Orange-fleshed sweet potato (*Ipomoea batatas*) extract attenuates lipopolysaccharide-induced inflammation in RAW264.7 cells via inactivation of MAPKs and IκB signalling

Yada Saokosol[1], Kemika Praengam[2], Monruedee Sukprasansap[2] & Siriporn Tuntipopipat[2]*

[1]*Graduate student in Master of Science Program in Nutrition, Faculty of Medicine Ramathibodi Hospital and Institute of Nutrition, Mahidol University, Thailand*;
[2]*Institute of Nutrition, Mahidol University, Salaya, Nakhonpathom 73170, Thailand*

ABSTRACT

Introduction: Orange-fleshed sweet potato (OFSP) is an excellent source of β-carotene. Due to its health benefits, β-carotene-rich plants are receiving attention. This study aimed to assess the inhibitory effect of the ethanol extract of steamed OFSP on lipopolysaccharide (LPS)-induced inflammation in murine macrophage cell line (RAW 264.7 cells). **Methods:** β-carotene, total phenolics and total flavonoids of OFSP were measured by high performance liquid chromatography (HPLC), the Folin-Ciocalteu assay and the aluminum chloride colorimetry, respectively. RAW264.7 cell monolayers were pre-treated with 0.5-2.0 mg/mL ethanol extract from steamed OFSP prior to co-incubation with or without LPS for 24 h. Culture media and cell lysate were collected to measure nitric oxide, interleukin-6 (IL-6), IL-1β, tumour necrosis factor-α, inducible nitric oxide synthase, cyclooxygenase-2, mitogen-activated protein kinases (MAPKs) and inhibitory kappa B (IκB), respectively. **Results:** The ethanol extract from steamed OFSP significantly suppressed LPS-induced production of such pro-inflammatory mediators by the inactivation of MAPKs and IκB signalling pathway. The ethanol extract from steamed OFSP contained 226 μg/g DW (dry weight) of β-carotene, 2.13 mg gallic acid equivalent/g DW of total polyphenolics and 0.24 mg quercetin equivalents/g DW of total flavonoids. **Conclusion:** These results indicated that bioactive compounds in steamed OFSP have anti-inflammatory potential.

Keywords: Orange-fleshed sweet potato, β-carotene, anti-inflammation, RAW264.7 cells, lipopolysaccharide

INTRODUCTION

Chronic inflammation has an association with the pathogenesis of obesity, metabolic syndrome and type 2 diabetes mellitus (Esser *et al.,* 2014). During an inflammatory response, various transcription factors including nuclear factor-kappa B (NF-κB) are activated resulting in up-regulated expression of pro-inflammatory mediators (Liu *et al.,* 2017). The over-expression of pro-inflammatory mediators during chronic

*Corresponding author: Siriporn Tuntipopipat, Ph.D.
Institute of Nutrition, Mahidol University, Salaya, Nakhonpathom, Thailand 73170
Tel: +66(0)-2800-2380; Fax: +66(0)-2441-9344; E-mail: siriporn.tun@mahidol.ac.th

inflammation has been hypothesised to be an initiating or aggravating factor for development of some chronic diseases (Aggarwal & Shishodia, 2004). The macrophage is an innate immune cell that plays an important role during inflammatory responses against noxious stimuli, including lipopolysaccharide (LPS). When macrophages are exposed to LPS, NF-κB and mitogen-activated protein kinases (MAPKs), including p-38, extracellular signal-regulated kinases (ERK1/2) and c-Jun N-terminal kinases (JNK) are activated, resulting in up-regulation of various pro-inflammatory mediators including tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Guha & Mackman, 2001).

Various dietary phytochemicals including carotenoids, phenolic acids and flavonoids in colourful dietary plants have demonstrated multiple benefits including antioxidant, anti-cancer and anti-inflammatory activities (Aggarwal *et al.,* 2009; Pan, Lai & Ho, 2010). The sweet potato (*Ipomoea batatas*) with various coloured tuberous roots is an important staple food in Sub-Saharan Africa, Asia and the Pacific Islands. In particular, the orange-fleshed sweet potato (OFSP) is a good source of several nutrients, β-carotene, phenolic acids and flavonoids (Park *et al.,* 2016). Being a rich source of β-carotene, previous studies have investigated its potential to improve vitamin A status in poor communities (Gurmu, Hussein & Laing, 2014; Jamil *et al.*, 2012). Recently, the impact of pasteurisation and sterilisation on its β-carotene content and bioaccessibility were evaluated in an OFSP-based baby puree (Dhuique-Mayer *et al.*, 2018). Currently, the health benefits of β-carotene, such as its antioxidant and anti-inflammatory activities are gaining interest. In addition, the effect of thermal processing on carotenoid stability, total phenolics and antioxidant capacity of OFSP cultivars have been evaluated (Donado-Pestana *et al.,* 2012). However, the anti-inflammatory activity of OFSP has never been investigated. This present study aims to assess anti-inflammatory activity of extract from OFSP in LPS-stimulated murine macrophage cell line (RAW264.7 cells).

MATERIALS AND METHODS

Chemicals and reagents

The chemicals that were used were analytical and high performance liquid chromatography (HPLC) grade. Enzyme-linked immunosorbent assay (ELISA) kits were purchased from BioLegend (San Diego, CA, USA). Antibodies against iNOS, COX-2, inhibitory kappa B (IκB) and MAPKs were purchased from Cell Signalling Technology (Danvers, MA, USA). Anti-IL-1β was purchased from Peprotech (USA). Carotenoid standards, anti–β-actin–horseradish peroxidase (HRP), secondary antibody, Dulbecco's Modified Eagle's Medium (DMEM), LPS (E. coli O11:B4), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma. Fetal bovine serum (FBS) was obtained from Merck (Darmstadt, Germany). Penicillin-streptomycin solution was purchased from Caisson labs (Smithfield, UT, USA). Trypsin-Ethylenediaminetetraacetic acid (EDTA) was purchased from Gibco (Grand Island, NY, USA).

Preparation of steamed OFSP

OFSP tubers were bought from three major distributors (10 kg from each distributor) at a wholesale market at Pathum Thani province, Thailand. Each distributor, in turn, had bought the OFSP from three different growing areas (Nakhon Ratchasima, Lopburi and Suphanburi province). The tubers were washed with tap water and peeled

prior to washing again with tap water. Approximately 1 kg of peeled OFSP from each distributor was steamed in a steaming basket over boiling water for 45 min, cooled down at 25°C and blended with an electric kitchen blender, prior to lyophilization by freeze dryer (GEA Lyophil GmbH, Germany) to preserve stability of phytochemicals. The dried samples were homogenised by a kitchen electronic blender (Philips). An equal amount of dry sample from each distributor was pooled together and thoroughly mixed prior to being packed in aluminum foil under vacuum and stored at -20°C until usage.

Carotenoid analysis

To 1 ml deionised (DI) water, 0.02±0.001g of dried sample was added. The resulting suspension was homogenised on ice by an ultrasonic processor (130 Watt, 20 kHz; Sonics & Materials, Inc., Newton, USA) for three cycles of 30 sec on/off pulsing (personal communication with Failla *et al.,* 2018 at the Ohio State University). Nine mL of absolute ethanol (resulting in a 90% final concentration) was added to the suspended sample. Separately, to another suspension, 10 mL of mixed solvent [hexane:acetone:ethanol (2:1:1)] (Kubola & Siriamornpun, 2011) was added. Both were thoroughly mixed for 2 min followed by sonication in an ultrasonic bath for 10 min (Daihan Scientific Co., Ltd., WUC-A02H, Korea) and centrifugation (Hettich, Rotina 38R, Tuttlingen, Germany) at 4,140 g at 25°C for another 10 min. The extraction procedures were repeated two times. The combined supernatants were evaporated until dry by using rotary evaporator (Buchi Rotavapor-Re-124, Switzerland) under vacuum at 38-40°C. The dry film was reconstituted with 1.5 mL methyl-tert-butyl-ether (MtBE) and 500 µL methanol before it was vigorously mixed and sonicated. The sample was then passed through 0.22 µm polytetrafluoroethylene

(PTFE) membrane filter and diluted to an appropriate concentration with MtBE:methanol (3:1, v/v) solution prior to analysis by HPLC. The carotenoid content was determined by the previously described method of Failla, Thakkar and Kim (2009) by using an HPLC system (Agilent 1100 series, Santa Clara, CA, USA) with photodiode array detector and separated in C30 reverse-phase column (YMC 150 mm x 4.6 mm ID, 5 µm, Japan) with a C18 cartridge guard column (4 mm x 3 mm ID, Phenomenex, USA) at 25 °C. Carotenoids were eluted at a flow rate of 0.6 mL/min with 20 µL injection volume. The 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. The eluted carotenoids were identified by comparison of their retention time and absorption spectra at 450 nm with carotenoid standards and quantified by comparison of the peak area of sample with those of standard curves of lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene.

Determination of total polyphenol and flavonoid content

To 2 mL deionised water was added 0.5±0.01g of dried sample and the suspension homogenized on ice by an ultrasonic processor for three cycles of 30 sec on/off pulsing. This was followed by the addition of 18 mL absolute ethanol and the mixture shaken at 25°C for 2 h before centrifugation at 1,400 *g* for 20 min. The supernatant was collected to measure total polyphenols and flavonoids.

The total polyphenol content was determined by the Folin-Ciocalteu assay (Alhakmani, Kumar & Khan, 2013). Twenty-five µL of extract or gallic acid (standards) or DI water (blank) were

each transferred into 96-well microplate and mixed with 50 µL of diluted Folin-Ciocalteu reagent (1:10) at 25°C for 5 min followed by addition of 200 µL of 7.5% sodium carbonate (Na_2CO_3) and incubated at 25°C for 2 h in the dark. The absorbance of the reaction mixture was measured at the wavelength of 760 nm. The total phenol content was calculated by reading off the absorbance of sample from the gallic acid standard curve and expressed as mg gallic acid equivalent per gram of dry weight (mg GAE/g DW).

Total flavonoid content was measured by the colorimetric method of Prommuak, De-Eknamkul & Shotipruk (2008). The 25 µL of extract was mixed with 75 µL of 90% ethanol, 5 µL of 10% aluminum chloride, 5 µL of 1 M potassium acetate and 140 µL of DI water. The mixture was incubated at 25°C for 30 min in the dark. Sample blank of extract and standard quercetin solutions were prepared in the same procedure by replacing aluminium chloride solution with DI water. The absorbance of the reaction mixture was measured at the wavelength of 415 nm and total flavonoid content was determined by comparing the absorbance of sample with those of quercetin standards and expressed as mg quercetin equivalents per gram dry weight (mg QE/g DW).

Sample extraction for cell treatment

Two sets of 0.50±0.02 g dried samples were suspended in 2 mL DI water and homogenised on ice by an ultrasonic processor for three cycles of 30 sec on/off pulsing. Eighteen mL absolute ethanol was added in a suspended sample (90% ethanol) and 20 mL of mixed solvent was added to another suspended sample (less polar extract) and mixed for 2 min before sonication in an ultrasonic bath for 10 min and centrifugation at 4,140 *g* for 10 min at 25°C. The extraction procedures were repeated three times. The supernatants were evaporated until dry by using a rotary evaporator at 38-40°C and kept at -20°C until use. Yields of 90% ethanol and mixed solvent extracts were 0.11±0.002 g and 0.05±0.002 g, respectively. The dried extract was reconstituted with 0.2% dimethyl sulfoxide (DMSO) and further diluted to designated concentrations with serum/phenol free medium before passing through a sterile 0.2 µm membrane filter for treatment with cell monolayers.

Cell growth and activation

Murine macrophage RAW264.7 cells were purchased from ATCC (Rockville, MD, USA). This cell line was established from a tumour induced by the Abelson murine leukemia virus derived from BALB/c mice. Stock RAW264.7 cells at passage number 6 were stored in liquid nitrogen. Cells with passage numbers of 10-20 were used in the experiments. The cells were grown in complete medium (DMEM supplemented with 10% FBS, 15 mM HEPES, 100 U/mL of penicillin and 100 µg/mL of streptomycin) at 37°C in humidified atmosphere of 5% CO_2/95% air. Cell monolayers were seeded for 24 h prior to incubation with or without non-toxic doses of extract or ferulic acid (FA), a well-known anti-inflammatory phenolic acid, in serum/phenol red free media for 1 h followed by co-culturing with or without 2 ng/mL LPS for another 24 h.

Cytotoxicity test

The cytotoxicity of the OFSP extracts were assessed by sulforhodamine B (SRB) assay (Vichai & Kirtikara, 2006), to select the non-toxic doses of the extract prior to conducting other experiments. Briefly, RAW264.7 cells were pre-treated with 0.5-2.0 mg/mL OFSP extracts from 90% ethanol or mixed solvent or 0.2% DMSO (vehicle control) or FA in serum/phenol red free media for 1 h prior to addition with or without 2 ng/mL LPS for another

24 h. Cell monolayers were washed with cold phosphate-buffered saline (PBS) prior to fixing with 50% trichloroacetic acid and then incubated at 4°C for 2 h. After excessive washing with DI water and air-drying, the cellular protein was stained with 7 mM SRB in 1% acetic acid for 20 min before extensively rinsing with 1% acetic acid to remove excess SRB and air-drying. The protein stained with SRB was solubilized with 10 mM Tris-hydro-methyl-aminomethane for 5 min on gyratory shaker. The absorbance was read at the wavelength of 500 nm and reference wavelength of 690 nm by a microplate reader. The absorbance was observed to be proportionate to the cell number. The absorbance of cells treated with LPS in the control vehicle was defined as 100% viability. The non-toxic doses of the extract were selected when the cells treated with extract and LPS had more than 90% cell viability relative to cells treated with LPS in the control vehicle.

Measurement of nitric oxide (NO), TNF-α and IL-6

After LPS stimulation, the culture media were collected to measure the nitrite concentration (a stable product of NO) by the Griess reagent, and, TNF-α and IL-6 by ELISA kits (BioLegend, San Diego, CA, USA).

Briefly, 100 μL of culture medium, standard sodium nitrite (NaNO$_2$) and DI water were each mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% *N*-(1-Naphthyl) ethylenediamine dihydrochloride in DI water) and incubated for 10 min at 25°C. The absorbance was measured at 520 nm. NO level was estimated from a NaNO$_2$ standard curve (y = 0.0226X + 0.0023, R^2 = 1).

Briefly, high-binding 96-well plates (NUNC, Roskilde, Denmark) were incubated overnight with capture antibody for mouse TNF-α and IL-6 at 25°C. After washing with 0.05% Tween-20 in PBS (PBST), the unbound sites were blocked with 1% bovine serum albumin (BSA) in PBS for 1 h at 25°C. After washing with PBST, culture medium or recombinant mouse TNF-α or IL-6 standards or DI water were added to each well and incubated for 2 h at 25°C prior to addition of biotinylated TNF-α or IL-6 antibodies to each well. After 1 h incubation and washing with PBST, the immune complex was detected with streptavidin HRP–tetramethylbenzidine detection system by incubating at 25°C for 30 min. Reactions were terminated with sulfuric acid (H$_2$SO$_4$) and the absorbance at 450 nm was measured by microtiter plate reader. Concentrations of TNF-α and IL-6 in samples were calculated from their standard curves. The equations of TNF-α and IL-6 standard curves were y = 0.0015X + 0.0481 (R^2 = 0.9978) and y = 0.0016X + 0.0092 (R^2 = 0.9981), respectively.

Western blot analysis

After LPS activation, the treated cell monolayers were washed with cold PBS and treated with ice-cold lysis buffer [50 mM Tris-hydrochloride pH 7.4, 150 mM sodium chloride (NaCl), 1 mM EDTA, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1% phosphatase inhibitor cocktail (Bio Basic Inc., Ontario, Canada) and 0.5% protease inhibitor cocktail (Sigma)] at 4°C for 30 min, on an orbital shaker. The supernatants of cell lysate were collected after centrifugation at 12,000 g at 4°C for 5 min. The protein concentration was determined by bicinchoninic acid assay. Samples [20 μg, 40 μg or 80 μg protein/well for IL-1β, iNOS, COX-2, IκB and MAPKs in loading buffer, respectively] were separated by 8% (for iNOS and COX-2), 10% (for IκB and MAPKs protein) or 12% (for IL-1β) of SDS-PAGE and transferred onto 0.45 μm nitrocellulose membranes (Whatman

GmbH, Dassel, Germany). Other procedures were followed as previously described (Tuntipopipat *et al.,* 2011).

Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 19.0 (SPSS Inc., Chicago, IL) was used to analyze data. All data were presented as mean±standard deviation (SD) from at least two separate experiments conducted on separate days. The statistical significance was determined by one-way analysis of variance (ANOVA) with Tukey's HSD (honestly significant difference) test for multiple comparisons to identify mean differences among treatment groups. Statistical significance was set at $p<0.05$.

RESULTS

Bioactive compounds in steamed OFSP extract

The HPLC chromatograms identified that β-carotene was the predominant carotenoid in OFSP extracted with 90% ethanol and solvent mixture of hexane, acetone and ethanol (in supplementary material). The β-carotene content of ethanol extract from steamed OFSP was 226±11 μg/g DW, while that of mixed solvent extract was 283.8±8.6 μg/g DW. Total polyphenol and flavonoid content in the ethanol extract of OFSP were 2.13±0.05 mg GAE/g DW and 0.24±0.01 mg QE/g DW, respectively.

Effect of 90% ethanol or mixed solvent extract from OFSP on cell viability

The cells treated with 0.5-2.0 mg/mL of 90% ethanol or mixed solvent extracts or ferulic acid followed by LPS treatment had a cell viability of more than 90% as compared with those treated with LPS alone. These results indicated that 0.5-2.0 mg/mL of 90% ethanol or mixed solvent extracts or ferulic acid did not show a significant effect on cell viability.

Comparison of 90% ethanol and mixed solvent extracts from steamed OFSP on LPS-induced NO production

RAW264.7 cell monolayers treated with ethanol extract from steamed OFSP at 0.5-2.0 mg/mL significantly inhibited LPS-induced NO production in a dose-dependent manner (Figure 1a) as compared to those treated with LPS alone ($p<0.05$), whereas cells treated with the extract from mixed solvent did not show any suppressive effect (Figure 1b). Thus, only the ethanol extract was used to assess other anti-inflammatory activities in the present study.

OFSP ethanol extract decreased NO production and iNOS expression

RAW264.7 cells exposed to LPS significantly produced NO while vehicle treated cells or the cells treated with ethanol extract alone had no significant effect (Figure 2a). Compared to the LPS treatment group, cell monolayers treated with OFSP ethanol extracts or ferulic acid significantly decreased LPS-induced NO production ($p<0.05$). NO is the product of enzyme iNOS. As expected, cells exposed to LPS up-regulated iNOS protein expression (Figure 2c) and the extracts significantly suppressed LPS-induced iNOS protein expression in a dose dependent manner when compared to LPS-only treatment group ($p<0.05$). Thus, the OFSP ethanol extract decreased NO secretion by inhibiting iNOS protein expression.

OFSP ethanol extract inhibited TNF-α, IL-6, IL-1β and COX-2 production

Exposure of RAW264.7 cells to LPS significantly produced TNF-α, IL-6 and IL-1β whereas the control vehicle or cells treated with extract alone had no significant effect (Figure 2b, d and f). Cell monolayers treated with OFSP ethanol extracts or ferulic acid before co-incubation with LPS significantly
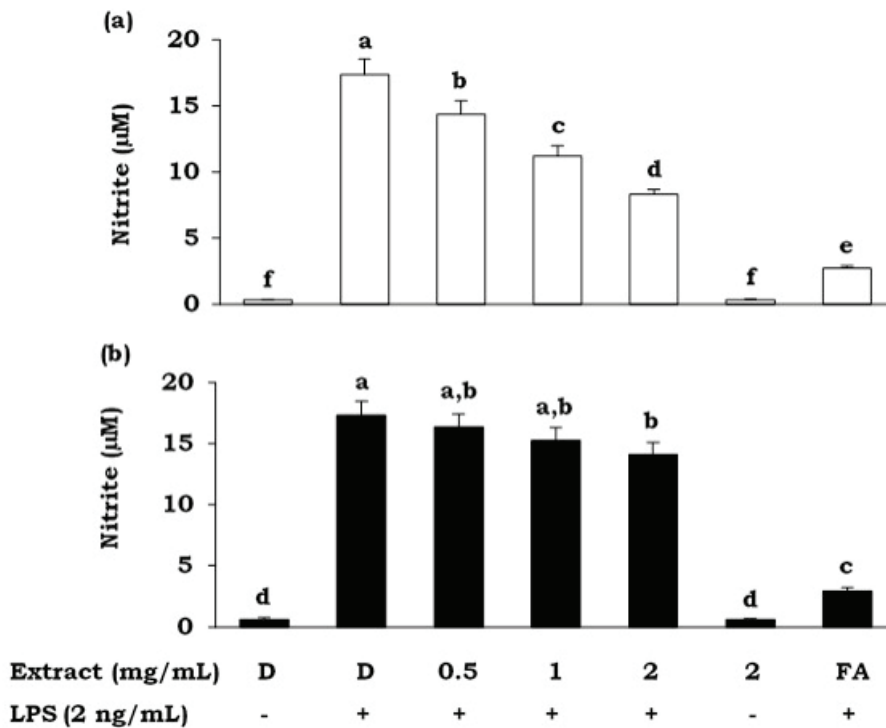
Figure 1. Effect of ethanol and mixed solvent extracts on LPS-induced NO production. Cells were pre-treated with 0.5-2.0 mg/mL of **(a)** ethanol or **(b)** mixed solvent extract from steamed OFSP or 0.2% DMSO (D) or 25 μM ferulic acid (FA) in serum/phenol red free media for 1 h, followed by 2 ng/mL LPS for 24 h. Ferulic acid was used as a control system. Nitrite in culture media was measured by Griess reagent. Data were expressed as mean±SD (n=6). Different letters above the error bars indicated significant differences among treatment groups (*p<0.05*)

decreased TNF-α, and IL-6 production and IL-1β expression when compared to LPS-only treatment group (*p*<0.05).

COX-2 is an inducible pro-inflammatory enzyme produced by macrophages during the inflammatory process. As expected, RAW264.7 cells exposed to LPS significantly enhanced COX-2 expression, whereas cells pre-treated with OFSP ethanol extracts or ferulic acid significantly inhibited LPS-induced COX-2 protein expression (Figure 2e) as compared to LPS-only treatment group (*p*<0.05). These results indicated that bioactive compounds in the ethanol extract of OFSP exerted anti-inflammatory activity by the suppression

of LPS-induced pro-inflammatory mediator production.

OFSP ethanol extract suppressed MAPKs phosphorylation

Cell monolayers treated with LPS for 24 h markedly activated phosphorylation of ERK1/2, JNK and p38 (Figure 3a-c) without effecting their total ERK1/2, JNK and p38. Cell monolayers treated with the OFSP extract significantly inhibited phosphorylation of ERK1/2 and JNK in a dose-dependent manner (Figure 3a, b) whereas phosphorylation of p38 was significantly inhibited only at 1-2 mg/mL of the OFSP extract (Figure 3c) as compared to LPS-only treatment
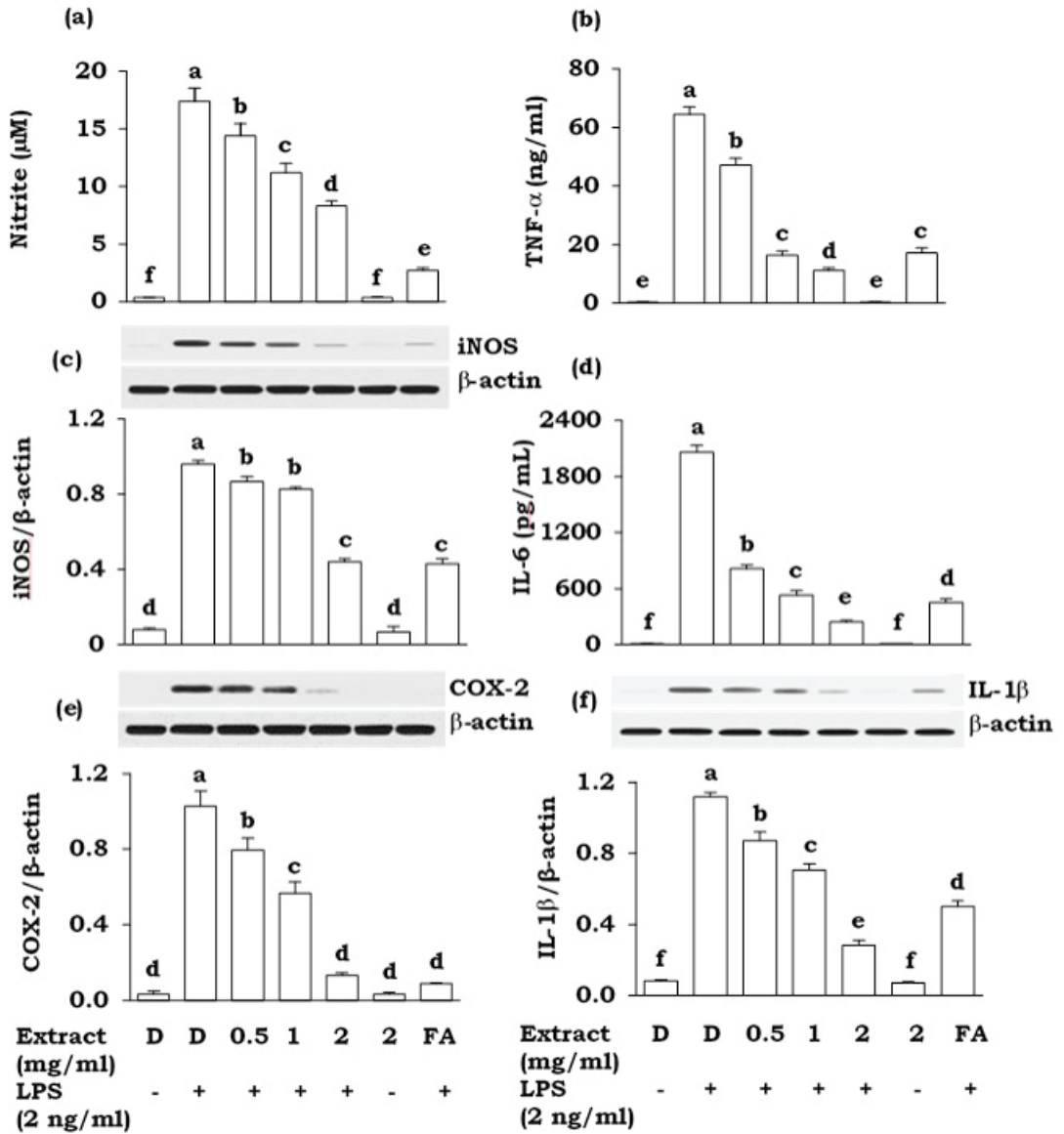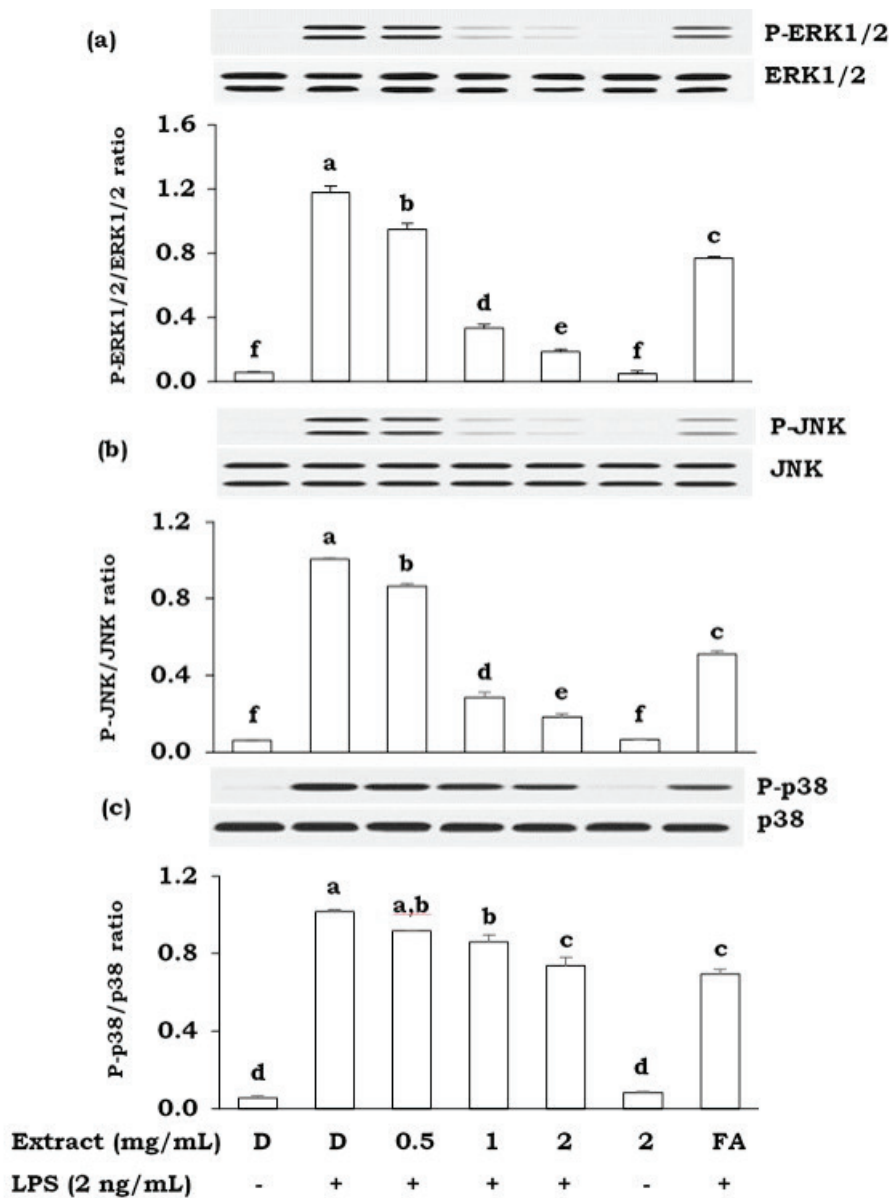
Figure 2. Ethanol extract from steamed OFSP inhibited LPS-induced NO, TNF-α, IL-6 production, iNOS, COX-2 and IL-β expression. Cells were treated with 0.5-2.0 mg/mL ethanol extract or 0.2% DMSO (D) or 25 μM ferulic acid (FA) in serum/phenol red free media for 1 h, followed by 2 ng/mL LPS for 24 h. Culture media were collected to measure **(a)** nitrite, **(b)** TNF-α and **(d)** IL-6 and cell lysates were collected to measure **(c)** iNOS/β-actin, **(e)** COX-2/β-actin and **(f)** IL-1β/β-actin. Data were expressed as mean±SD (n=6). Different letters above the error bars indicated significant differences among treatment groups (*p<0.05*)

group (*p*<0.05). Ferulic acid treated cells also significantly suppressed phosphorylation of ERK1/2, JNK and p38 (Figure 3a-c). Thus, the steamed OFSP ethanol extract inhibited pro-inflammatory mediator expression by blocking MAPKs phosphorylation.
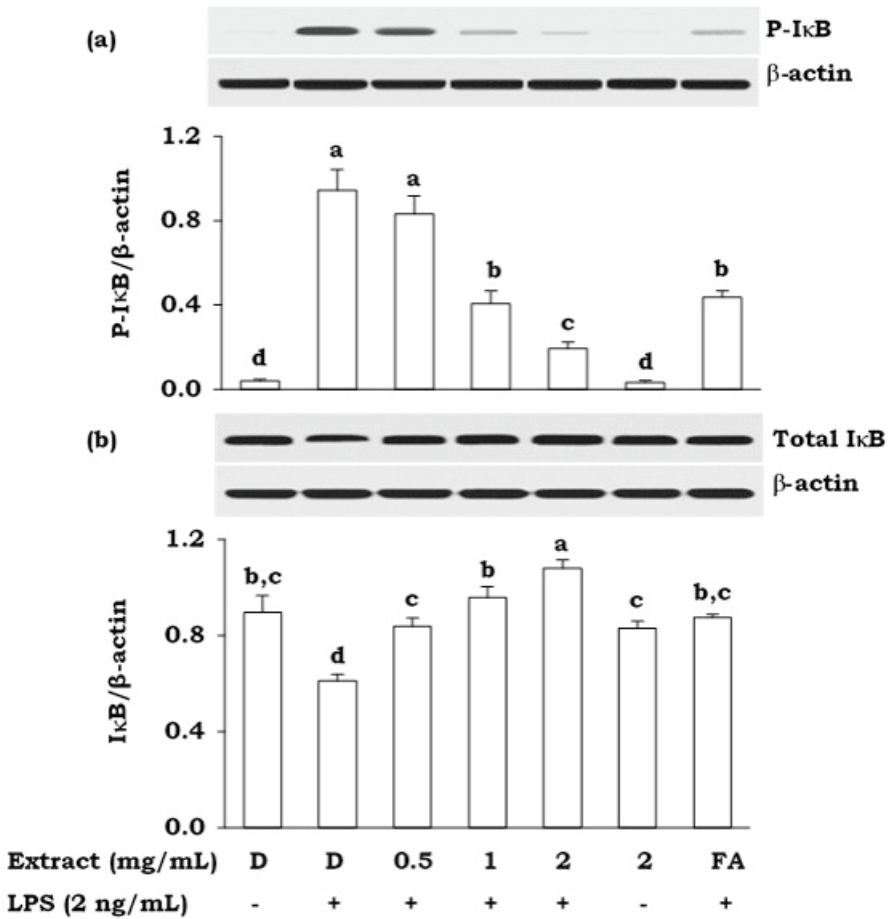
Figure 3. Ethanol extract from steamed OFSP inhibited LPS-activated MAPKs signalling. Cells were treated with 0.5-2.0 mg/mL ethanol extract or 0.2% DMSO (D) or 10 μM ferulic acid (FA) in serum free media for 1 h, followed by 2 ng/mL LPS for 24 h before collecting cell lysate to measure **(a)** phospho-ERK1/2 and total ERK1/2, **(b)** phospho-JNK and total JNK and **(c)** phospho-p38 and total p38. Data were expressed as mean±SD (n=4). Different letters above the error bars indicated significant differences among treatment groups (*p<0.05*)

OFSP ethanol extract inhibited IκB phosphorylation and degradation
LPS activated RAW264.7 cells induced IκB phosphorylation and degradation

(Figure 4a, b). Pretreatment of cell monolayers with OFSP extract significantly inhibited LPS-activated IκB phosphorylation and degradation at 1-2

Figure 4. Ethanol extract from steamed OFSP inhibits LPS-activated IκB phosphorylation and degradation. Cells were pre-treated with 0.5-2.0 mg/mL ethanol extract or 0.2% DMSO (D) or 25 μM ferulic acid (FA) in serum free media for 1 h, followed by 2 ng/mL LPS for 24 h before collecting the cell lysates to measure **(a)** phospho-IκB/β-actin and **(b)** total IκB/β-actin. Data were expressed as mean±SD (n=6). Different letters above the error bars indicated significant differences among treatment groups ($p<0.05$)

mg/mL (Figure 4a, b) when compared with LPS-only treatment group ($p<0.05$). These results indicated that steamed OFSP ethanol extract inhibited pro-inflammatory mediator production via suppression of IκB phosphorylation and degradation.

DISCUSSION

OFSP is a good source of macro- and micro- nutrients and various phytochemicals (Wang, Nie & Zhu,

2016). The present study found that OFSP extract contained β-carotene, polyphenols and flavonoids, which were consistent with data from previous studies (Failla *et al.,* 2009; Tang, Cai & Xu, 2015). β-carotene is the predominant carotenoid, which is consistent with a previous study (Failla *et al.,* 2009). The ethanol extract contained 226 μg/g DW of β-carotene while the mixed solvent extract contained 284 μg/g DW of β-carotene. A previous study in

Taiwan (Liu, Lin & Yang, 2009) reported the presence of 127-258 μg/g DW of β-carotene in mixed solvent extract of OFSP (Tainung 66 variety). However, the amount of mixed solvent extract in this study was slightly higher than that of the Taiwanese variety.

The ethanol extract from steamed OFSP contained 2.13 mg GAE/g DW of total polyphenolic compounds. This figure was lower than the Chinese variety (5.19 GAE/g DW) (Tang *et al.,* 2015) but was significantly higher than the Brazilian variety (1.05-1.56 mg GAE/g DW) (Donado-Pestana *et al.,* 2012). Our study demonstrated that the total flavonoid content of steamed OFSP extract was 0.24 mg QE/g DW which was slightly lower than the Taiwanese variety which had 0.33 mg QE/g DW of total flavonoids (Huang, Chang & Shao, 2006). The amount of β-carotene, polyphenol and flavonoid compounds in OFSP is dependent on many factors such as the variety, environmental conditions and agricultural management. In addition, the solvent used for extraction and the method used for the measurement of carotenoids by HPLC also influenced the measurable β-carotene content as reported in previous studies (Rautenbach *et al.,* 2010). Therefore, it is difficult to compare our results for the amounts of bioactive compounds present in OFSP with those reported in other studies.

Besides β-carotene, other compounds found in OFSP which have demonstrated anti-inflammatory activity include phenolic acids such as caffeic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, FA, sinapic acid and flavonoids including quercetin, myricetin, kaempferol and luteolin (Ambriz-Pérez *et al.,* 2016; Pan *et al.,* 2010; Li, Hong & Zheng, 2018). As OFSP contains various bioactive compounds such as phenolic acids, flavonoids (polar organic compounds) and carotenoids (non-polar organic compounds), the comparative effect of its extracts from ethanol (polar) and hexane:acetone:ethanol mixture (non-polar) on LPS-induced NO production in RAW264.7 cells were investigated to select the potent solvent. The results revealed that the ethanol extract significantly inhibited NO production, whereas the mixed solvent extract did not show any suppressive effect (Figure 1a, b). Although mixed solvent extract contained a higher amount of β-carotene than the ethanol extract, its suppressive effect on LPS-induced NO production did not correlate with the β-carotene content. It implied that organic compounds in mixed solvent extract did not play major role in suppressive effect or mixed solvent extract may contain interfering compounds that neutralize such an inhibitory effect. Conversely, the polar organic compounds in the ethanol extract may play an important role in this inhibitory effect. Therefore, the ethanol extract was selected to further assess anti-inflammatory activity in the present study.

The ethanol extract also decreased NO secretion by inhibiting iNOS protein expression (Figure 2a, c). NO is a vital free radical that plays an important role in the progression of inflammation. It is synthesized by the oxidation of L-arginine to L-citrulline through the activity of NOS (Phaniendra, Jestadi & Periyasamy, 2015). iNOS is up-regulated during inflammation leading to NO generation. LPS can stimulate macrophages to produce NO, which is mediated by activation of transcription factor "NF-κB". Our results also demonstrated that OFSP ethanol extract inhibited the production of TNF-α and IL-6 (Figure 2b, d) and suppressed IL-1β and COX-2 expressions (Figure 2f, e). These pro-inflammatory cytokines and enzymes play crucial roles in activating the acute phase of immune response. They promote tissue damage in the pathogenesis of

chronic inflammatory diseases and facilitate tumour progression and invasiveness. Additionally, prolonged overexpression of such pro-inflammatory mediators is mainly associated with the loss of apoptosis, uncontrolled cell proliferation, growth, metastasis, neovascularization and angiogenesis, leading to development of pathogenesis of various inflammatory diseases.

In addition, pre-treatment of RAW264.7 cells with OFSP extract inhibited LPS-induced pro-inflammatory protein expression by suppressing the phosphorylation of MAPKs including ERK, JNK and p38 (Figure 3a-c) and IκB activation (Figure 4a, b). These inflammatory effects of LPS are widely known as an important inducer for triggering the phosphorylation of MAPKs, resulting in NF-κB activation. MAPKs are serine/threonine protein kinases that mediate intracellular signalling related with the regulation of biological processes and cellular activities such as cellular stress, inflammatory responses, gene induction, cell proliferation, differentiation, survival, apoptosis and transformation (Kim & Choi, 2010). Additionally, JNK activation has been associated with the regulation of cellular functions such as cell proliferation, survival and differentiation. JNK is activated by LPS, environmental stress, growth factors and inflammatory cytokines such as IL-1β and TNF-α. JNK plays an important role in the transcriptional regulation of many inflammatory mediators such as IL-2, iNOS and COX-2. Short-term JNK activation can promote cell survival, but prolonged JNK activation induces cellular apoptosis (Arndt *et al.,* 2004). Furthermore, p38 activation has been implicated in the production and activation of inflammatory mediators for initiating leukocyte recruitment and activation. Also, p38 plays a key role in regulating the expression of various

genes related to inflammation such as those encoding TNF-α, IL-1β, IL-6, IL-8, iNOS and COX-2 (Guha & Mackman, 2001; Neuder *et al.,* 2009).

The master transcription factor "NF-κB" is a dimer protein that is bound to IκB protein in cytoplasm during quiescent state. When macrophages are exposed to LPS, NF-κB is activated and IκB undergoes phosphorylation and degradation by the action of the IκB kinase (IKK) complex. NF-κB becomes free and is translocated into the nucleus, which then binds to responsive elements and induces many genes encoding inflammatory mediators including TNF-α, IL-1β, IL-6, COX-2 and iNOS (Liu *et al.,* 2017). The present results indicated that the suppressive effects of OFSP extract on pro-inflammatory mediator expression were mediated partly by inactivation of MAPKs (Figure 3a-c) and IκB (Figure 4a, b) signalling pathway. Therefore, the suppression of these signalling pathways may reveal the potent activity of our extract as an inhibitor of inflammatory mediators and cytokines.

The bioactive compounds present in the steamed OFSP extract may act synergistically to suppress inflammatory mediator production observed in the present study. According to a previous report, mouse peritoneal macrophages treated with phytonutrient mixture (lycopene or Lyc-O-Mato and carnosic acid, lutein, and/or β-carotene) revealed a synergistic inhibition of LPS-induced TNF-α, NO, prostaglandin E_2 (PGE_2) and superoxide production derived from down-regulation of iNOS, COX-2 and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in both messenger RNA (mRNA) and protein expression. A combination of phytonutrients is known to exhibit an anti-inflammatory effect by the 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 (Hadad & Levy, 2012). This study identified and quantified the predominant carotenoid in the extract bioactive compound in OFSP, namely β-carotene. Supporting evidence indicated that synthetic β-carotene inhibited LPS-stimulated COX-2, iNOS and TNF-α gene expression in RAW264.7 cells (Kawata *et al.,* 2018). β-carotene treatment inhibited the production and expression of various pro-inflammatory mediators in LPS stimulated RAW 264.7 cells by suppressing the phosphorylation and degradation of IκB/NF-κB pathway (Li *et al.,* 2018). However, phenolic acids including caffeic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, *p*-coumaric acid, FA and sinapic acid and flavonoids namely, quercetin, myricetin, kaempferol and luteolin were also found in OFSP (Park *et al.,* 2016) which might exert anti-inflammatory activity along with β-carotene. Due to the potent anti-inflammatory effect that were observed in the present study, OFSP may be an alternative promising functional food for preventing or reducing the risk of inflammatory diseases.

CONCLUSION

The present study indicated that the ethanol extract of steamed OFSP inhibited the production and expression of several pro-inflammatory mediators by suppressing the MAPKs and IκB activation on LPS-induced murine macrophage cell line. These findings clearly demonstrated that OFSP had anti-inflammatory potential, and that regular consumption of OFSP may reduce risk of inflammatory diseases. 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 an alternative food supplement to prevent or alleviate severity of inflammatory diseases.

Authors' contributions

YS, conducted the study, performed data analysis and interpretation, and prepared draft of the manuscript; KP, gave advice on the analytical methods and data analysis; MS, reviewed and edited the manuscript; ST, designed the study, gave advice on the analytical methods and data analysis, edited and overviewed the manuscript.

Conflict of interest

The authors declare that they have no conflicts of interest.

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