J. Toxicol. Pub. Health
Vol. 22, No. 3, pp. 229-235 (2006)

Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Anti-oxidative, Nitric Oxide Inhibitory Activities and Irritation Test of the Fermented *Opuntia humifusa* Cladodes

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Received June 26, 2006; Accepted September 13, 2006

ABSTRACT. *Opuntia humifusa* is a member of the Cactaceae family. In the present study, the antioxidant, nitric oxide (NO) inhibitory activities and potential irritation response of the fermented *Opuntia humifusa* cladodes (FOH) were investigated for cosmetic use. Antioxidant activities were tested using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and xanthine oxidase assay, we found that FOH could scavenge DPPH free radicals and inhibit xanthine oxidase activity in a dose dependent manner, with IC_{50} of 2599.46 μg/ml and 721.38 μg/ml, respectively. To investigate the possible anti-inflammatory effects of FOH, RAW 264.7 macrophages were pretreated with FOH (0~400 μg/ml) for 30 min and then treated with LPS for 24 h. We found that cell number did not vary significantly with the treatment of FOH, and FOH did not show any inhibitory effects on LPS-induced NO production. After application of FOH to rabbits for skin and eye irritation test, the experimental sites did not show any response compared to the control. FOH were considered to be a non-irritant to the skin and eye. Based on the above information, we suggest that FOH can be considered to be a non-irritant base cosmetic material for safely use.

Keywords: *Opuntia humifusa*, DPPH, Xanthine oxidase, Nitric oxide, Irritation.

INTRODUCTION

The cactus *Opuntia* (genus *Opuntia*, subfamily *Opuntioideae*, family *cactaceae*) is a xerophyte producing about 200~300 species and is mainly growing in arid and semi-arid zones. Traditionally and still today, cactus plants serve as sources for fruits and vegetables as well as for medicinal and cosmetic purposes (Stintzing and Carle, 2005). For achieving these purposes, two kinds of *Opuntia* spp. (*O. humifusa* and *O. ficus-indica*) have been cultivated in Korea.

O. ficus-indica has been reported that it has radical scavenging activity towards superoxide and hydroxyl anions (Lee *et al.*, 2002; Dok-Go *et al.*, 2003), but it can not grow in cold weather. *O. humifusa* can be cultivated in Korean winter, even with temperatures reaching below -20°C (Goldstein and Nobel, 1994). Till now, there is little information on biological activity of *O. humifusa* despite a lot of practical applications.

There may be not a new material, although Cho *et al.* (2006) had reported the radical scavenging and anti-inflammatory activity of extracts from *O. humifusa* recently. In previous study, the ferment of *O. humifusa* cladodes (FOH) showed the inhibition on proliferation of CaSki and SiHa cells in a dose response manner, while the juice extracted from raw *O. humifusa* cladodes did not show such effects (Choi *et al.*, 2005). It indicated that *O. humifusa* cladodes after fermentation could exert novel biological activities.

Overproduction of free radicals can cause oxidative damage to biomolecules (e.g., lipids, proteins, and DNA), eventually leading to many chronic diseases, such as atherosclerosis, cancer, diabetes, aging, and other degenerative diseases in humans (Halliwell, 1994; Poulson *et al.*, 1998; De Souza *et al.*, 2004). Free radi-

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cals may also be a contributory factor in a progressive decline in the function of the immune system (Pike and Chandra, 1995). Many investigations indicated that antioxidants like some flavonoids could modulate the LPS-mediated cytokine production and the release of inflammatory mediators such as nitrite oxide (NO) and prostaglandin E_2 (Gerhauser *et al.*, 2003; Saha *et al.*, 2004). Moreover, in cosmetic industry, consumption of antioxidants from plant materials that inhibit free radical formation or accelerate their elimination is important to protect the aging process of skin. Therefore, in the present work, we have screened the anti-oxidative, NO inhibitory activities *in vitro* and the irritation activity in rabbits for making a novel biomaterial obtained by fermentation for cosmetics.

MATERIALS AND METHODS

Preparation of the fermented liquid of *O. humifusa* cladodes (FOH)

O. humifusa was collected in October from the province of Asan, Korea. The fresh cladodes juice (1,000 kg) was extracted by an extruder under high pressure, and then homogenized to make cladodes juice. For fermentation, *Saccharomyces cerevisiae* (ATCC 7754) purchased from ATCC (American Type Cell Collections, USA) was cultured and harvested for using as seed culture. The cladodes juice (1,000 kg) was added with black sugar by 5% (w/v) and *S. cerevisiae* by 10% (v/v; 10^9 CFU/ml) and then fermented in 3-ton tank at 30°C for 10 days. The fermented liquid was filtered by diatomite, and the filtrate was lyophilized.

Assays for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH free radical scavenging activity of FOH was assessed using the method described by Abe *et al.* (1998) with slight modification. Samples were diluted with distilled water (DDW) to make trial samples of different concentrations. The reaction mixture contained 1 ml of 0.5 mM DPPH-ethanol solution, 0.9 ml of 10 mM acetate buffer (pH 5.6) and 0.1 ml of either test samples with different concentration respectively or DDW (control). The mixture reacted at room temperature for 30 min, and then the absorbance values were measured at 517 nm and converted into the percentage antioxidant activity, which was expressed as the percent decrease in the absorbance compared with the control. The experiments were performed in triplicate.

Assays for the inhibition of xanthine oxidase (X.O.)

Samples were diluted with 50 mM phosphate buffer (pH 7.4) to make trial samples of different concentrations. The reaction mixtures containing 100 μl of xanthine water solution (1.3 mM), 40 μl of X.O. solution (0.0741 units/ml), 100 μl of sample, and phosphate buffer for adjusting the final volume to 2 ml. The inhibition of X.O. activity was evaluated by measuring the formation of uric acid from xanthine with a spectrophotometer at 295 nm for 3 min. The reaction mixture without sample was measured as control. X.O. inhibitory activity was calculated as (C-S)/C × 100%, where C and S are the activities of the enzyme without and with test material. The experiments were performed in triplicate.

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay for the measurement of cell proliferation

MTT assay was performed to exclude the possibility that the observed nitric oxide inhibition was falsely positive due to cytotoxicity. To determine the cell viability, 20 μl of MTT solution (2 mg/ml) was added to 180 μl of cell suspension for 4 h. After sucking the supernatant out, the insoluble formazan product was dissolved in 200 μl DMSO. Then, optical density (OD) of the culture wells was measured using VERSA max microplate reader, (Molecular Devices Corp., Sunnyvale, CA) at 570 nm.

Measurement of nitrite

RAW264.7 cells (5×10^4 cells/ml) were pretreated with FOH for 30 min, and then treated with or without LPS (1 μg/ml) for 24 h. To measure nitrite, 50 μl of the cell-free culture media were mixed with equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, 2% phosphoric acid) and incubated for 5 min at room temperature (light protected). The absorbance was measured using an ELISA reader at 540 nm. The nitrite level in each sample was calculated from a standard curve generated with sodium nitrite (0~100 μM in cell culture medium).

Skin irritation study in rabbit

Four adult rabbits (male) of the New Zealand strain, weighing 2.0 kg (mean) were selected. Prior to dosing the application sites were prepared by clipping the hair from the saddle area of the rabbits. Two abraded areas located diagonally for each rabbit were prepared by making minor epidermal incisions with hypodermic needle. Dried FOH was dissolved in DDW to yield 50 mg/ml of sample solution. The sample solution was applied in a quantity of 0.5 ml under a 2-square-centimeter surgical gauze patch on an intact skin area and an abraded skin area on each rabbit, and 0.5 ml of DDW

was also applied under gauze patch on the rest skin test areas to serve as control. After application of the patches, the trunk of each rabbit was wrapped with bandage, and the animals were restrained for 24 h. At the end of exposure period, the patches were removed and the reactions were scored at 24 h and 72 h following application.

Ocular irritation study in rabbit

Four adult rabbits (male) of the New Zealand strain, weighing 2.0 kg (mean) were selected. Dried FOH was dissolved in DDW to yield 50 mg/ml of sample solution. 0.1 ml of sample solution was applied to the conjunctival sac of the left eye of each test rabbits and 0.1 ml of DDW was applied to the right eye served as control. The upper and lower eyelids were gently held together for few seconds and then released. Examination for gross signs of eye irritation was made at 1, 2, 3, 4 and 7 days following application.

RESULTS

Anti-oxidative activity of FOH

The DPPH radical scavenging activity of FOH were examined at six different concentrations (0, 175, 350, 700, 1,400 and 2,800 μg/ml) as showed in Table 1. All samples at different concentrations of FOH showed the radical scavenging properties to different extent, and DPPH was reduced gradually with the increasing on concentration of FOH. The concentration of the FOH required for inhibiting DPPH radical formation by 50% (IC_{50}) was 2599.46 μg/ml and the IC_{50} of positive control material, ascorbic acid, was 13.74 μg/ml.

The effects of FOH on the inhibition of X.O. were examined at seven different concentrations as showed in Table 2. All samples at different concentrations of FOH showed the inhibition activities on X.O. to different extent, and the formation of uric acid from xanthine was reduced gradually with the increasing on concentration of FOH. FOH inhibited X.O. activity by 50% (IC_{50}) at a concentration of 721.38 μg/ml and the IC_{50} of positive control material, ascorbic acid, was 445.51 μg/ml.

Table 1. Effects of FOH on DPPH free radical scavenging activity

Concentration (μg/ml)	Free radical scavenging activity (%)
0	0.000
175	4.928 ± 0.570
350	9.843 ± 0.752
700	17.767 ± 0.281
1400	27.661 ± 5.418
2800	53.673 ± 1.063
IC_{50} (μg/ml)	2599.46
IC_{50} (μg/ml) of Ascorbic acid	13.74

Values were expressed as Means ± S.D. of 3 independent experiments. IC_{50} value was determined by linear regression analysis.

Table 2. Effects of FOH on inhibitory activity of xanthine oxidase

Concentration (μg/ml)	Inhibition of xanthine oxidase (%)
0	0.0
70	5.75 ± 1.99
140	7.28 ± 4.65
280	11.49 ± 3.04
560	43.3 ± 2.39
1120	78.54 ± 4.65
1400	82.76 ± 11.08
IC_{50} (μg/ml)	721.38
IC_{50} (μg/ml) of Ascorbic acid	445.51

Values were expressed as Means ± S.D. of 3 independent experiments. IC_{50} value was determined by linear regression analysis.

Effects of FOH on the production of NO

Effects of FOH on cell viability were investigated by using MTT assay. We found that cell numbers did not vary significantly by the treatment with FOH at the concentration used in this study, similar to that of cells treated with vehicle alone (data not shown).

To investigate the effects of FOH on LPS-induced NO production, RAW264.7 macrophages were pretreated with FOH (0~400 μg/ml) for 30 min and then treated with LPS (1 μg/ml) for 24 h. We found that cells released 1.46 μM of nitrite in resting state after incubation for 24 h, and no significant difference on the production of nitrite was found between the cells treated

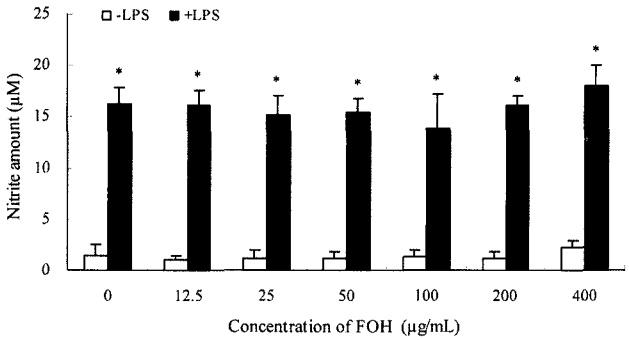

Fig. 1. Effects of FOH on nitric oxide (NO) production of RAW264.7 macrophage cells stimulated by LPS (1 μg/ml). Data were expressed as mean ± SD of three independent experiments. *Represents $p < 0.05$ compared with the media alone treated group.

with FOH (0~400 μg/ml) alone and the control (Fig. 1). The cells significantly increased nitrite production up to 16.20 μM by stimulated with LPS (1 μg/ml) alone, whereas FOH did not show any inhibitory effects on nitrite production stimulated by LPS in the present study (Fig. 1).

Skin irritation study in rabbit

All 4 rabbits survived for the duration of the study and exhibited a gain in body weight. No overt signs of toxicity were seen in any of the animals during the course of the study. After the application of FOH to rabbits, there is a light green staining at the treated skin sites, which did not affect evaluation of the skin responses. No edema, erythema, eschar formation was observed in test or control sites in any rabbits (Table 3). The primary irritation index did not equal or exceed the limit values considered to indicate a significant inflammatory response to treatment. Therefore, FOH was considered to be a non-irritant to the skin.

Ocular irritation study in rabbit

After the application of FOH to rabbit eyes (ocular membrane), all of the rabbit eyes were normal. No abnormal changes like lacrimation, reddening, swelling, or pus formation were observed up to 7 days after exposure (Table 4). Therefore, FOH was considered to be a non-irritant to the eye.

DISCUSSION

Cactus (*Opuntia*) has been used for many years as a common functional food and as medicine by Native Americans and Mexicans (Tesoriere *et al.*, 2004). Simi-

Table 3. Effects of FOH on skin irritation in rabbits

Site	Control site								Test site							
Change	Erythema & eschar				Edema				Erythema & eschar				Edema			
Phase (hrs)	Intact		Abraded		Intact		Abraded		Intact		Abraded		Intact		Abraded	
	24	72	24	72	24	72	24	72	24	72	24	72	24	72	24	72
No.1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No.2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
No.4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean score	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mean score	0				0				0				0			
Total	0								0							
P.I.I.	0								0							

P.I.I.: primary irritation index = total/4

Table 4. Effects of FOH on eye irritation in rabbits

Animal No.	Tissue	Time after application					
		Day 1	Day 2	Day 3	Day 4	Day 7	A.O.I.
1	Cornea	0	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctiva	0	0	0	0	0	0
2	Cornea	0	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctiva	0	0	0	0	0	0
3	Cornea	0	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctiva	0	0	0	0	0	0
4	Cornea	0	0	0	0	0	0
	Iris	0	0	0	0	0	0
	Conjunctiva	0	0	0	0	0	0
M.O.I.		0	0	0	0	0	0
Day-7 I.O.I.		0					

M.O.I.: Mean Ocular irritation Index: I.O.I.: Individual Ocular irritation Index: A.O.I.: Acute Ocular irritation Index.

larly, in Chinese traditional medicine, cactus fruit is considered a weak poison and used as medicine for treatment of inflammation and pain. Moreover, it has even been used as a detoxification agent for snake bite (Zou *et al.*, 2005). Researchers found that cactus contains saponins, alkaloids, flavonoids, polypeptides, β-sitosterol, pectin, carotenes, betalains, ascorbic acid, quercetina and quercetin derivatives, all of which have antioxidant activity (Park *et al.*, 2001; Zou *et al.*, 2005).

Antioxidants have been of growing interest recently since they are supposed to reduce oxidative damage (Zhu *et al.*, 2004) that involved in many diseases such as ischaemic diseases (Ji *et al.*, 2003; Sun *et al.*, 2005), inflammation (Wang *et al.*, 2004), Alzheimer's diseases (Christen, 2000), cancer, and diabetes (Anderson *et al.*, 2004; Stanner *et al.*, 2004). DPPH has been used widely to evaluate antioxidant activities because the model of scavenging DPPH radical can detect a large number of samples in a short period and is sensitive enough to the compounds at low concentrations (Russo *et al.*, 2005). In our previous report (Cho *et al.*, 2006), we compared the DPPH radical scavenging activity of water, chloroform, methanol, ethyl acetate and butanol extract from *Opuntia humifusa*. Among them, the IC_{50} of chloroform and ethyl acetate extract was 247.5 μg/ml and 48 μg/ml, respectively. However, water, butanol and methanol extract did not show the DPPH scavenging activity. In the present study, we found FOH has free radical scavenging activities (IC_{50}, 2599.46 μg/ml) to some extent, showing a concentration dependent manner although its effects were lower than that of the conference material, ascorbic acid (IC_{50}, 13.74 μg/ml). The result indicates that other materials having free radical scavenging activity have to supply FOH for cosmetic products.

The free radical scavenging and antioxidant activity found in *Opuntia* spp. are believed to be mainly associated with the presence of ascorbic acid, flavonoids, quercetins and betalains constituents in it (Butera *et al.*, 2002; Dok-Go *et al.*, 2003; Galati *et al.*, 2003; Stintzing *et al.*, 2005; Cho *et al.*, 2006). Flavonoids are well known antioxidants and attracted a tremendous amount of interest among researchers as possible therapeutic agents for diseases mediated by free radicals. Moreover, flavonoids are also effective inhibitors of several enzymes including XO, cyclooxygenase, and lipooxigenase (Nguyen *et al.*, 2004; Hoorn *et al.*, 2002). The betalains are one of the most important natural colorants used in food systems. Recently, some health effects such as antiradical and antioxidant activity of betalains have been reported too (Kanner *et al.*, 2001; Cai *et al.*, 2003).

Considering DPPH is a synthetic radical and the xanthine oxidase inhibitors are known to be therapeutically useful for the treatment of gout or other XO-induced diseases (Insel, 1990), we also investigated the xanthine oxidase inhibitory activity of FOH using the xanthine/xanthine oxidase system. It has been found that FOH could also inhibit xanthine oxidase activity (IC_{50}, 721.38 μg/ml) in a concentration dependent manner. These results were similar to the recent study conducted by Cho *et al.* (2006) in which water extract of *O. humifusa* was not found antioxidative activities on DPPH and xanthine oxidase, but not in its ethyl acetate fraction. In the present study, we did not use the solvents that can extract them. From the above results, DPPH scavenging and xanthine oxidase activity of FOH could be produced by the fermentation of microorganism.

To exclude the possibility that the inhibitory effect of FOH in NO production was due to the cytotoxicity effect of FOH, we carried out a cell viability test using the MTT assay. The results showed that all concentrations of FOH did not show toxicity (data not shown) as well as NO production (Fig. 1), even at 400 μg/ml.

NO can be induced during macrophage activation and has been recognized as an important messenger in diverse pathophysiological functions, including neuronal transmission, vascular relaxation, immune modulation and cytotoxicity against tumor cells (Fidler and Kleinerman, 1993). However, overproduction of NO can be harmful and result in deleterious consequences such as septic shock, neurotoxicity, and inflammatory diseases (Lowenstein *et al.*, 1994). In order to be used as a base material for cosmetic product such as mask pack and soap, FOH should not induce or should inhibit the NO production. We, therefore, intended to determine whether FOH was capable of NO production in RAW 254.7 cells, or not.

Cho *et al.* (2006) reported that chloroform and ethyl acetate fractions of *O. humifusa* showed nitric oxide inhibitory activity whereas water extract did not. In the present study, FOH did not induce or inhibit the NO production in RAW 264.7 cells in the presence or absence of LPS, even at 400 μg/ml (Fig. 1). The result shows that FOH may be used as a base cosmetic material, indicating a non-inflammatory activity.

We had further investigated to confirm whether FOH has irritation *in vivo*, or not. To do that, we evaluated the local toxicity by rule of KFDA in rabbits. After the application of FOH to rabbits, the animals did not show any skin responses such as edema, erythema, and eschar formation in test or control sites (Table 3). FOH was considered to be a non-irritant to the skin. After the

application of FOH to rabbit eyes (ocular membrane), all of the rabbit eyes were normal. The result indicates that FOH was a non-irritant to the eye.

In summary, FOH had low free radical scavenging activity in DDPH assay but inhibited free radical production in XO assay. Also, FOH did not induce NO production in the presence and absence of LPS at various dose in RAW 264.7 cells. Furthermore, FOH did not show local toxicity of skin and eye in rabbits. Based on the above information, we suggest that FOH can be considered to be a non-irritant base cosmetic material for safely use.

ACKNOWLEGMENTS

This study was supported by Technology Development Program for Agriculture and Forestry, Ministry of Agriculture and Forestry (105036-3) and the Regional Industry Technology Support Business (Daegu). Zhi-qiang Chang was supported by Brain Korea 21 program.

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