RESEARCH ARTICLE

Inhibitory Effects of *Opuntia humifusa* on 7, 12-Dimethylbenz[a]anthracene and 12-O-tetradecanoylphorbol-13acetate Induced Two-stage Skin Carcinogenesis

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Abstract

Opuntia humifusa, member of the Cactaceae family, was previously demonstrated to have radical scavenging, anti-inflammatory and anti-proliferative effects in *in vitro* models. It was suggested that O. humifusa could function in the prevention of carcinogenesis. To investigate the in vivo chemopreventive effect of O. humifusa, mice were fed a diet containing either 1% or 3% following 7, 12-dimethylbenz[a] anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) induction of skin carcinogenesis. Significant decrease in the numbers of papilloma and epidermal hyperplasia were observed in mice fed with O. humifusa, compared to the control group. O. humifusa also upregulated high total antioxidant capacity and level of phase II detoxifying enzyme such as superoxide dismutase and glutathione S-transferase activity in the skin. Lipid peroxidation activity level was measured in skin cytosol and significantly inhibited in 3% OH fed group compared to the control group. These results suggest that O. humifusa exerts chemopreventive effects on chemical carcinogenesis in mouse skin and that prevention effects are associated with reduction of oxidative stress via the modulation of cutaneous lipid peroxidation, enhancing of total antioxidant capacity especially in phase II detoxifying enzyme system and partial apoptotic influence.

Key words: Opuntia humifusa - prickly pear fruit - skin cancer - oxidative stress

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Introduction

Skin cancer is the most frequently diagnosed cancer and represents an important public health problem due to its high incidence and medical costs (Hara-Chikuma et al., 2008). The major etiologic factor leading to the development of skin cancer is thought to be exposure of the skin to solar ultraviolet (UV) radiation (Armstrong et al., 2001). The direct absorption of UV to skin induces DNA damage, gene mutation, inflammatory responses, and also causes production of reactive oxygen species (ROS). ROS, which consist of superoxide anion (O^{2-}) , hydrogen peroxide (H_2O_2) , hydroxyl radical and organic peroxides, are produced from normal metabolic reactions in living system (Inoue et al., 2003). Under a sustained environmental stress, ROS are over-produced for a long time and can be an important source of damage to cell structures, including lipids and membranes, proteins and nucleic acids, consequently result in oxidative stress (Poli et al., 2004; Fang et al., 2009; Khandrika et al., 2009). Furthermore, the accumulation of oxidative stress during the life cycle accelerates the DNA mutations or induces DNA damage, genome instability and cell proliferation (Halliwell et al., 2007; Parmar et al.,

2010). Also, this repeated active oxidative damage has been linked to all three stages of tumor development, i.e., initiation, promotion and progression (Scandalios et al., 2005; Visconti et al., 2009). Hence, reducing intracellular oxidative stress or blocking ROS generation may represent an effective strategy for preventing skin carcinogenesis.

Opuntia humifusa is a member of the Cactaceae family that has been cultivated for a long time in South Korea. Cactus pear fruit (Opuntia spp.) has been extensively used for centuries as a traditional natural coloring agent (Tesoriere et al., 2008) and employed as a popular folk remedy to stimulate the immune system in medicinally (Bisson et al., 2010). The pharmacological properties of O. humifusa have been explored for several years. It has been reported that extracts from O. humifusa have radical scavenging activity and anti-inflammatory activity via blocking with expression of inducible nitric oxide synthetase (iNOS), interleukin-6 (IL-6) and interleukin 1- β (IL-1 β) in lipopolysaccharide (LPS)activated RAW264.7 cells (Cho et al., 2006). Also, O. humifusa partitioned extracts showed anti-proliferative effects by inducing of G1 arrest, non-apoptotic cell death and reducing of ROS production in human glioblastoma

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cell line (Hahm et al., 2010). Although these studies may suggest potential chemopreventive activity of *O*. *humifusa* on in vitro models, its chemopreventive effects in in vivo skin carcinogenesis model have not previously been explored.

The development of skin cancer is a stepwise process, consisting of initiation, promotion and progression in experimental animals and possibly in human cancer, including induction and propagation (Meeran et al., 2009). A commonly used two-stage model of skin carcinogenesis in mice involves initiation by treatment of the skin with 7, 12-dimethylbenz[a]anthracene (DMBA) followed by promotion through treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA). In normal skin, benign squamous lesions termed papilloma result from a single initiating dose of a carcinogen such as DMBA followed by several weeks of repetitive applications of an effective tumor promoter such as TPA (Abel et al., 2009).

Therefore, the aim of this study was to investigate the effects of dietary *O. humifusa* on TPA promoted skin tumor development in DMBA-initiated mouse skin to assess the possibility of its application in skin cancer preventive agent.

Materials and Methods

Animals

Specific pathogen-free (SPF) 6-week-old, female BALB/c mice were purchased from DBL (Daejeon, Korea). All animals were housed in an air-conditioned room, maintained under standard conditions at $24 \pm 2^{\circ}$ C and relative humidity of $50 \pm 10\%$, and allowed free access to their particular diet and tap water ad libitum.

O. humifusa preparation

O. humifusa used in the present study was harvested in October from local farm in Chonnam province, Republic of Korea. Fresh *O. humifusa* fruit were cleaned thoroughly, chopped into small pieces in a biosafety hood and lyophilized. Following lyophilization, the fruits were further processed into fine powder. The powder was stored at 4°C until incorporated into diets.

Experimental design

The mice were randomized into three groups with eight mice in each group. The control group received a commercial, nutritionally complete, standard pellet form feed (Feedlab, Gyeonggi, Korea). The experiment groups received the same standard pellet form feed contained with either 1% (w/w) *O. humifusa* (1% OHfed group) or 3% (w/w) *O.humifusa* (3% OH-fed group). *O. humifusa* containing 1 and 3% experimental diet were prepared as previously described (Mittal et al., 2003). In carcinogenesis studies, supplemented diet was provided to the mice starting 3 weeks before initiation with DMBA. The dorsal skin area of the female BALB/c mice was shaved with electric clippers 2 days before the start of the experiment. Each mouse initiated with **4656** *Asian Pacific Journal of Cancer Prevention, Vol 13, 2012*

two topical applications of 25 μ g of DMBA (Sigma-Aldrich, St Louis, MO, USA) in 100 μ l acetone at an interval of 72 hr. After 1 week, the mice were treated containing 5 μ g of TPA (Sigma-Aldrich) in 100 μ l acetone twice a week for 19 weeks from the start of TPA treatment. All animal procedures were approved by the Institutional Animal Care and Use Committee of Chonnam National University (Approval number: CNU IACUC-YB-2010-1). During the experiment, all mice were weighed weekly until the end of the experiment (data not shown).

Detection of tumor growth

The mice of each group were carefully examined once a week for counting and recording the incidence of papilloma and the number of papilloma. Skin papillomas exceeding 1 mm in diameter that persisted for at least 1 week or more were defined as tumors and recorded. Two different experts who were not concerned with the research team conducted all enumerations. At the end of the experiment, all mice were screened for the cumulative number of tumors.

Histopathological analysis

At the end of the experiment, all mice in each group were sacrificed. For the epidermal hyperplasia study, the dorsal shaved areas of the skin were collected and washed with cold phosphate buffered saline (PBS), then immediately fixed in 10% buffered neutral formalin and embedded in paraffin. Tissue sections 5 μ m in thickness were prepared and stained with hematoxylin and eosin according to standard histology procedure. Epidermal hyperplasia was determined as the mean vertical epidermal thickness of 10 different locations by microscopic examination.

Deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-nick end labeling (TUNEL) assay

Apoptotic cells in skin were visualized by the TUNEL method, using an in situ peroxidase-based cell death detection kit (Roche, Penzberg, Germany). The deparaffinized tissue sections were dewaxed with xylene and rehydrated through graded alcohols, and permeabilized using 0.1% Triton X-100 (Sigma-Aldrich) for 10 min. For the TUNEL staining assay, the sections were treated with 3% hydrogen peroxide for 8 min to block endogenous peroxidases. The sections were then incubated with a TUNEL reaction mixture containing the terminal deoxynucleotidyl transferase and fluoresceindeoxyuridine triphosphate, at 37°C for 1 hr. Peroxidase was added and the slides were incubated at 37°C for 30 min. 3, 3 - Diaminobenzidine (DAB) substrate solution was added for 5 min and each slide was counterstained with hematoxylin for 1 min. Sections of the skin were used for counting labeled and unlabeled nuclei in cells. The apoptotic index (AI) was determined as the percentage of labeled nuclei with respect to the total number of nuclei counted in five different areas of the sections under a light microscope.



Figure 1. Inhibition of Skin Carcinogenesis by *O*. *humifusa* on DMBA-TPA Induced Two-stage Skin Carcinogenesis. a) Changes of papilloma numbers per group were presented as mean \pm SD (n = 8). b) Representative pictures; * p<0.05 and ** p<0.01 versus control group

Estimation of total antioxidant capacity in skin cytosol The capacity of the antioxidants in the sample to convert Cu+2 to Cu+1 is compared with that of Trolox and is quantified as µM Trolox equivalents. Therefore the µM Trolox equivalents can be assessed for estimation of total antioxidant capacity in the skin cytosol. For the determination of trolox equivalent antioxidant capacity (TEAC), Total Antioxidant Power kit (Oxford, Michigan, MI, USA) was used. Briefly, the skin tissue was collected, washed with a cold PBS to remove any red blood cells and clots. Then skin was weighed out 100mg and homogenized in cold RIPA buffer (Sigma-Aldrich) using Precellys® 24 homogenizer (Cayman, Ann harbor, MI, USA). And skin homogenate was centrifuge at 3,000 x g for 12 min at 4°C. Supernatant was analyzed according to the manufacturer's protocol. Free radical scavenging activity level was expressed as mM Trolox equivalent/ mg of protein.

Estimation of lipid peroxidation activity in skin cytosol

Thiobarbituric acid reacting substances (TBARS) content in the skin cytosol was assessed as an indicator of lipid peroxidation. For the determination of TBARS, a commercial TBARS assay kit (Cayman Chemical) was used. Briefly, the skin tissue was collected and washed with cold PBS. Then, skin was weighed and 100 mg samples were homogenized in RIPA buffer (Sigma-Aldrich) using Precellys[®] 24 homogenizer (Cayman Chemical) and sonicated for 15 sec at 40 V over ice. The skin homogenate was centrifuged at 1,600 x g for 10 min at 4°C. Supernatant was analyzed according to the manufacturer's protocol. The level of lipid peroxidation was expressed as μ M malondialdehyde (MDA)/mg of protein.

Estimation of SOD activity level in skin cytosol

SODs are metalloenzymes that play a key role in the

cellular antioxidant defense mechanism. SOD activity in the skin cytosol was estimated using a SOD Assay kit (Cayman Chemical) according to the manufacturer's protocol. Briefly, the skin tissue was collected, washed and homogenized in 20 mM HEPES (Sigma-Aldrich) buffer using Precellys[®] 24 homogenizer (Cayman Chemical). The skin homogenate was centrifuge at 1,500 x g for 5 min at 4oC, and the supernatant was analyzed according to the manufacturer's protocol. The level of SOD level was expressed as U/mg of protein.

Estimation of GST activity in skin cytosol

GSTs are ubiquitous and multifunctional enzymes, which play a critical role in cellular detoxification. GST activity in the skin cytosol was estimated using a GST Assay kit (Cayman Chemical) according to the manufacturer's protocol. Briefly, the skin tissue was collected and washed with cold PBS to remove any red blood cells and clots. Then, skin was weighed and 100 mg samples were homogenized in RIPA buffer (Sigma-Aldrich) using Precellys[®] 24 (Cayman Chemical) and sonicated for 15 sec at 40 V over ice. The skin homogenate was centrifuged at 10,000 x g for 15 min at 4°C, and the supernatant was analyzed according to the manufacturer's protocol. The level of GST level was expressed as mmol/min/mg of protein.

Statistical analysis

The data are expressed as mean \pm standard deviation (SD) and the mean of the different parameters were compared between groups by analysis of variance (ANOVA). In particular, the Mann-Whitney U nonparametric test was used for the determination of microscopic lesion score (ordinal data) in tissues. All statistical analyses of data were performed using SPSS version 19.0 software (SPSS, Chicago, IL, USA).

Results

Effects of O.humifusa on average skin papillomas

Skin papilloma development began at 11 weeks in control group and at 12 weeks in both the 1% OH-fed and 3% OH-fed groups. The papilloma numbers were elevated in all groups. The number of papilloma in the mice at different weeks is shown in Figure 1a. At the end of the experiment, papilloma numbers of each mouse in the 3% OH-fed group (4.60 ± 2.61) were significantly reduced (p<0.01) compared to the control group (10.80 ± 4.66) and the 1% OH-fed group (5.80 ± 1.48) were also significantly reduced (p<0.05) compared to the controls.

Effects of O.humifusa on epidermal hyperplasia

At the end of the experiment, vertical epidermal thickness of the skin was measured to assess effects of epidermal hyperplasia on the mouse carcinogenesis. The vertical epidermal thickness of the skin was 56.73 ± 6.46 µm in the control group mice, but it was significantly reduced (p<0.001) in the 1% OH-fed group (38.93 ± 2.56 µm) and 3% OH-fed group (31.37 ± 2.59 µm) (Figure



Figure 2. Epidermal Hyperplasia of the Mice with DMBA-TPA Induced Two-stage Skin Carcinogenesis. a) Epidermal hyperplasia was evident by the increasing of the epidermal thickness between control and 1% OH-fed group, 3% OH-fed group at the end of the experiment (p<0.001). b) Representative sections of the skin stained with H&E in the control mice, 1% OH-fed group and 3% OH-fed group 200x. Scale bars denote 100 μ m. Bar diagram indicates mean \pm SD (n = 8); **** p<0.001 versus control group; ## p<0.01 versus 1% OH-fed group



Figure 3. Effects of *O. humifusa* on Total Antioxidant Level and Lipid Peroxidation in Skin Cytosol. a) TEAC value in the 3% OH-fed group significantly increased (p<0.01) compared to the control group at the end of the experiment; b) Generation of MDA was used as a marker of lipid peroxidation. The level of lipid peroxidation in the 3% OH-fed group was significantly decreased (p<0.01) compared to the control group; Data are mean \pm SD (n = 8); ** p<0.01 versus control group; # p<0.05 versus 1% OH-fed group

2a).

Effects of O.humifusa on antioxidative capacity in skin cytosol

The capacity of total antioxidant level in skin cytosol of the and 3%-OH fed group (6.57 ± 0.57 mM Trolox/mg of protein) was significantly increased (p<0.01) compare to the level of control group (5.57 ± 0.49 mM Trolox/mg of protein), although 1%-OH fed group (6.06 ± 0.59 mM Trolox/mg of protein) did not reach statistical significance, but showed a increasing tendency compared than in the control group mice (Figure 3a).

Effects of O humifusa on lipid peroxidation activity level in skin cytosol

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Figure 4. Effects of *O. humifusa* on SOD and GST Activity Level in Skin Cytosol. a) SOD; b) GST. Data are mean \pm SD (n = 8); ** p<0.01 versus control group. ***p<0.001 versus control group. #p<0.05 versus 1% OHfed group.

The lipid peroxidation activity level in skin cytosol of the 3% OH-fed group (13.44 ± 5.65 μ M MDA/mg of protein) were significantly decreased (p<0.01) compared to the level in the control group of mice (25.01 ± 4.89 μ M MDA/mg of protein). Also, the lipid peroxidation activity level in skin cytosol of the 1% OH-fed group (20.69 ± 6.16 μ M MDA/mg of protein) showed a decreasing tendency compared than in the control group mice (Figure 3b).

Effects of O.humifusa on SOD activity level in skin cytosol

SOD activity level in skin cytosol of the 3% OH-fed group (0.43 \pm 0.02 U/mg of protein) were significantly increased (p<0.001), compared to the level in the control group of mice (0.36 \pm 0.05 U/mg of protein) (Figur 4a). Although the SOD activity level in skin cytosol of the 1% OH-fed group (0.38 \pm 0.02 U/mg of protein) showed increasing tendency, this effect did not reach statistical significance.

Effects of O.humifusa on GST activity level in skin cytosol

GST activity level in skin cytosol of the 1% OHfed group ($0.51 \pm 0.11 \text{ mmol/min/mg}$ of protein) were significantly increased (p<0.01) and 3% OH-fed group ($0.57 \pm 0.11 \text{ mmol/min/mg}$ of protein) also showed significant increasing (p<0.001), compared to the level in the control group of mice ($0.32 \pm 0.10 \text{ mmol/min/mg}$ of protein) (Figure 4b).

Effects of O.humifusa on induction of apoptosis

AI of skin tumors by TUNEL assay indicates the level of programmed cell death in the skin tissue. TUNEL-positive cells present in sections from skin tissues were enumerated. The AI was increased significantly in the 1% OH-fed group (12.10 ± 1.21) (p<0.01) and the 3% OH-fed group (15.30 ± 2.80) (p<0.001) compared to the control group (7.78 ± 1.91) (Figure 5a).

Discussion

It has been widely documented that many antioxidants

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Figure 5. Effects of *O. humifusa* on Induction of Apoptosis in Mice with DMBA-TPA Induced Two-Stage Skin Carcinogenesis. TUNEL assay was performed using the DAB-peroxidase reaction. a) Both the 1% OHfed group and the 3% OH-fed group showed significantly increased AI (p<0.01 and p<0.001 respectively) compared to the control group. b) 1% OH-fed group and 3% OH-fed group. Scale bars denote 100 μ m. Bar diagram indicate mean \pm SD of eight mice. ** p<0.01 and *** p<0.001 versus control group; # p<0.05 versus 1% OH-fed group

are promising agents with the potential action to inhibit carcinogenesis (Kumar et al., 2010). Mittal et al. (2003) reported that proanthocyanidins from grape seeds have the potential activity to prevent photocarcinogenesis and malignant conversion of papillomas to carcinomas in mice, and this prevention is closely associated with the inhibition of photo-oxidative damage of lipids, and reduction in tissue fat levels. Also, selenium containing compounds appear to have a chemopreventive activity through modulation of cutaneous lipid peroxidation, phase II detoxifying enzyme system and nitric oxide production (Das et al., 2004). Therefore, the present study investigated the anti-oxidative effect of *O. humifusa* and evaluated the possible inhibitory effect in skin carcinogenesis.

During the tumor promotion stage, repeated application of TPA can trigger the production of squamous papilloma and epidermal hyperplasia, which is the pre-neoplastic lesion of skin carcinoma (Shelton et al., 2000; Abel et al., 2009). These features have been used routinely as quantitative markers of pre-neoplastic lesions during carcinogenesis via the measurement of the level of epidermal thickness and the counting of papilloma number (Cibin et al., 2011; Ko et al., 2011). The present study showed that the number of skin papilloma was significantly decreased in dietary feeding with 3% *O. humifusa* compared to the control group. Also, both dietary feeding of 1% and 3% *O. humifusa* produced a highly significant decrease of epidermal hyperplasia compared to the control group.

Also, it was well established that application of TPA leads to excessive ROS production and decline

the ROS detoxification enzymes, such as SOD and GST in epidermal cells (Das et al., 2012). Especially, SOD is generally regarded as one of the most effective intracellular enzymatic antioxidants that protect cells against free radical stress by catalytic action of the dismutation of O₂- to H₂O₂ and molecular oxygen. GST also plays an essential role in detoxifying and transport action by catalyzing and elimination of GSH, the active metabolites of carcinogen compounds. Impaired SOD activity together with a decrease level of cellular GST content could make the tissue highly susceptible to lipid peroxidation and O₂- toxicity (Das et al., 2004; Arya et al., 2011). Hence, papilloma and squamous cell carcinoma with decreased level of SOD and GST, leads to pro-oxidant state of carcinogenesis (Reuter et al., 2010). In the present study, both SOD and GST activities increased in mice fed 3% O. humifusa compared to the control group. Also, 1% O. humifusa-fed mice showed increase of GST activity level compared to the control group. This finding is similar to previous reports that selenium containing compound inhibit the increased papilloma formation of croton oil induced two-stage mouse skin carcinogenesis via enforcement of SOD and GST activity, modulation of cutaneous lipid peroxidation and nitric oxide (Das et al., 2004; Arya et al., 2011).

The ROS production leads to not only decline the ROS detoxification enzymes, but also attack the cellular components involving polyunsaturated fatty acid residues of phospholipids, which trigger the initiation of lipid peroxidation (Reed, 2011; Rauchová et al., 2012). As a result of lipid degradation, malondialdehyde (MDA) and other aldehydes are formed in the biological system. Increased levels of MDA can cause chaotic cross-linkage between proteins and nucleic acids, which leads to alteration of different biochemical pathways and gene expression, resulting in tumor promotion (Valko et al., 2006; Biswas et al., 2010). Therefore, elevated level of MDA indicates the risk factors associated with cancer promoting effects (Cibin et al., 2011). In this study, there was a significant decrease in MDA levels by dietary feeding of O. humifusa, 3%, compared to the control group. A similar observation was also noted with proanthocyanidins from grape seeds on the inhibition of skin photocarcinogenesis concomitant with reduction in lipid peroxidation (Mittal et al., 2003).

In the cancer promoting stage, ROS can attribute to abnormal gene expression, blockage of cell-to-cell communication and modification of second-messenger systems, resulting in a decrease in apoptosis of the initiated cell population (Reuter et al., 2010). In the present study, AI was decreased significantly in the control group compared to the *O. humifusa*-fed mice. This result indicates a possible role for *O. humifusa* in the inhibition of tumor growth in mouse carcinogenesis via the induction of apoptosis. The mechanism(s) of the effects of dietary feeding with *O. humifusa* on apoptosis of tumor cells require(s) further study of the expression of apoptosis related genes that regulate the cell cycle and

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apoptotic pathways.

Taken together, this present findings suggest that dietary supplementation of *O. humifusa* confers chemopreventive effects in terms of reduction of papilloma formation and epidermal hyperplasia in a mouse skin carcinogenesis model. These inhibitory activities could be associated with the reduction of lipid peroxidation, protection against depletion of SOD and GST and a partial apoptotic effect. However, *O. humifusa* contains a complex array of compounds. Therefore, more detailed studies with precise knowledge of the major component(s) of *O. humifusa* on cellular anti-oxidant system and apoptosis could lead to new strategies for cancer chemoprevention.

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