Original article

Antioxidant Potential of Ethanol Extract from Orange Fleshed Sweet Potato *(Ipomoea batatas)* in Murine Macrophage RAW264.7 Cell Line

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ABSTRACT

Imbalance between pro-oxidant and antioxidant homeostasis is a vital risk factor for development of several chronic diseases. Orange fleshed sweet potato (OFSP) is an excellent source of β-carotene, which has been demonstrated antioxidant potential. The present study evaluated the antioxidant effect of ethanol extract from steamed OFSP in murine macrophage cell line (RAW264.7 cells). RAW264.7 cell monolayers were pretreated with 0.5-2.0 mg/mL ethanol extract from steamed OFSP for 1 h prior to co-incubation with or without LPS for another 24 h. Cell lysate were collected to determine reactive oxygen species (ROS), glutathione (GSH), heme oxygenase-1 (HO-1) and malondialdehyde (MDA), respectively. The extract contained significant content of β-carotene, total phenolic and flavonoids. The results indicated that the steamed OFSP extract significantly decreased LPS-induced ROS production in RAW264.7 cells without cytotoxicity. The extract also decreased MDA content but enhanced GSH content and HO-1 expression in RAW264.7 cells. The present data provided that bioactive compounds in steamed OFSP have antioxidant potential. It may be useful as an alternative plant food to reduce risk factor of oxidative stress-related chronic diseases.

Keywords: Orange fleshed sweet potato, Antioxidant, Murine macrophage cells

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Introduction

Oxidative stress is an imbalance between reactive oxygen species (ROS) or free radicals and antioxidants[1]. Free radicals are generated from endogenous and exogenous sources. Endogenous ROS are by-products of cellular metabolic activities in mitochondria-catalyzed electron transport reactions and are produced by neutrophils and macrophages during inflammatory response[2] while exogenous sources are environmental agents such as ultraviolet radiation, smoking, alcohol, ischemia-reperfusion injury and chronic infection[3]. Although low-moderate ROS concentrations play beneficial physiological roles in cellular responses against noxious agents, overproduction of free radicals during chronic inflammatory response causes oxidative damage of vital macromolecules, including lipid bilayer of cell membranes, proteins and nucleic acids[4]. Such oxidative damage of macromolecules contributes to pathological development of some chronic diseases such as cancer, hypertension and diabetes mellitus[5].

Dietary phytochemicals in vegetables and fruits have been shown to exhibit antioxidant effect[6] in vitro and in vivo[7]. Due to their multiple biological functions, phytochemicals are increasing interest in food industry for development of healthy functional food products. Orange fleshed sweet potato (OFSP) is a good source of carbohydrate, dietary fiber, vitamins, minerals and some phytochemicals including phenolic acids, flavonoid and carotenoids in particular, β-carotene[8-10]. Due to its high content of β-carotene, provitamin A activity was extensively investigated[10]. Currently, the health benefits of β-carotene, besides being a source of vitamin A, are gaining interest such as antioxidant capacity that plays an important protective role against cellular free radical-mediated damage[11-13]. Therefore, the present study aims to assess antioxidant properties of ethanol extract from steamed OFSP against oxidative stress in murine macrophage cell line (RAW264.7 cells).

Materials and methods

Preparation of steamed OFSP

Orange fleshed sweet potato tubers were randomly bought from 3 major distributors in wholesale market: Talaad-Thai market

(Pathum Thani, Thailand). Equal amount of sample from each distributor was pooled and aliquoted for steaming. They were washed with tap water and peeled prior to washing again with tap water. Approximately 1 kg of peeled OFSP was steamed in a steamer until cooked. Steaming duration depended on weight and size of OFSP. After cooking, they were cooled down at room temperature, mashed with spoon and blended with electric blender prior to lyophilization until dry. The freeze dried samples were homogenized and kept in aluminum foil bag under vacuum and stored at -20 °C until use.

Carotenoids analysis

Dried sample of 0.02 g was suspended with 1 ml of deionized water (DI) and homogenized on ice by Ultrasonic Liquid Processor for 3 cycles of 30 sec on/off pulsing (personal communication with Mark L. Failla et al.). Either 9 mL of absolute ethanol (90% ethanol final concentration) was added to suspended sample and thoroughly mixed by a vortex mixer for 2 min. This was followed by sonication for 10 min and then centrifuged at 4,140 g at 25 °C for 10 min. The extraction procedures were repeated 2 times. The combined supernatants were evaporated using a rotary evaporator under vacuum at 38-40 °C until dry. The dried extract film was reconstituted with 1.5 mL methyl-tert-butyl-ether (MtBE) and 500 µL methanol and vigorous mixed by vortex mixer for 1 min prior to sonication in ultrasonic bath for 1 min. Suspended sample was filtered through 0.22 µm polytetrafluoroethylene membrane filters prior to analysis by high performance liquid chromatography (HPLC). Carotenoids content was determined by modified method as previously described[14]. Carotenoids content was analyzed by reversed phase HPLC system with photodiode array detector. Carotenoids were separated in C30 reverse-phase column (YMC 150 mm x 4.6 mm ID, 5 µm, Serial No., 114FA70081, Japan) with a C18 cartridge guard column (4 mm x 3 mm ID, Phenomenex, USA) at 25 °C. Carotenoids were eluted by gradient elution at a flow rate of 0.6 mL/min with 20 µL injection volume. Mobile phases consisted of 98% methanol in ammonium acetate buffer [Solvent A] and MtBE [Solvent B]

with the following solvent gradient profile: 80% A for 0-1 min, 60% A for 1-10 min, 40% A for 10-20 min, 25% A for 20-30 min and 80% A for 30-37 min. Carotenoids were identified by comparison of retention time and absorption spectra at 450 nm with those of pure standards. Quantification of carotenoids was calculated by comparison the peak area of sample with standards curve of lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene. The amount of carotenoids was expressed as microgram per gram of dry weight (µg/g DW).

Analysis for total polyphenol and flavonoid contents

Freeze dried sample of 0.5 g was suspended with 2 mL DI and homogenized on ice by Ultrasonic Liquid Processor for 3 cycles of 30 sec on/off pulsing and 18 mL of absolute ethanol was added and shaken at 25 °C for 2 h before centrifugation at 1,400 g for 20 min Total polyphenol and total flavonoid were measured in the supernatant. Total polyphenol content in the OFSP extracts were determined by Folin-Ciocalteu assay[15]. Total polyphenol content was calculated against gallic acid calibration curve and expressed as milligram of gallic acid equivalent per gram of dry weight (mg GAE/g DW). Total flavonoid content was measured by aluminum chloride colorimetric method[16]. Total flavonoid content was measured at wavelength 415 nm and expressed as milligram quercetin equivalents per gram dry sample (mg QE/g).

Sample extraction for cell treatment

Dried sample (0.5 g) was suspended with 2 mL DI and homogenized on ice by Ultrasonic Liquid Processor for 3 cycles of 30 sec on/off pulsing. Either 18 mL absolute ethanol was added in suspended sample (90% ethanol final concentration) and mixed for 2 min before sonication in ultrasonic bath for 10 min and centrifuged at 4,140 g for 10 min at 25 °C. The extraction procedures were repeated for another 3 times. Supernatants were evaporated by rotary evaporator at 38-40 °C until dry and kept at -20 °C until use. Yield of 90% ethanol extract was 0.11 ± 0.002 g. The dried extract was re-solubilized with 0.2% dimethyl sulfoxide (DMSO) to final concentration and further diluted to designated concentrations with

serum/phenol free medium before filtered through a sterile 0.2 µm membrane filter prior to addition to the RAW264.7 cell monolayer.

Cell culture growth and activation

Murine macrophage RAW264.7 cell line (TIB 71) was obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were grown in complete medium consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 15 mM HEPES, 100 U/mL of penicillin and 100 µg/mL of streptomycin at 37 °C in a humidified CO_2 incubator. Cells (7.5 × 10^5 cells/mL) were seeded for 24 h and incubated with or without non-toxic doses of extract or 10 µM ferulic acid (FA), a well-known antioxidant phenolic acid[17], for 1 h prior to co-culture with or without 2 ng/mL LPS for 24 h. This experimental set was conducted for determination of intracellular ROS. Another set of experiment was performed without LPS activation by incubation the cell monolayer with or without extract or FA for 24 h prior to collection the cell lysate for measurement of MDA, GSH and HO-1.

Cytotoxicity test

Cytotoxicity of OFSP extracts were assessed by sulforhodamine B (SRB) assay[18] prior to conducting other experiments. Absorbance of LPS in vehicle control was defined as 100% viability. The acceptable viability values of cells treated with extract and LPS should be more than 90% relative to the vehicle control with LPS.

Measurement of intracellular ROS

Intracellular ROS level of the treated cells was evaluated as previously described[19]. After stimulation with LPS for 24 h, the treated cells were washed with warm basal medium (37 °C) prior to addition with 2′, 7′ dichlorofluorescein diacetate in basal medium and incubated at 37 °C in the dark for 30 min. Cells were lysed with 0.5% Triton X-100 in cold phosphate buffered saline (PBS) for 20 min, and then cell lysate was transferred to 96 wells black plate for immediately measured fluorescent signal at excitation/emission wavelength of 485/528 nm. The level of ROS in treated cells was presented as an arbitrary unit and described as percentage of vehicle control.

Determination of malondialdehyde (MDA) and glutathione (GSH)

Monolayers of RAW264.7 cells were incubation with 0.5-2.0 mg/mL OFSP extract or 0.2% DMSO or 10 μM FA in serum free media for 24 h. The monolayers of treated cells were harvested according to a previously described method[20]. The MDA level in cellular extracts were measured as thiobarbituric acid reactive substances (TBARS), following method as previously described[21]. The protein concentration of cellular extract was measured by Bradford assay. The level of MDA was expressed as pmol/mg protein. Glutathione content in harvested treated cells were measured according to presviuosly described protocol[22,23]. Results were expressed as percentage of vehicle control.

Determination of heme oxygenase-1 (HO-1) protein expression by Western blot analysis

After incubation for 24 h, treated cell monolayers were washed with cold PBS and harvested with ice-cold lysis buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetra-acetic acid (EDTA), 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% phosphatase inhibitor cocktail and 0.5% protease inhibitor cocktail] at 4 °C for 30 min on orbital shaker. Supernatants of cell lysate were collected after centrifugation at 12,000 g at 4 °C for 5 min. Protein concentration was determined by bicinchoninic acid assay. Forty microgram of protein/well of samples were separated by 10% of sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto 0.45 μm nitrocellulose membranes (Whatman GmbH, Dassel, Germany). Other procedures were followed as previously described[24]. Briefly, the membranes were washed with Tris-buffered saline containing 0.1% Tween-20 (TBST) and blocked with 5% non-fat dry milk in TBST for 1 h. After that, the membrane were washed and incubated overnight with specific primary antibody (Anti-HO-1 antibody) in 5% bovine serum albumin in TBST at 4 °C. After washing, membranes were reacted with anti-mouse-horseradish peroxidase conjugated secondary antibody for 2 h. The membranes were washed and incubated with chemiluminescent for 5 min and exposed to X-ray film. The density of

targeted protein was scanned and quantified by Image J program. Results were shown as relative ratio of intensity between HO-1/β-actin proteins.

Statistical analysis

SPSS (version 19.0, SPSS Inc., Chicago, IL) was used to analyze the data. All data were presented as mean ± SD from at least three independent experiments. The statistical significance was analyzed by one-way analysis of variance (ANOVA) with post-hoc Tukey's HSD for multiple comparisons to identify mean difference among treatment groups. Statistical significance was set at $P < 0.05$.

Results

Bioactive compounds in steamed OFSP extract

β-carotene was a predominant carotenoid in OFSP extract (Fig. 1). The β-carotene content of the extract was 226.3 µg/g DW. Total polyphenol and flavonoid contents in the extract were 2.13 mg GAE/g DW and 0.24 mg QE/g DW, respectively.

Steamed OFSP extract inhibits LPS-induced intracellular ROS production

To investigate LPS-induced ROS production in RAW264.7 cells, detection of fluorescent oxidative product of 2′, 7′ dichlorofluorescein diacetate was used to examine ROS production in cells. Exposure of the cells with LPS significantly produced ROS whereas cells exposed to vehicle control or the extract alone had no significant effect (Fig. 2). Cell monolayers treated with OFSP extracts before co-incubation with LPS significantly inhibited LPS-induced ROS production in dose-dependent manner (Fig. 2). While, ferulic acid treated RAW264.7 cells also decreased ROS formation (Fig. 2). These results demonstrated that steamed OFSP extract exhibited strong antioxidant activity by reduction of ROS formation.

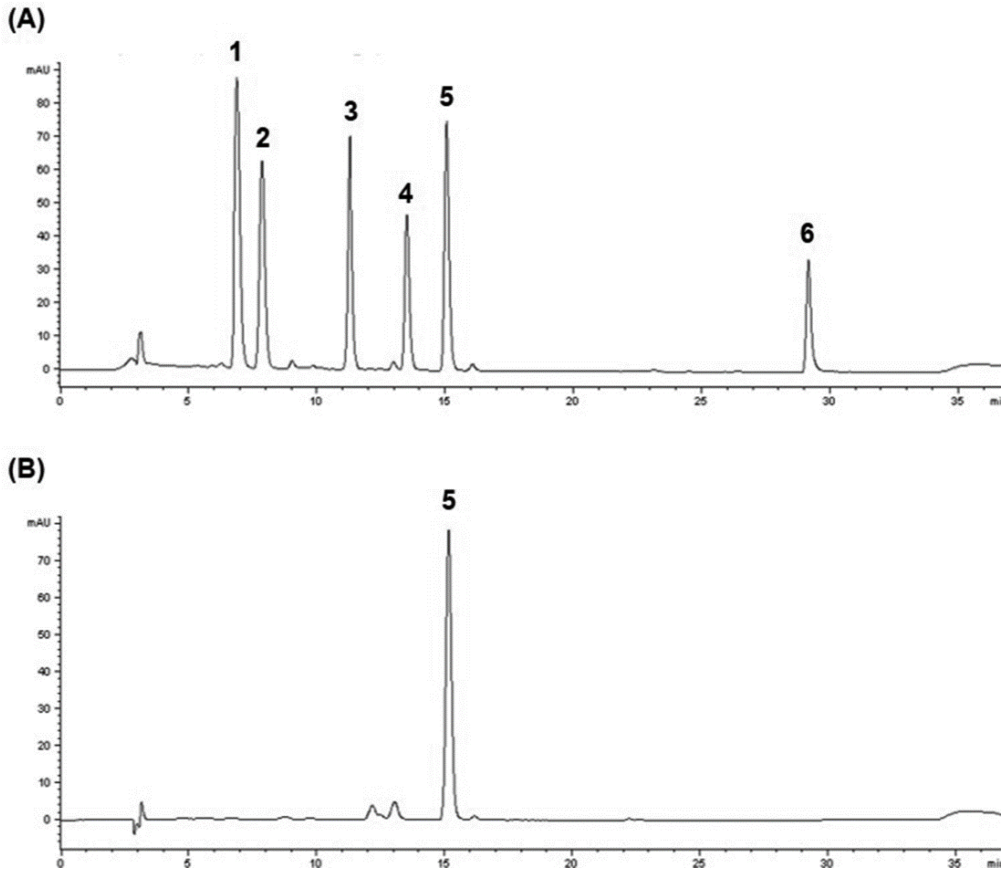

Figure 1. HPLC chromatograms of carotenoids. **(A)** Carotenoid standards [(1) lutein, (2) zexanthin, (3) β-cryptoxanthin, (4) α-carotene, (5) β-carotene and (6) lycopene] and **(B)** 90% ethanol extract from steamed OFSP

Steamed OFSP extract decreases MDA level

Malondialdehyde (MDA) is a principal product of polyunsaturated fatty acid peroxidation. This aldehyde is a highly toxic molecule and its interaction with DNA and proteins has mutagenic and atherogenic effect[25]. RAW264.7 cells treated with OFSP extract for 24 h significantly decreased MDA concentration in a dose-dependent manner (Fig. 3)

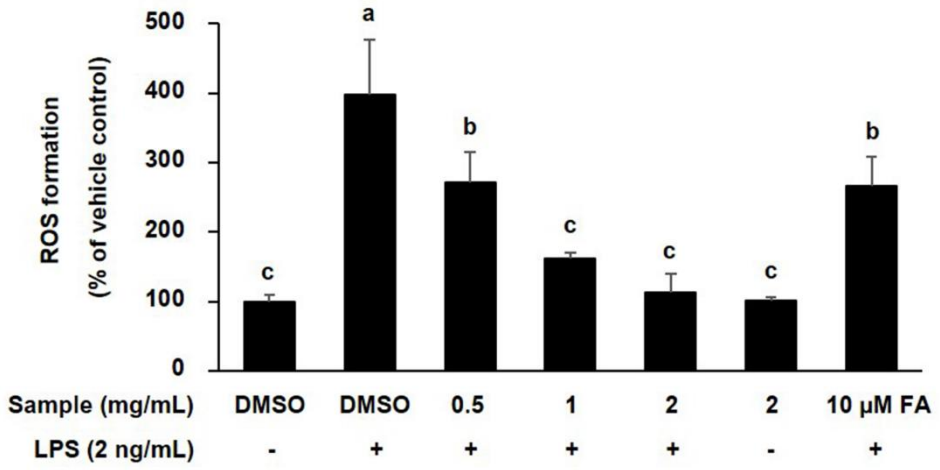

Figure 2. Ethanol extract of steamed OFSP inhibited LPS-activated intracellular ROS formation in RAW264.7 cells. Cells were pretreated with steamed OFSP extract (0.5-2.0 mg/mL) or 0.2%DMSO or 10 μM ferulic acid (FA) for 1 h, followed by 2 ng/mL LPS for 24 h. Ferulic acid used as a control system. After LPS activation, cells were collected to measured intracellular ROS concentration. The formation of ROS in treated cells was presented as percentage of vehicle control. Data were expressed as means ± SD (n = 6). Different letters above the error bars indicated significant differences among treatment groups ($p < 0.05$)

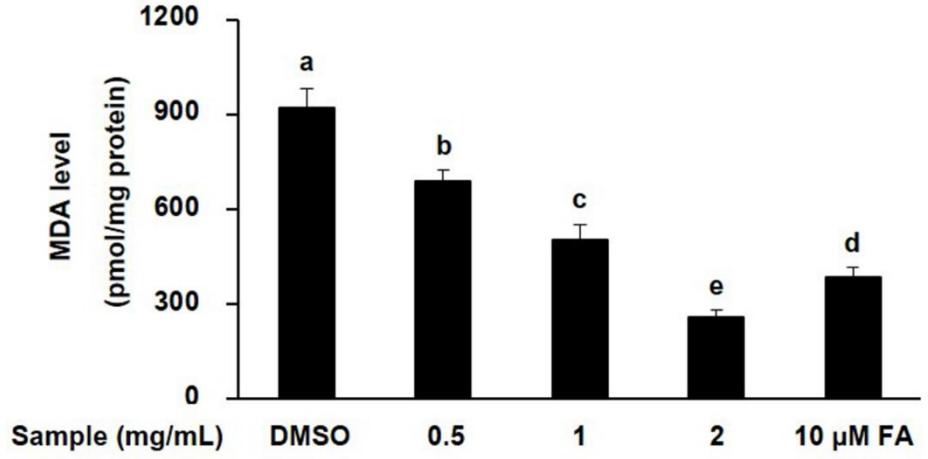

Figure 3. Ethanol extract of steamed OFSP decreased MDA formation in RAW264.7 cells. Cells were incubated with steamed OFSP extract (0.5-2.0 mg/mL) or 0.2%DMSO or 10 μM ferulic acid (FA) for 24 h. After incubation, cells were collected to measure MDA formation. Data were expressed as means ± SD (n = 6). Different letters above the error bars indicated significant differences among treatment groups ($p < 0.05$)

Steamed OFSP extract enhances intracellular GSH level

Glutathione (GSH) is the most important low molecular weight antioxidant synthesized in cells. GSH plays critical roles in protecting cells from oxidative damage and toxicity of xenobiotic electrophiles and maintaining redox homeostasis. Cell monolayer treated with OFSP extract for 24 h significantly enhanced GSH level in a dose-dependent manner relative to vehicle control (Fig. 4)

Steamed OFSP extract enhances HO-1 expression

One such stress-response protein is HO-1, this enzyme involving the antioxidant, anti-inflammatory, anti-apoptotic, anti-proliferative and immunomodulatory effects on vascular cells[26]. To examine whether OFSP induces HO-1 expression, the Western blot analysis was performed to determine the levels of HO-1 protein. We found that OFSP extract upregulated HO-1 expression in a concentration-dependent manner (Fig. 5)

Discussion

Orange fleshed sweet potato (OFSP) is a good source of macro-micro nutrients and various phytochemicals[8-10]. Steamed sweet potato is the most popular in Thai population. Several researches revealed the profiles of nutrients and some bioactive compounds of OFSP[9, 27-28]. The present study found that steamed OFSP extract contained β-carotene, phenolic and flavonoid contents which are consistent with several studies[27-29]. Moreover β-carotene was identified as a predominant carotenoid in the extract. The content of nutrients and non-nutrients found in OFSP depends on variety, environmental conditions during growing period and agricultural managements[30]. OFSP is a β-carotene rich plant; therefore many previous studies have emphasized its potential for improvement of vitamin A status[31, 32]. However, the present study focused on the health benefits beyond vitamin A precursors of OFSP such as antioxidant activity because some carotenoid, phenolic and flavonoid compounds in OFSP have been demonstrated antioxidant property[11, 33, 34].

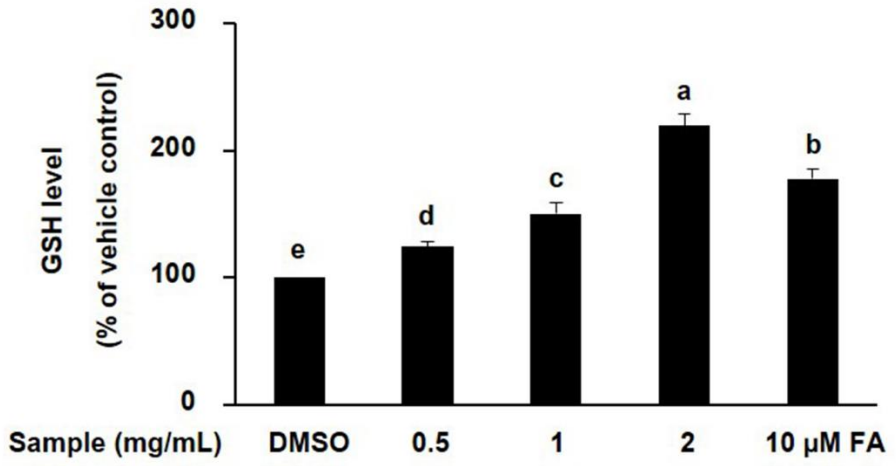

Figure 4. Ethanol extract of steamed OFSP enhances intracellular GSH level in RAW264.7 cells. Cells were incubated with steamed OFSP extract (0.5-2.0 mg/mL) or 0.2%DMSO or 10 μM ferulic acid (FA) for 24 h. After incubation, cells were collected to measure GSH level. Results were expressed as percentage of vehicle control. Data were expressed as means ± SD (n = 6). Different letters above the error bars indicated significant differences among treatment groups ($p < 0.05$)

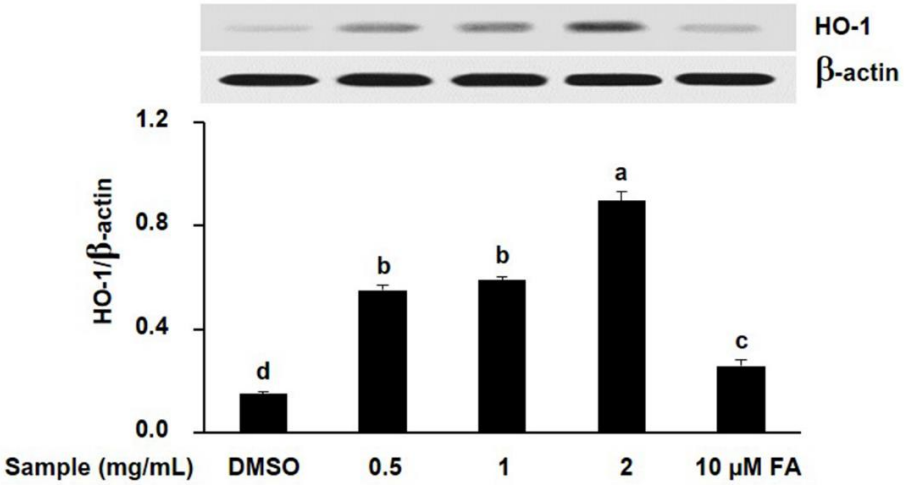

Figure 5. Ethanol extract of steamed OFSP enhances HO-1 expression in RAW264.7 cells. Cells were incubated with steamed OFSP extract (0.5-2.0 mg/mL) or 0.2%DMSO or 10 μM ferulic acid (FA) for 24 h. After incubation, cells were collected to measure the protein levels of HO-1 and β-actin by immunoblot. Data were expressed as means ± SD (n = 6). Different letters above the error bars indicated significant differences among treatment groups ($p < 0.05$).

OFSP contains various bioactive compounds; therefore, our preliminary study started to compare effect of ethanol and hexane/acetone/ethanol (mixed solvent or less polar solvent) from steamed OFSP on LPS-induced NO production in RAW264.7 cells. The ethanol extract from steamed OFSP significantly inhibited NO production, but the mixed solvent extract did not show suppressive effect. Although the mixed solvent extract contained a greater amount of β-carotene (284 µg/g DW) than 90% ethanol extract (226 µg/g DW), the suppressive effect on LPS-induced NO production did not correlate with the β-carotene content. This implied that β-carotene or lower polar compounds in the mixed solvent extract might not play major role on such suppressive effect or the mixed solvent extract might contain interfering compounds from OFSP to neutralize such inhibitory effect. Based on these results, only ethanol extract was used to assess antioxidant activity in the study.

In this study, two types of experimental conditions in RAW264.7 cells including oxidative stress status and normal status were used. In oxidative stress model, cells were induced oxidative stress by LPS addition while the normal model, cells were not induced with LPS. Both models could elucidate the objectives of this study that desire to determine the antioxidant potential of OFSP.

A previous study revealed that LPS markedly induced ROS production and oxidative stress, whereas treatment with N-acetyl cysteine (a well-known antioxidant agent) showed protection against oxidative stress induced apoptosis and mitochondrial dysfunction[35]. It has been shown that the OFSP extract reduced LPS-induced ROS accumulation in SH-SY5Y human neuroblastoma cells[36]. Likewise, the present results indicated that OFSP extract effectively inhibited LPS-activated intracellular ROS generation in RAW264.7 cells that were linked to the potent antioxidant activity of the extract. Excessive generation and accumulation of ROS such as hydroxyl radical and peroxynitrite can degrade lipids of cell membrane through lipid peroxidation reaction. This reaction generates

MDA, a highly toxic molecule[25]. This aldehyde can cause mitochondrial dysfunction observed in many chronic diseases such as atherosclerosis, cancer and diabetes[37]. The study in macrophage RAW264.7 cells indicated that ferrous sulphate and hydrogen peroxide were induced lipid peroxidation, whereas treatment with dried plum polyphenols extract showed reduction in MDA production[38]. Accordingly, pretreatment of RAW264.7 cells with OFSP extract exerts antioxidant activity by decreasing MDA level. Furthermore, OFSP extract obviously upregulated the levels of endogenous antioxidant proteins, GSH and HO-1 which played crucial role as a detoxifying agent and free radical scavenger, preventing oxidative damage of the cells[39]. Reduction of cellular GSH concentration has been shown as an early event in the apoptotic cascade induced by mitochondrial apoptotic signaling and oxidative stress. Moreover, the reduction of cellular GSH levels has been attributed to mechanisms of GSH oxidation promoted by ROS[40]. HO-1 possesses anti-inflammatory, antioxidant, anti-apoptotic and anti-proliferative effects on the vasculature. It is upregulated by various stimuli such as oxidized LDL, heme, hydrogen peroxide, cytokines and endotoxin. Thus, it plays important role in the protective response to oxidative stress.

This study identified and quantified the bioactive compound in OFSP, namely β-carotene which was the predominant carotenoid in the extract. Supporting evidences indicated that β-carotene directly blocked the intracellular production and accumulation of ROS in LPS-stimulated RAW264.7 cells[41]. It also upregulated phase II detoxifying enzyme such as HO-1 gene expression in RAW264.7 cells[42]. In addition, antioxidant activity of β-carotene in clinical study indicated that supplementation with 10 mg of β-carotene once a day for 12 weeks reduced oxidative stress and improved GSH level in chronic lead exposure workers[43]. Moreover, the bioactive compounds present in the steamed OFSP extract may act synergistically to suppress oxidative stress observed in the present study. According to a previous report, mouse peritoneal macrophages treated with phytonutrient mixture revealed a synergistic inhibition of LPS-induced superoxide production derived from downregulation of NADPH oxidase

in both mRNA and protein expression. Moreover, the combination of phytonutrients exhibit anti-inflammatory effect by synergistic inhibition of LPS-induced internal superoxide production leading to a marked reduction in ERK and NF-ΚB activation, probably due to their antioxidant activities[44]. Due to its potent antioxidant effect observed in the present study, OFSP may be an alternative promising functional food for the prevention or reduction of oxidative stress-related chronic diseases.

Conclusion

The study clearly demonstrated that steamed OFSP extract exerts the potent antioxidant properties in murine macrophage cell line, regular consumption of steamed OFSP may prevent or alleviate severity of oxidative stress-related chronic disease. However, this study is an *in vitro* experimental model. Further studies in animal models and humans are needed to confirm whether OFSP can be used as a food material for decrease risks of oxidative stress-associated chronic diseases.

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Conflict of interests

The authors declare that they have no conflicts of interest.

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