manner. At the BuOH fraction concentrations of 250 and 500 μg/mL, the DPPH scavenging activity was 63.4% and 87.1%, respectively. In addition, the BuOH fraction scavenged ·OH radicals effectively. At the BuOH fraction concentrations of 10, 25, and 50 μg/mL, ·OH radical scavenging activity was 87.7%, 87.8% and 89.7%, respectively. These results indicate that the BuOH fraction from bitter melon has a marked protective effect against the strong and toxic ·OH radical, and is an effective free radical scavenger that can protect against radical-induced oxidative damage.

We further investigated the antioxidative activity of the BuOH fraction from bitter melon in a cellular system using LLC-PK_1 renal epithelial cells. LLC-PK_1 renal epithelial cells are susceptible to oxidative stress. Therefore, experimental model of oxidative damage on LLC-PK_1 cells exposed to free radicals would be useful for searching for agents that can provide effective protection from free radicals. A generator-induced cellular oxidative model was employed to investigate the protective effects of the BuOH fraction of bitter melon against oxidative damage (22). Consistent with the present results, several studies demonstrated that cellular oxidative stress in LLC-PK_1 cells was induced by free radical generators such as SIN-1, SNP, pyrogallol, and 2,2'-azobis(2-amidinopropand) dihydrochloride (23,24).

To evaluate the protective activity of the BuOH fraction against free radical-induced oxidative stress, pyrogallol and SIN-1 were used. As shown in Fig. 3, O_2^- generated by pyrogallol decreased cell viability to 42.8% compared to 100% viability in non-treated cells. When

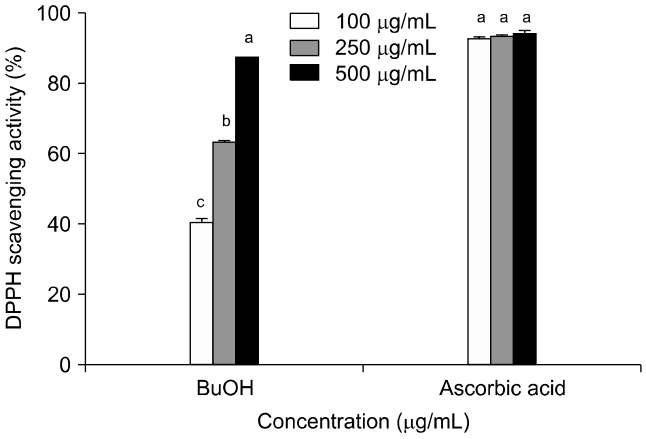

Fig. 1. DPPH scavenging activity of the BuOH fraction from bitter melon. Values are mean±SD. [a-c]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

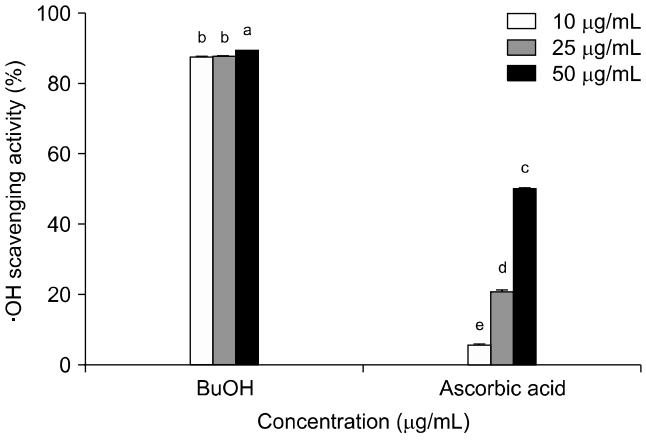

Fig. 2. Hydroxyl radical scavenging activity of the BuOH fraction from bitter melon. Values are mean±SD. [a-e]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

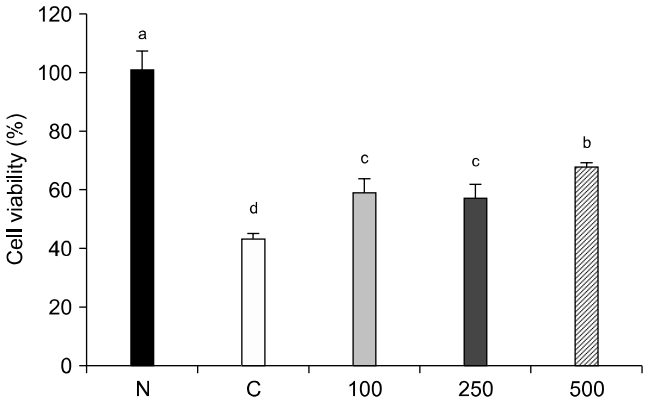

Fig. 3. Effect of the BuOH fraction from bitter melon on viability of LLC-PK_1 cells treated with pyrogallol. N, normal; C, pyrogallol-treated control; 100, 250, and 500 are the BuOH fractions represented as μg/mL. Values are mean±SD. [a-d]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

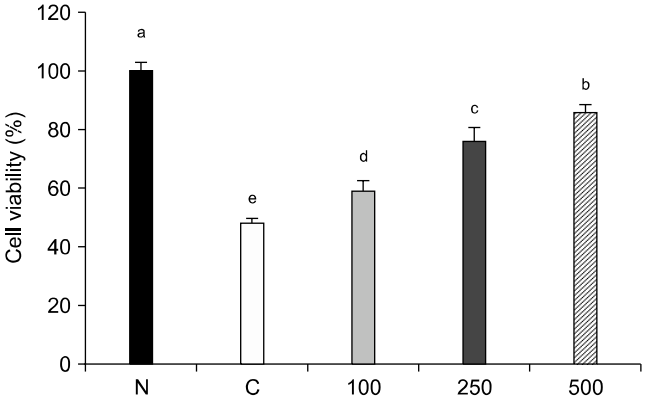

Fig. 4. Effect of the BuOH fraction from bitter melon on viability of LLC-PK_1 cells treated with SIN-1. N, normal; C, SIN-1-treated control; 100, 250, and 500 are the BuOH fractions represented as μg/mL. Values are mean±SD. $^{a-e}$Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

the BuOH fraction from bitter melon was treated at concentrations of 100, 250, and 500 μg/mL, cell viability was increased to 58.4%, 56.7%, and 67.2%, respectively. O_2^- reacts rapidly with NO to produce the more toxic $ONOO^-$. $ONOO^-$ is protonated and forms peroxynitrous acid (ONOOH) under physiological conditions and easily decays to give strong oxidants such as nitrogen dioxide, nitryl cations, and ·OH. $ONOO^-$ and its decomposition products contribute to antioxidant depletion, alterations of protein structure, and oxidative damage observed in human diseases (25-28). SIN-1 simultaneously generates both NO and O_2^-, which then combine rapidly to form $ONOO^-$, and is therefore widely used experimental models (29). While cell viability declined to 48.1% after treatment of SIN-1, treatment with the BuOH fraction from bitter melon increased cell viability in a dose-dependent manner (Fig. 4). In particular, at the concentration of 500 μg/mL, cell viability increased to more than 86%. From these results, the BuOH fraction acted against O_2^- and $ONOO^-$-induced oxidative stress and recovered cell viability in a dose-dependent manner in LLC-PK_1 cells.

Oishi reported blood glucose and serum neutral fat-lowering effects of the saponin fraction (BuOH-soluble fraction) as an active fraction of bitter melon (30). In addition, Kobori showed that the BuOH-soluble fraction of bitter melon extract strongly suppresses LPS-induced TNF alpha production in RAW 264.7 cells (31). Our present study also supports that the BuOH fraction of bitter melon is an active fraction.

In conclusion, the BuOH fraction from bitter melon showed strong direct scavenging activities against DPPH and O_2^- in concentration dependent manners. In addition, it attenuated the oxidative stress induced by O_2^- and $ONOO^-$ through elevations of cell viability. Therefore, this study suggests that bitter melon, especially its BuOH fraction, would be a promising agent with protective activities against oxidative stress induced by free radicals. Further investigations using an *in vivo* model and for identification of active components in bitter melon are needed.

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