Anti-oxidative and Anti-inflammatory Effects of *Protulaca Oleracea* on the LPS-stimulated AGS Cells

Chae Hyun Kim, Pyeong Beom Park[1], Seung Ryeol Choe[1], Tae Heon Kim[2], Jong Kil Jeong, Kwang Gyu Lee[1], Chang Hyun Lee[2], Han Sol Jeong[1]*

Department of Prescription, College of Oriental Medicine, Dongshin University,
1: Department of Pathology, 2: Department of Anatomy, College of Oriental Medicine, Woosuk University

Protulaca oleracea, a widely distributed weed, has been reported to exhibit different health promoting effects. The objective of this study was to evaluate the anti-oxidative and anti-inflammatory effects of *P. oleracea* on LPS-stimulated AGS cells. The cytotoxicity of *P. oleracea* in AGS cells was examined by MTT assay. The anti-oxidative effects of *P. oleracea* were examined by DPPH assay. RT-PCR was carried out to examine the effect of *P. oleracea* in the mRNA expression of different inflammatory mediators. MTT assay revealed that *P. oleracea* have almost no cytotoxity in AGS cells. DPPH radical scavenging activities were better than butylated hydroxyl toluene (BHT). The mRNA expression of different endogenous anti-oxidative enzymes (SOD2, GPx3 and catalase) were preserved by *P. oleracea* in AGS cells. The nitric oxide production and expression of iNOS in LPS stimulated RAW264.7 were suppressed in *P. oleracea* treated groups. Based on these findings, *P. oleracea* has protective anti-oxidant and anti-inflammatory effects.

Key words : p*ortulaca oleracea*(*p. oleracea*), SOD(superoxide dismutase), GSH(glutathione), ROS(reactive oxygen species), iNOS(inducible nitric oxide synthase)

Introduction

P. oleracea(Portulacaceae) is listed in the World Health Organization as one of the most widely used medicinal plants and it has been given the term 'Global Panacea'[1], and it is widespread as a weed and has been ranked the eight most common plants in the world[2]. *P. oleracea* L. (Chinese name Ma-Chi-Xian) is widely used in China not only as an edible plant, but also as a traditional Chinese herbal medicine for alleviating pain and swelling[3]. Also in Korea, *P. oleracea* can be found growing wild, but rarely cultivated[4].

P. oleracea is well-known as a strong free radical scanvenger and there has been reported that the administration of *P. oleracea* has numerous beneficial effects against many diseases. The ameliorative effects of a *P. oleracea* against oxidative stress may have been proposed as a key mechanism for its beneficial effects[5,6].

In the present study, we investigate the effects of *P. oleracea* on the LPS stimulated AGS and RAW 264.7 cells. We showed that *P. oleracea* inhibited the LPS induced inflammatory mediator including iNOS, TNF-α and has anti-oxidative effects.

* To whom correspondence should be addressed at : Han Sol Jeong, Department of Pathology, College of Oriental Medicine, Woosuk University, Samnye-eup, Wanju-gun, Chonbuk, Korea
· E-mail : jhsol33@paran.com, · Tel : 063-290-1539
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Materials and Methods

1. Plant material Extraction and fractionation

P. oleracea was purchased from Omni-herb (Dae-gu, Korea) and identified by Department of prescription, College of Oriental Medicine, Dong Shin University. A voucher specimen was deposited at the herbarium of College of Oriental Medicine, Dong Shin University. The dried *P. oleracea* (5 kg) were extracted thrice with 90% ethanol (5 L) by maceration at room temperature for 24 h. The extracts were filtered and dried at 40℃ under vacuum.

2. Cell culture

The AGS gastric cancer cells and were obtained from the Korean Cell Line Bank (KCLB). The cells were maintained in RPMI 1640 (Gibco BRL) supplemented with 100 U/ml of penicillin (Gibco BRL) and 100 μg/ml of streptomycin (Gibco BRL) and a 10% FBS (Gibco BRL). The mouse monocytic-macrophage cell line RAW264.7 (KCLB) was cultured in DMEM containing 10% FBS and antibotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). Cells were

grown at 37℃ and 5% CO_2 in humidified air.

3. MTT assay

The cytotoxic effect of crude extract of *P. oleracea* in AGS cells was determined by using MTT (Sigma-aldrich, USA) assay. AGS cells (1×10^5 cells/ml) were seeded in a final volume of 100 μl of 10% FBS medium per well in 96 well plates after verifying cell viability by a trypan blue dye exclusion assay. After 4 h, the cells were starved for 6h in 0.4% FBS medium. Then, the complete medium was replaced with 10% FBS medium and treated with various concentrations of *P. oleracea* for 24 hours. The cells were labelled with 50 μl of MTT (1 mg/ml) for 4 h. The formazan crystals thus formed were dissolved in 150 μL of 10% SDS in 0.01 N HCl and the absorbance of each well was read at 570 nm using an microplate reader.

4. DPPH assay

The antioxidant activities of various fractions were assessed by the method of Mondal et al.[7] with some modifications. The reaction mixture contained 1 ml of 0.1 mM DPPH in ethanol, 0.95 ml of 0.05 M Tris HCl (pH 7.4), and 50 μl of the test samples or de-ionised water. The mixture was shaken vigorously and left to stand in the dark at room temperature for 30 min. The absorbance of the resulting solution was measured at an optical density of 517 nm against blank samples without DPPH. BHT was used as the positive control. All results were run in duplicate and averaged. The percent inhibition of the DPPH radical by the samples was calculated according to the following formula

% Inhibition = [(A0 - At)/A0] x 100

where A0 is the absorbance of the DPPH radical without antioxidant at t = 0 min and At is the absorbance of the antioxidant at t = 30 min.

5. Measurement of nitrite

The concentration of nitrite, a stable metabolite of NO, was measured in the culture supernatant of RAW 246.7 cells stimulated with LPS (1 μg/ml) using Griess reagent. RAW 264.7 ($1x10^6$ cells/ml) were pre-treated with various concentrations of *P. oleracea* for 18h before stimulation with LPS (1 μg/ml) for 24h. The culture supernatant of volume 100 μl was transferred into 96 well microplates and mixed with an equal volume of Griess reagent at room temperature. After incubation for 30 min, the absorbance was recorded at 540 nm by a microplate reader. The concentrations of nitrite were calculated by a regression analysis using serial dilutions of sodium nitrite as a standard.

6. Western blot analysis

The whole cell lysates from the cells under diverse conditions were prepared by washing with ice-cold PBA followed by lysis using a RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris (pH 8.0), 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, 1 μg/ml aprotinin, leupeptin, pepstatin). The protein concentration was determinded using a Bio-Rad protein assay kit. An equal amount of the proteins was loaded, separated by SDS-PAGE, and transferred to a PVDF membrane. After blocking with PBS-0.1% Tween20 (PBST) containing 1% skim milk and 1% BSA for 1h, the membrane was incubated overnight at 4℃ with the primary antibodies against i-NOS (Santa Cruz Biotechnology. Inc., Santa Cruz, CA). After washing in 1×PBST for 1h (3 times × 20 min), the membranes were incubated with HRP-conjugated secondary antibodies and the immunobands were visualized using an enhanced chemiluminescence detection system (Amersharm-Pharmacia Biotech, Buckinghamshire, England).

7. RT-PCR

Whole cell lysates from cells under diverse conditions were prepared by washing with ice-cold PBS. And total RNA was extracted with Trizol reagent (Invitrogen). The RNA concentration was determined by measuring the absorbance at 260 nm (A260) in a spectrophotometer, and the ratio of absorbance at 260 nm to that at 280 nm was 1.8 or higher. The integrity of RNA was checked by visual on an agarose gel. cDNA was synthesized from 1 μg of total RNA as a template in a 20 μL reaction mixture containing 5× first strand buffer, 0.1 M DTT, 10 mM dNTP, 200 unit M-MLV reverse transcriptase (Invitrogen, USA). The reaction was incubated at 42℃ for 1 hour and then inactivated at 95℃ for 5 minutes. After inactivation, the cDNA was stored at -20℃ until use. RT-PCR was performed by coamplification of the genes with a reference gene (18S ribosomal RNA) by use of the cDNA template and corresponding gene-specific primer sets. The primer sequences were as follows: (sense) AGC ACT AGC AGC ATG TTG AG and (antisense) ACT TCT CCT CGG TGA CGT TC for SOD2; (sense) GTA CGG AGC CCT CAC CAT T and (antisense) AGC CCA GAA TGA CCA GAC C for GPx3; (sense) CCC TCT CAT CCC AGT TGG T and (antisense) TAG TTG GCC ACT CGA GCA C for Catalase; (sense) ACG GCA TGT GAG GAT CAA AA and (antisense) GTA GCC AGC ATA GCG GAT GA for iNOS (human); (sense) CCA AAC GAT GTT GTA CCC GA and (antisense) CAG TTG GAG GAG AGA CGG TA for TNF-alpha; (sense) CTC TCT CAC CTC TCC TAC TCA C and (antisense) ACA CTG CTA CTT CTT GCC CC for

IL-1β. PCR was carried out in a total volume of 20 μL containing 1 μL of cDNA solution, 25 μM of sense primers, and 25 μM of antisense primers, 1×PCR buffer, 2.5 mM $MgCl_2$, and 2.5 unit Taq. DNA polymerase (TaKaRa Korea, Korea). The RT-PCR exponential phase was determined after 30-32 cycles to allow quantitative comparisons among the cDNAs developed from identical reactions. Thermal cycling with the following temperature profile was used for amplification: an initial denaturation step of 95℃ for 3 min, followed by 30 cycles of denaturation at 95℃ for 30 s, annealing at 55-59℃ for 30 s, elongation at 72℃ for 30 s and a final extension step of 72℃ for 5 min. The control PCR was performed for 30 or 32 cycles with 1 μL of cDNA solution to allow quantitative comparisons among the cDNAs developed from identical reactions with primers for GAPDH. Amplified products were resolved on 1% agarose gels, stained with ethidium bromide, visualized with Gel Doc System (Bio rad, USA).

8. Data expression and statistical analysis

Data are expressed as means±SEM (standard error of means). Statistical differences between means were determined by the Student's t-test. Differences between multiple groups were tested using analysis of variance (ANOVA) for repeated measures. P-value below than 0.05 was considered as significant data.

Results

1. Effect of *P. oleracea* on cytotoxicity of AGS cells

Time and dosage effect of *P. oleracea* extract on the cytotoxicity of AGS cells are shown in Fig. 1. Viability of cell treated with *P. oleracea* at concentrations of 0.1 0.5 and 1 μg/ml for 24 hrs was 93.2±2.1%, 91.1±3.6% and 107.1±5.1% of control value, respectively. This result indicated that *P. oleracea* extract have almost no cytotoxicity in AGS cells.

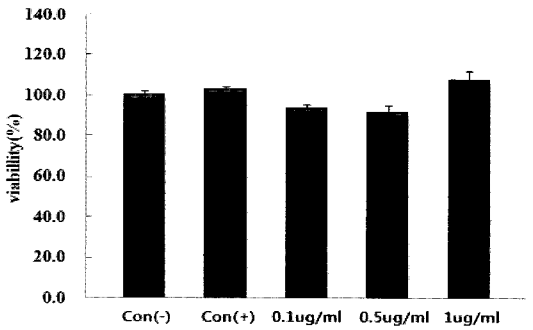

Fig. 1. Effect of *P. oleracea* on cytotoxicity of AGS cells. AGS cells were seeded at a density of 1x10^5 cells/ml in 0.1 ml medium in 96-well plate. Various concentrations of *P. oleracea* were treated for 24 hrs. After solubilization of formazan thus formed, absorbance was recorded at 570 nm using microplate reader.

2. DPPH free radical scanvenging activity

The free racidal scanvenging activities of the extract and water fractions of *P. oleracea* were determined with the DPPH assay, and the results are shown in Fig. 2. The percentage DPPH radical scanvenging activities were dose dependent. Almost concentrations showed better performance against the DPPH radical than BHT which was used as the positive control.

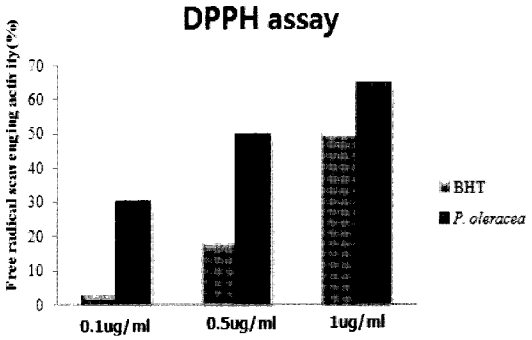

Fig. 2. DPPH free radical scavenging activity of water fraction of *P. oleracea*. Anti-oxidation effect of *P. oleracea* was evaluated by DPPH free radical scavenging assay. Almost all concentrations of the extract showed better performance against the DPPH radical than BHT which was used as positive control.

3. Inhibitory effects against NO production

To investigate the molecular mechanisms of anti-inflammatory activity of *P. oleracea*, the effects of *P. oleracea* on NO production in LPS-activated RAW 264.7 cells were tested. The accumulated NO, estimated by the Griess method, in the culture medium was used as an index for NO synthesis from LPS-activated RAW 264.7 cells. Cells were pre-incubated in 24 well tissue culture plates (2×10^5 cells/well) with 0.1, 0.5 and 1 mg/ml *P. oleracea* for 1h and stimulated with 1 μg/ml of LPS for 24 hrs. LPS-stimulated cells without *P. oleracea* (control) produced 30.1 μM of NO. When the cells were treated with 0.1. 0.5 and, 1 mg/ml *P. oleracea*, the cells produced 27.16±0.27, 19.25±0.04 and 12.36±0.04 μM NO, respectively. *P. oleracea* suppressed NO release into culture supernatant in a dose-dependent manner(Fig. 3).

4. Protein immunoblots

The expression of iNOS and production of NO in LPS induced macrophage were determined by using western blot and Griess reagent respectively. The pre-treatment of *P. oleracea* inhibited the expression of iNOS and production of NO dose dependently in LPS induced macrophage as shown in Fig. 4.

5. RT-PCR

The mRNA expression of endogenous anti-oxidant enzymes and pro-inflammatory mediators in LPS induced AGS

cells were determined by using RT-PCR. The pre-treatment of *P. oleracea* preserved the mRNA expression of GPx3, catalase and SOD2 in LPS induced AGS cells. Also, pre-treatment of *P. oleracea* inhibited the expression of TNF-α while the expression of IL-1β and COX-2 remained unchanged in LPS induced AGS cells as shown in Fig. 5.

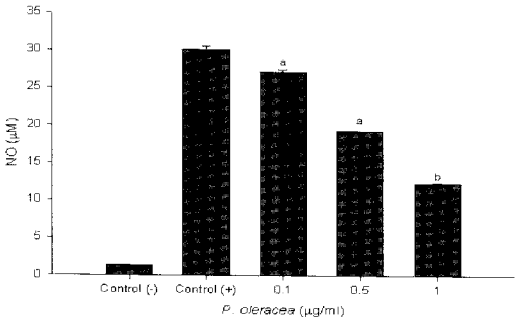

Fig. 3. Inhibitory effects of *P. oleracea* in NO production in LPS-activated RAW 264.7 cells. NO production in LPS-induced RAW 264.7 cells were determined by using Griess reagent. When the cells were treated with 0.1, 0.5 and 1 mg/ml of *P. oleracea*, the cells produced 27.16±0.27, 19.25±0.04 and 12.36±0.04 μM of NO respectively. *P. oleracea* suppressed NO release into culture supernatant in a dose-dependent manner.

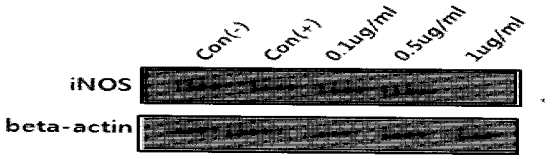

Fig. 4. Effect of *P. oleracea* on iNOS expression in LPS stimulated RAW 264.7. Western blot was carried out to determine iNOS expression in LPS stimulated RAW 264.7. The treatment of *P. oleracea* dose dependently inhibited the expression of iNOS.

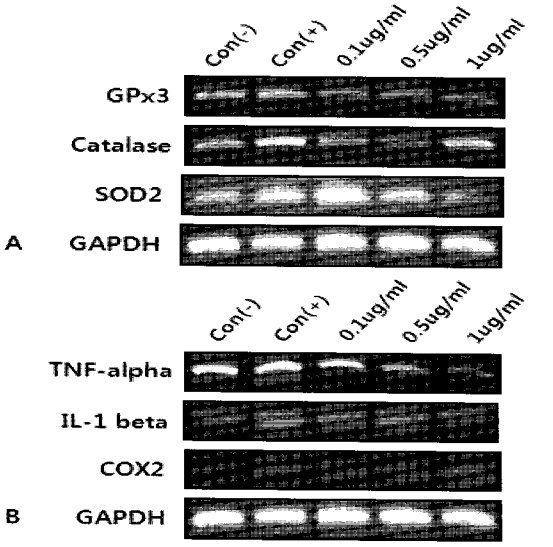

Fig. 5. Effect of *P. oleracea* on the mRNA expression of endogenous enzymes and inflammatory mediators. RT-PCR was carried out to determine the mRNA expression of endogenous enzymes(A) and inflammatory mediators(B) in LPS stimulated AGS cells. The treatment of *P. oleracea* preserved the mRNA expression of endogenous enzymes while inhibited the expression of TNF-α. The expression of IL-1β and COX2 remained unchanged.

Discussion

The use of herbal products in the purpose of prevention or treatment of several chronic diseases has been practiced traditionally in various ethnic societies worldwide[8]. However, the restraining effect and involved mechanisms to the gastritis of herbal products have not been cleared yet. *P. oleracea* (Portulacaceae) which has been ranked the eight most common plants in the world[2] is listed in the World Health Organization as one of the most used medicinal plants and it has been given the term 'Global Panacea'[1]. Portulacae Herba is sour in flavor, cold in nature, nonpoisonous and attributive to large intestine and liver meridians. Medicinal properties of *P. oleracea* include away heat, relieving toxin, cooling the blood, treating dysentery, stopping bleeding, relieving swelling and treating stranguria. It is used for dysentery, eczema, erysipelas, snake-bite poisoning, and hemorrhoids with blood[9-12]. The reported pharmacological effects of this plant include antibacterial[13,14], anti-inflammatory and analgesic effects[15], skeletal muscle-relaxant[16], potassium channel opening effect[17], relaxant effect on smooth muscle[18], wound-healing activities[19] and it is important in preventing heart attacks and strengthening the immune system[20] and reducing the occurrence of cancer[21]. Different chemical constituents have been reported in *P. oleracea*. Some of them include ω-3 fatty acids (α-linolenic acid), α-tocopherol, ascorbic acid, β-carotene, glutathione[32], flavonoids, coumarins[22], monoterpene glycoside[23] and alkaloids. It is excellent source of anti-oxidants such as vitamins A, C and E and β-carotene[6].

This study was designed to evaluate the anti-oxidative and anti-inflammatory effects of *P. oleracea* in vitro. Cytotoxicity was not observed in our in vitro assay in AGS cells. Inflammation is a defensive response that begins after cellular injury, which may be caused by microbes, physical agents (burns, radiation, trauma), chemicals (toxins, caustic substances), necrotic tissue and/or immunological reactions[24]. Inflammation involves a complex web of intercellular cytokine signals. Activated monocytes and/or macrophages release a variety of inflammatory mediators such as TNF-α, IL-1β and NO[25]. In our in vitro assays, LPS induced AGS cells expressed TNF-α and IL-1β. Pretreatment with *P. oleracea* suppressed the mRNA expression of inflammatory mediators TNF- α and IL-1 β dose dependently while the expression of COX-2 remained unchanged in LPS stimulated AGS cells. Free radicals are produced in the mitochondria of cell during biological oxidation and if not quenched rapidly, they damage the lipid membrane by peroxidation. Various antioxidant enzymes like SOD, Catalase and Glutathion peroxidase control the

accumulation of free radicals. Any imbalance in the activity of these enzymes normally leads to faulty disposal of free radicals and its accumulation. These reactive oxygen species besides lipid peroxidation are also responsible for oxidation of bases in cellular DNA and consequent mutagenic, cytotoxic and crosslinking outcomes. Resulting net uncontrolled expression of certain genes effects multiplication of cells leading to malignancy[26]. In our in vitro assays, we observed that pretreatment with *P. oleracea* preserved the mRNA expression of Catalase while Glutathion peroxidase remained unchanged in LPS stimulated AGS cells. SOD2 which is located in mitochondria transforms toxic superoxide into hydrogen peroxide and oxygen[27]. In our experiment, pretreatment of *P. oleracea* increased the expression of SOD2 mRNA at a concentration of 0.1ug/ml in LPS stimulated AGS cells.

Scientists have confirmed that *P. oleracea* contains more ω -3-fatty acids than any other green leafy vegetable[28]. In our study, the free radical scavenging activities of *P. oleracea* was found to be better than BHT suggesting the low risk of gastritis with dietary supplementation of antioxidants like β -carotene and vitamin A. Oxidation and inflammation are inter-related for pro-inflammatory mediators are highly expressed whenever there is oxidative stress. It has been proposed that reactive oxygen and nitrogen species may play a role in gastritis. The reactive nitrogen species are derived from the synthesis of NO, stimulated by iNOS in a variety of cell types including activated macrophages and neutrophils. Increased iNOS activity has been observed in patients with chronic gastritis and gastric cancer. Furthermore, recent studies have revealed that H. pylori infection leads to the formation of nitrotyrosine, which may contribute to DNA damage and apoptosis in gastric mucosa[29]. iNOS is detected in inflammatory cells in all types of gastritis and NO produced by iNOS has been claimed to be involved in gastritis; however, its characteristics are largely unknown[30]. In our study, *P. oleracea* dose dependently inhibited the expression of iNOS and NO production in LPS stimulated RAW 264.7 cells. The early inflammatory response is regulated predominately by the macrophage component of the immune system and is characterized by an increased production of pro-inflammatory cytokines, mediated, in part, through NFκB activation[31]. Previous studies have demonstrated that ω-3 fatty acid emulsions inhibit LPS-mediated TNF-α expression in macrophage, though the mechanisms of its action are still unknown[32]. It is hypothesized that ω-3 FA may modulate NFκ B transcriptional activator proteins, a principal pathway for macrophage pro-inflammatory cytokines elaboration[31]. The inhibitory effect on LPS and INF-γ induced NO production was shown by the extracts of *P. oleracea* in a concentration dependent manner[33]. Thus, the results from our experiment suggest that *P. olaracea* can be a potential natural resource for the management of gastritis although the mechanism of action involved in the treatment remains to be explored.

Acknowledgements

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