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Full Length Research Paper

Protective effects of the active fraction of bitter melon (*Momordica charantia)* on nitric oxide-induced oxidative stress in LLC-PK_1 cell

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In this study, *in vitro* and cellular nitric oxide (NO)-generating systems were used to investigate the NO-scavenging effects of the butanol (BuOH) fraction, the active fraction of bitter melon (*Momordica charantia*). The BuOH fraction of bitter melon showed NO scavenging activities as concentration-dependent manners. These results suggest that BuOH fraction of bitter melon would be a promising antioxidant through scavenging NO. Furthermore, under LLC-PK_1 cellular model, the cells showed declines in viability and increases NO formation against oxidative stress induced by sodium nitroprusside (SNP), generators of NO. However, BuOH fraction of bitter melon significantly and dose-dependently inhibited cell cytotoxicity and NO formation. In addition, the over-expressions of cyclooxygenase-2 and inducible NO synthase induced by SNP were observed, but BuOH fraction of bitter melon down-regulated the expression level of both genes. These results indicate that BuOH fraction of bitter melon would have a protective activity against NO-induced oxidative stress.

Key words: Bitter melon, nitric oxide (NO), LLC-PK_1 cell, antioxidative effect, inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2).

INTRODUCTION

Bitter melon *(Momordica charantia)* belongs in the bitter gourd, and it is an important cultivated food crop and widely used as vegetable in Asia. Several biological effects of bitter melon have been reported, such as hypoglycemic effects, anti-rheumatic, anti-inflammatory, antiseptic, and anti-diabetic remedies (Leatherdale et al., 1981; Anila and Vijayalakshmi, 2000). In addition, bitter melon also has been reported to have other medicinal properties such as anticarcinogenic, hypocholesterolemic, antiviral, cytotoxic, hypoglycemic, and dissipate melancholia and antimutagenic properties (Lotikar and Rajarama, 1966). We previously reported the protective effects of methanol extract and their four fractions from bitter melon against oxidative stress and found that the butanol (BuOH) fraction has the strongest activity among them (Sin et al., 2011). In addition, the main constituents of bitter melon which are responsible for these effects are such as triterpene, proteid, steroid, alkaloid, inorganic, lipid, and phenolic compounds (Grover and Yadav, 2004). However, the protective activity from reactive nitrogen species (RNS) has not been investigated.

Excessive accumulation of reactive oxygen species (ROS) and RNS is associated with many chronic degenerative diseases, such as cardiovascular disease, diabetes, cancer, Alzheimer's as well as the aging process (Halliwell, 1997; Bokov et al., 2004). In biological systems, the inactivation and removal of free radicals depend on the activity of antioxidants. Oxidative stress is

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the result of an imbalance between excessive production of free radicals and depletion of antioxidative defense systems to neutralize and eliminate ROS and RNS. These stressors are considered important mediators of cell injuries, including DNA damage, lipid oxidation, protein modification, and alterations of cellular receptor functions (Valko et al., 2007).

Nitric oxide (NO), which exhibits an enormous range of beneficial functions in organisms, including regulation of vascular tone, ventilation, hormone secretion, inflammation and immunity, as well as neurotransmission, is also suspected to be cytotoxic or cytostatic to host cells and to act as a toxic radical (Akaike et al., 1998; Moncada et al., 1991; Paller et al., 1984). In addition, recent experiments revealed that the toxicity and damage caused by NO in tissues and cells are multiplied enormously if it reacts with O_2^- to yield peroxynitrite ($ONOO^-$), an extremely reactive radical (Virag et al., 2003).

Antioxidants that prevent cellular damage induced by ROS and RNS are considered effective therapeutic agents for degenerative diseases (Ames et al., 1993). Although, several synthetic antioxidants showed preventive effects against oxidative stress-related diseases, their toxicity and side effects are of concern (Wichi, 1988).

Therefore, to elucidate the antioxidative effects of active fraction from bitter melon against RNS, we investigated the protective effects of BuOH fraction from bitter melon against NO under *in vitro* and cellular system.

MATERIALS AND METHODS

Reagent

Bitter melon was obtained from Hamyang, Korea. Sodium nitroprusside (SNP), a metabolic generator of NO, was purchased from Wako Pure Chemical Industries (Osaka, Japan). 3-(4, 5-dimethyl-2-thiazo lyl)-2, 5-diphenyl-2*H* tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). LLC-PK_1 porcine renal epithelial cells were provided by ATCC (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Invitrogen Co. (Grand Island, NY, USA).

NO scavenging activity

NO was generated from SNP and measured by the Griess reaction according to the method of Sreejayan and Rao (1997). SNP (5 mM) in phosphate buffered saline was mixed with different concentrations of the BuOH fraction of bitter melon and was incubated at 25°C for 150 min. The amount of NO produced by SNP was assayed by measuring the accumulation of nitrite, using a microplate assay method based on the Griess reaction.

Cell culture

Commercially available LLC-PK_1 cells were maintained in 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 for 5 days with 0.05% trypsin-ethylenediamminetetraacetate acid (EDTA) in PBS.

Radical generation

Generator-induced cellular oxidative model was employed to investigate the protective effect of the BuOH fraction of bitter melon from oxidative damage (Yokozawa et al., 2003). After confluence had been reached, the cells were plated into 96-well plate at 10^4 cells/ml and allowed to adhere for 2 h. SNP was performed to generate NO. After 24 h incubation, the BuOH fraction of bitter melon was treated with various concentrations in the test well for 24 h.

MTT cytotoxicity assay

Cell viability was assessed using the MTT colorimetric assay (Carmichael et al., 1987). MTT solution (1 mg/ml) was added to each 96-well culture plate and was incubated for 4 h at 37°C, and then the medium containing MTT was removed. The incorporated formazan crystals in the viable cells were solubilized with 100 µl dimethyl sulfoxide (DMSO) and the absorbance at 540 nm of each well was read using microplate reader.

Assay of NO levels

The amount of NO production was assayed by measuring the accumulation on nitrite, using a microplated assay method based on the Griess reaction (Sreejayan and Rao, 1997). Briefly, 100 µl of culture supernatant was allowed to react with 100 µl of Griess reagent, and then it was incubated at room temperature for 5 min. The optical density at 540 nm of the samples was measured using a microplate reader.

RNA extraction and reverse transcription (RT)-polymerase chain reaction (PCR)

Total RNA was isolated using a Trizol reagent (Invitrogen, Carlsbad, CA), and 2 µg of RNA from each BuOH fraction of bitter melon were reverse transcribed using 5X buffer, superscript II, 0.1 M dithiothreitol (DTT), 2.5 mM dNTP in a total reaction volume of 20 µl. cDNA was synthesized from 2 µg of the total RNA by incubation at 37°C for 2 h with AMV reverse transcriptase (Amersham Corporation, Illinois, USA) and 5 pmol each of inducible nitric oxide synthase (iNOS) or cyclooxygenase-2 (COX-2) forward and reverse primers, with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers included in the same PCR reaction as an internal control for efficiency of RT and amount of RNA. Each PCR cycle consisted of a denaturation step (94°C, 1 min), an annealing step (53°C, 43 s), and an elongation step (75°C, 2 min). There were a total 35 cycles, followed by an additional extension step (72°C, 5 min). Primers to specifically amplify the genes interested are shown in Table 1. The amplified PCR products were then separated on 1% agarose gel and ethidium bromide (Sigma, St. Louis, MO, USA), and the intensity of the bands was determined by scanning.

Statistical analysis

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

Table 1. Primers and conditions used in PCR.

mRNA	Primer sequence	PCR conditions
iNOS	F: CCT-CCT-CCA-CCC-TAC-CAA-GT R: CAC-CCA-AAG-TGC-CTC-AGT-CA	53°C, cycle: 35
COX-2	F: AAG-ACT-TGC-CAG-GCT-GAA-CT R: CTT-CTG-CAG-TCC-AGG-TTC-AA	53°C, cycle: 35
GAPDH	F: TCA-TGA-AGT-GTG-ACG-TTG-ACA-TCC-GT R: CCT-AGA-AGC-ATT-TGC-GGT-GCA-CGA-TG	60°C, cycle: 35

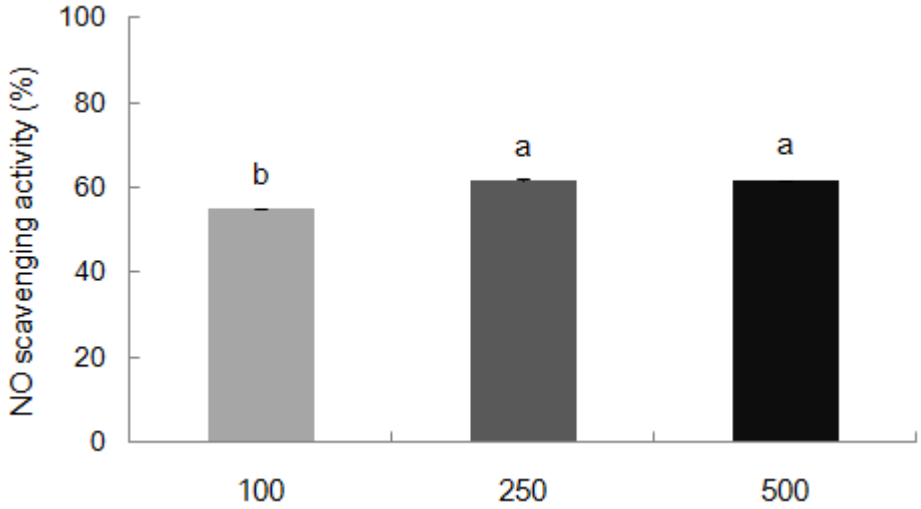

Figure 1. Nitric oxide scavenging activity of BuOH fraction from bitter melon. 100, BuOH fraction 100 µg/ml; 250, BuOH fraction 250 µg/ml; 500, BuOH fraction 500 µg/ml. Values are mean ± SD. [a,b]Means with the different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

RESULTS

NO radical scavenging activity

The NO scavenging activities of the BuOH fraction of bitter melon are as shown in Figure 1. The BuOH fraction of bitter melon scavenged NO effectively in a dose-dependent manner. At the dose of 500 µg/ml, NO radical scavenging activity was 61.62%.

Protective effect against SNP-induced oxidative stress

The protective effects of the BuOH fraction of bitter melon against NO under a cellular system are as shown in Figures 2 and 3. Cell viability through generation of NO by SNP was markedly decreased to 23.47% as compared to cells not treated with SNP. The BuOH fraction of bitter melon, however, exerted an increase in cell viability in a dose-dependent manner. Cell viability was elevated to 76.84 and 102.21% when 100 and 500 µg/ml of the BuOH fraction of bitter melon was treated after NO generation (Figure 2).

The cellular NO levels in SNP treated LLC-PK_1 cells were increased by 15 folds from 1.33 nmole/mg proteins to 19.61 nmole/mg. However, the BuOH fraction of bitter melon decreased NO levels dose-dependently. At the concentrations of 100 and 500 µg/ml, NO level was decreased by 93% (18.33 nmole/mg protein) and 75% (14.72 nmole/ mg protein), respectively (Figure 3).

Effect of BuOH fraction on mRNA and protein expressions of COX-2 and iNOS

Figure 4 shows the effect of the BuOH fraction of bitter melon on the expression of mRNA related to oxidative

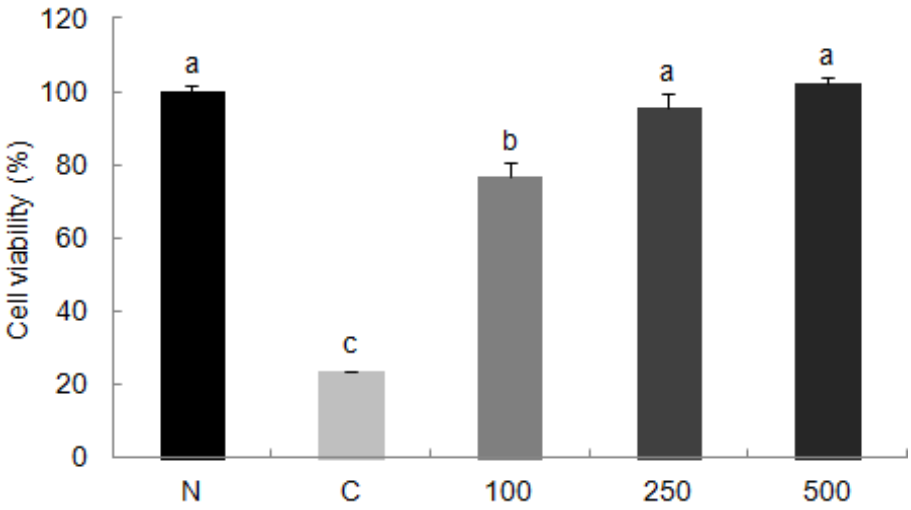

Figure 2. Effect of BuOH fraction from bitter melon on viability of LLC-PK$_1$ cell treated with SNP. N, normal; C, SNP-treated control; 100, BuOH fraction 100 μg/ml; 250, BuOH fraction 250 μg/ml; 500, BuOH fraction 500 μg/ml. Values are mean ± SD. [a,b,c]Means with the different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

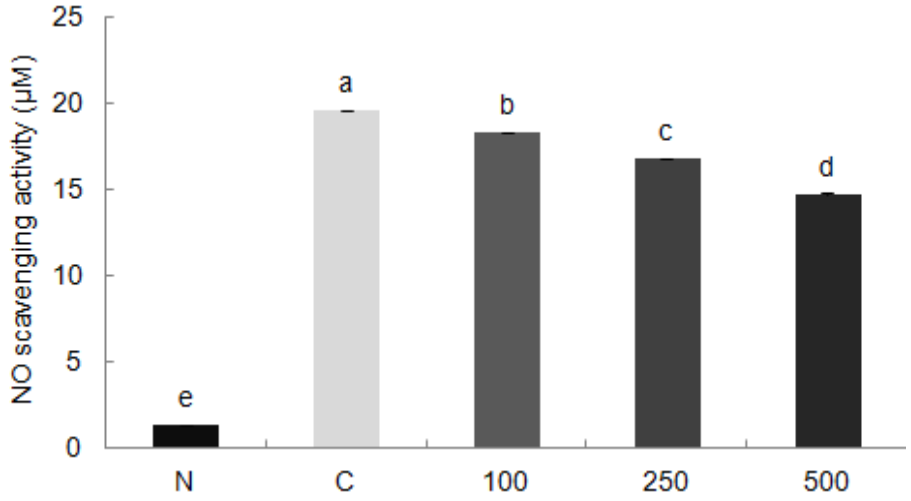

Figure 3. Effect of BuOH fraction from bitter melon on NO generation activity of LLC-PK$_1$ cell treated with SNP. N, normal; C, SNP-treated control; 100, BuOH fraction 100 μg/ml; 250, BuOH fraction 250 μg/ml; 500, BuOH fraction 500 μg/ml. Values are mean ± SD. [a,b,c,d,e]Means with the different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

stress. mRNA expression of COX-2 was elevated in SNP treated LLC-PK$_1$ cells and that of iNOS was also increased by NO. However, the treatment of the BuOH fraction of bitter melon markedly decreased the expression of both genes.

DISCUSSION

It has become fairly clear in recent years that NO acts as a part of the host defense mechanism. NO is generated in greatly increased amounts during infection and inflammation through immunological stimulation and shows cytotoxic or cytostatic activity against viruses and invasive organisms (Akaike et al., 1998). However, such an increase in NO may also have adverse effects on host cells, thus causing tissue injury (Moncada et al., 1991). For example, excess NO produced during ischemia-reperfusion is considered to act as a toxic radical and to cause renal dysfunction, as does O_2^- (Paller et al., 1984;

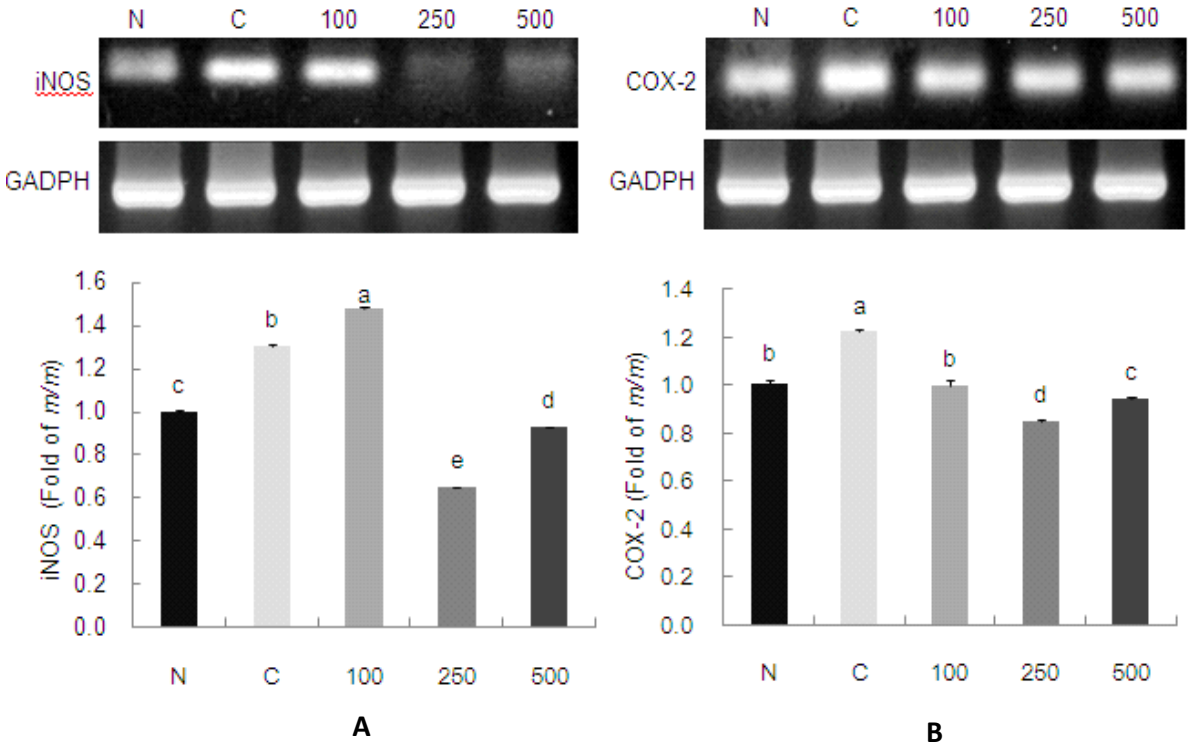

Figure 4. Effect of BuOH fraction from bitter melon on mRNA expression of iNOS (A) and COX-2 (B) under SNP induced oxidative stress in LLC-PK$_1$ cell. N, normal; C, SNP-treated control; 100, BuOH fraction 100 μg/ml; 250, BuOH fraction 250 μg/ml; 500, BuOH fraction 500 μg/ml. [a,b,c,d,e]Means with the different letters are significantly different ($P < 0.05$) by Duncan's multiple range test.

Yu et al., 1994). Also, anti-infective and anti-inflammatory actions can be achieved through inhibition of formation of pro-inflammatory mediators, such as prostaglandins, ROS, and NO (Robak and Gryglewski, 1996). In addition, NO is involved in many physiological processes and has both beneficial and detrimental effects. Although, appropriated levels of NO production are necessary to protect the body systems, sustained levels of NO cause tissue damage and various physiological conditions including neuronal degeneration, neurotransmission, and macrophage cytotoxicity. Furthermore, it works as a main precursor of $ONOO^-$, which is cytotoxic and decompose into ·OH (Valko et al., 2007). Therefore, the production of NO must be tightly regulated.

NO scavenging activities of methanol extract and their four fractions from bitter melon were previously reported and it was found out that BuOH fraction has the strongest activity among them (Sin et al., 2011). Also, in this study, the BuOH fraction of bitter melon demonstrated and showed strong NO radical scavenging activities as dose dependent manner (Figure 1). The present results of NO scavenging effect support that the BuOH fraction of bitter melon has antioxidative effects through direct scavenging NO.

Also, we employed cellular model system to investigate the protective effect of the BuOH fraction of bitter melon using LLC-PK$_1$ renal epithelial cells which are susceptible to free racial (Schena et al., 2001). Our results showed that SNP led to oxidative damage to LLC-PK$_1$ cells through decreases in viability and elevation in NO formation. However, treatment of the BuOH fraction of bitter melon resulted in increase in cell viability with dose dependence (Figure 2). The BuOH fraction of bitter melon also showed decreases of SNP-induced NO formation (Figure 3). These findings imply the protective effect of the BuOH fraction of bitter melon on NO-induced oxidative damage.

The proinflammatory enzymes, including COXs and iNOS, are known to be involved in the pathogenesis of many chronic diseases associated with oxidative stress. COXs have been found in two forms, COX-1 and COX-2. COX-1, a housekeeping enzyme, is constitutively expressed in almost all mammalian tissues, but COX-2 is barely detectable under normal physiological conditions. As shown in this study, NO induced the expression of COX-2 protein, and this was concentration-dependently inhibited by treatment with the BuOH fraction of bitter melon (Figure 4). However, the level of NO released from iNOS under stimulation, such as high glucose conditions or under inflammatory conditions, is significantly elevated and forms the potent free radical $ONOO^-$ by combining with O_2^- (Spencer et al., 1997). Therefore, an increase in iNOS production has been proposed to be responsible for multiple organ dysfunctions (Thiemermann et al., 1995)

and selective iNOS inhibition has been shown to attenuate or prevent this syndrome (Liaudet et al., 1998). This study showed that the mRNA expression of iNOS induced by SNP was down-regulated by the treatment of the BuOH fraction of bitter melon. In this study, NO induced iNOS and COX-2 expressions, but the BuOH fraction of bitter melon inhibited the expressions of iNOS and COX-2 (Figure 4). These results suggest that the BuOH fraction of bitter melon alleviates oxidative stress by inhibiting the expressions of iNOS and COX-2 enzymes.

Taken together with this evidence and results, it seems likely that the BuOH fraction of bitter melon showed strong direct scavenging activities against NO as concentration dependent manners. In addition, it attenuated the oxidative stress induced by NO through elevation in cell viability and inhibition of NO formation by down-regulation of mRNA expressions of COX-2 and iNOS. This suggests that active component of bitter melon may be present in the BuOH fraction. Further studies on the active components from BuOH fraction and the metabolism of the components *in vivo* are needed to elucidate the mechanism of protective effect against oxidative stress of bitter melon.

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