ผลของสารสกัดเอทานอลจากเนื้อและเยื่อหุ้มเมล็ดของผลฟักข้าว ต่อภาวะต้านการอักเสบในเซลล์ไลน์แมคโครฟาจของหนูชนิด RAW264.7 ที่เหนี่ยวนำด้วยไลโปโปลิแซคคาร์ไรด์

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บทคัดย่อ

ผลฟักข้าวมีสารพฤกษเคมีหลากหลายชนิดซึ่งมีหน้าที่ทางชีวภาพหลายอย่าง รวมทั้งฤทธิ์ในการต้านการอักเสบ การศึกษานี้ทำการทดสอบฤทธิ์การต้านการอักเสบของสารสกัดเอทานอลจากเนื้อและเยื่อหุ้มเมล็ดฟักข้าวในเซลล์ไลน์แมคโครฟาจของหนูที่เหนี่ยวนำการอักเสบด้วยไลโปโปลิแซคคาร์ไรด์ จากการวิเคราะห์สารออกฤทธิ์ชีวภาพพบว่าเยื่อหุ้มเมล็ดมีปริมาณ เบต้าแคโรทีน ไลโคปีน total phenolic และ total flavonoid สูงกว่าเนื้อฟักข้าว เมื่อนำสารสกัดมาเลี้ยงเซลล์ก่อนกระตุ้นด้วยสารไลโปโปลิแซคคาร์ไรด์ พบว่าสามารถยับยั้งการแสดงออกของตัวชี้วัดการอักเสบ ได้แก่ NO, iNOS, COX-2, TNF-α และ IL-6 ได้ดี ($p < 0.05$) ซึ่งศักยภาพการยับยั้งแปรผันตามปริมาณสารสกัดที่มากขึ้นโดยไม่เป็นพิษกับเซลล์ ผ่านกลไกการยับยั้งการกระตุ้นโปรตีน IKB-α และ MAPKs (p38 ERK1/2 และ JNK) โดยสารสกัดจากเยื่อหุ้มเมล็ดมีศักยภาพในการลดการอักเสบดีกว่าสารสกัดจากเนื้อฟักข้าว ผลการศึกษาเหล่านี้แสดงให้เห็นถึงคุณประโยชน์ต่อสุขภาพของผลฟักข้าวที่มีนัยสำคัญ อย่างไรก็ตามคุณสมบัติต่อสุขภาพดังกล่าวควรมีการศึกษาต่อในสัตว์ทดลองและในมนุษย์ที่เป็นโรคที่มีภาวะการอักเสบเรื้อรังต่อไป

คำสำคัญ: ผลฟักข้าว การต้านการอักเสบ ไลโปโปลิแซคคาร์ไรด์ เซลล์แมคโครฟาจ

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Ethanol Extract of Aril and Pulp from *Momordica cochinchinensis* Fruit Exhibits Anti-inflammatory Effect in LPS-induced Macrophage RAW264.7 Cells

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Abstract

Gac (*Momordica cochinchinensis* Spreng.) fruit contains several bioactive compounds exhibiting multiple biological functions including anti-inflammatory activity. In the present study, ethanol extracts of pulp and aril from gac fruit were compared for their anti-inflammatory effects in lipopolysaccharide (LPS)-induced murine macrophage cell line (RAW264.7 cells). Aril extract had greater amounts of β-carotene, lycopene, total flavonoids and phenolic compounds than pulp extract. Prior treatment cell monolayer with pulp and aril extract significantly suppressed LPS-induced NO, iNOS, COX-2, TNF-α, and IL-6 expressions ($p < 0.05$) by dose-dependently without a cytotoxic effect. The suppressive effect was mediated partly by inhibiting phosphorylation and degradation of IκB-α and phosphorylation of mitogen-activated protein kinases (p38, ERK1/2 and JNK). The aril extract exhibited a greater anti-inflammatory effect than the pulp extract. These data support a significant health benefit effect of gac fruit. However, this functional activities warrant further study in both animal and humans inflicted with inflammatory-associated chronic diseases.

Key words: Gac fruit, Anti-inflammation, LPS, macrophage RAW264.7 cells

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Introduction

Gac (*Momordica cochinchinensis*) fruit has long been consumed in Asia. The ripe fruits have deep orange or red skin. Gac fruits contain various bioactive compounds, such as β-carotene, lycopene, essential fatty acids, lutein, α-tocopherol (vitamin E), phenolic compounds and flavonoids[1-3]. Red aril has been reported to contain β-carotene five times greater than those of carrots, as well as lycopene content eight times greater than in tomatoes[4-6]. The pulp of green fruits and the aril of ripe fruits also contain significant amounts of phenolic compounds and flavonoids[2]. Due to high carotenoid content and other functional phytochemicals, both aril and pulp of gac fruits have multiple biological activities including pro-vitamin A, antioxidant, anti-cancer and anti-atherogenic effects[7].

Chronic inflammation is proposed to be a crucial mechanism underlying the pathophysiology of Metabolic syndrome (MetS)[8] and other chronic diseases[9]. A previous study found that metabolic syndrome patients exhibit characteristics of a pro-inflammatory state[10]. During inflammatory response, macrophages are induced by several stimuli resulting in the release of several pro-inflammatory mediators, including nitric oxide (NO), cyclooxygenase-2 (COX-2), tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), IL-6, etc.[11]. Excessive expression of such pro-inflammatory mediators contributes in the pathogenesis of many inflammatory diseases[12, 13]. NF-κB, a master transcription factor, is a dimer protein associated with IκB-α protein in cytoplasm. Upon activation by inducers, including LPS, IκB-α protein is phosphorylated, ubiquitinated, and subsequently degraded in the proteasomes. Consequently, NF-κB is released and translocates into the nucleus and binds to promotor regions that regulate transcription of more than 200 genes including pro-inflammatory mediator genes, such as pro-inflammatory cytokines, chemokines, COX-2, and iNOS[14]. Mitogen-activated protein kinase, or MAPKs, is another pathway playing a critical role in regulating expression of inflammatory mediators and cytokines induced by inducers that modulate severity of inflammatory diseases[15]. Consequently, both NF-κB and MAPK pathway are important molecular targets for the development of potential anti-inflammatory agents.

Several pure bioactive compounds, such as β-carotene[16], lycopene[17], flavonoids[18] and phenolic compounds[19] are reported to have anti-inflammatory effects *in vitro* and *in vivo*. Due to the high content of such compounds in gac fruit pulp and aril, the present study aimed to assess anti-

inflammatory activity of extracts from gac fruit pulp and red aril in LPS-induced murine macrophage cell lines (RAW264.7 cells)

Materials and methods

Chemicals

Dulbecco's Modified Eagle's Medium (DMEM), LPS (Escherichia coli O11:B4), anti-β-actin–conjugated HRP, and species-specific HRP conjugated secondary polyclonal antibodies were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum was purchased from Millipore (US Origin, EmbryoMax® ES Cell Qualified FBS). Penicillin and streptomycin were obtained from Invitrogen (Grand Island, NY, USA). Primary antibodies against iNOS (Abcam, Cambridge, UK), COX-2, phospho-p38, total p38, phospho-JNK, total JNK, phospho-ERK1/2, total ERK1/2, phospho-IκB, and total IκB were purchased from Cell Signaling Technology (Danvers, MA, USA). Chemical and reagents were analytical and HPLC grade.

Preparation of dry sample

M. cochinchinensis (gac fruit) was purchased from three representative farms in Kamphaengsaen district, Nakhonpathom province of Thailand. The skin was peeled and the pulp and aril (seed membrane) were carefully separated and homogenized using a kitchen blender. An equal amount from each farm was pooled prior to boiling in deionized water (DI) at a ratio 1:1 (w/v) for 20 min and let cool to room temperature and then lyophilization. The dry sample was ground using a kitchen blender, packed in vacuum-aluminum foil, and kept at -20 °C until analysis.

Analysis for carotenoids

Dried samples of 0.02 g were suspended with 1 mL DI and homogenized by Vibra cell^{TM} Ultrasonic Liquid Processor on ice. Further extraction protocol was added with 9 mL of absolute ethanol (90% final concentration) or 10 mL of hexane:acetone:ethanol (2:1:1)[2] to obtain different types of active compounds and thoroughly mixed for 1 min and sonicated for 10 min and then centrifuged at 4,140 g for 10 min at 25°C. The supernatants were combined and solvent was evaporated to dryness. The residue was re-solubilized in 2 mL of the mobile phase and filtered through a 0.22 μm PTFE membrane prior to analysis by HPLC. Carotenoids were separated by a C30 reverse-phase column (YMC carotenoid 150 x 4.6 mm ID, 5μm, Serial No., 114FA70081, Japan) with a C18 cartridges guard column (Phenomenex C18, 4 x 3 mm, Torrance, USA). The mobile phase consisted of a mixed solvent of 98% methanol containing 2%

ammonium acetate pH 4.6 and 100% methyl tertiary-butyl ether. The carotenoids content was separated by gradient elution programs at a flow rate of 0.6 mL/min with an injection volume of 20 µL and the absorbance was read at 450 and 470 nm as previously described[20].

Analysis of total phenolics and total flavonoids content

The extract sample was modified from a previous protocol[2]. A dry sample of 0.5 g was suspended with 2 mL DI and homogenized by Vibra cell™ Ultrasonic Liquid Processor on ice and then 18 mL of absolute ethanol added (90% final concentration) was added and shaken on an orbital shaker at 25 °C for 2 h. The mixture was centrifuged at 1,400 g for 20 min and the supernatant was transferred into an amber vial. Total phenolics and total flavonoid contents were measured in the supernatant.

1. Determination of total phenolics content

The total phenolics content was determined by using Folin-Ciocalteu reagent[21]. Briefly, 25 µL of extract, standard (gallic acid) or DI (blank), were added into a clear 96-well plate; 50 µL of diluted Folin-Ciocalteu reagent (dilute with deionized water 1:10) was added and allowed to stand at 25 °C for 5 min and then 200 µL of 7.5% Na_2CO_3 solution was added into each well and incubated at 25 °C for 2 h. Absorbance was measured at 760 nm by Microplate Reader (BioTek® Instruments, Vermont, and USA). The total phenolic content of the extracts was calculated and expressed as gallic acid equivalents per gram of dry weight (mg GAE/gDW).

2. Determination of total flavonoids content

Total flavonoids content was determined by using the Aluminium chloride colorimetric method[22]. Briefly, 25 µL of the extract or standard quercetin or DI was added into each well of a 96-well plate and then 75 µL 90% ethanol, 5 µL of 10% aluminium chloride solution, 5 µL of 1M potassium acetate solution and 140 µL DI were added to each well. Sample blank of extract and standard quercetin were performed in the same procedure by replacing aluminium chloride solution with DI. The absorbance was immediately measured at 415 nm using a Microplate Reader (BioTek® Instruments, Vermont, and USA). Results were expressed as mg quercetin equivalents in 1 g of dried sample (mg QE/g).

Sample extraction for cell culture treatment

Dried samples of 0.5 g were suspended with 2 mL DI and homogenized

by Vibra cell™ Ultrasonic Liquid Processor on ice. Absolute ethanol 18 mL was added (90% final concentration) and thoroughly mixed for 1 min, sonicated for 10 min and then centrifuged at 4,140 g for 10 min at 25°C. The supernatant was transferred to an amber round bottomed flask. The sample was further extracted 3 times with 20 mL of 90% ethanol. The combined supernatant was evaporated to dryness before being kept in -20ºC until use. The dried extract was re-solubilized with 0.2% Dimethyl sulfoxide (DMSO) and further diluted to a designated concentration with phenol red and serum free medium and filtered through a sterile 0.2 µm filter (cellulose acetate) prior to addition to the RAW264.7 cell monolayer.

Growth and activation of cells

Murine macrophage RAW264.7 cells were grown in DMEM supplemented with 10% fetal bovine serum, 15 mM N-(2-hydroxyethyl) piperazine-N′-(2-ethanesulfonic acid), 100 U/mL of penicillin, and 100 µg/mL of streptomycin at 37 °C in humidified atmosphere of 5% CO_2/95% air. Cells were used for experimentation between passages 10 and 20. Cells (7.5×10^5 cells/mL) were seeded for 24 h and incubated with extract for 1 h prior to co-culture with LPS (2 ng/mL) in serum-free medium for another 24 h.

Cell viability

Cytotoxic doses were determined by sulforhodamine B assay (SRB)[23]. In brief, pretreated cell monolayer with or without extract prior to stimulation with LPS for 24 h were washed with phosphate buffered saline (PBS) and fixed with cold trichloroacetic acid at 4 °C for 2 h. Cell monolayers were stained with SRB in acetic acid for 20 min and solubilized with Tris-hydro-methyl-aminomethane at pH 10. Absorbance at 500 nm was measured, with absorbance of the vehicle control defined as 100% viability.

Nitric oxide measurement

NO level was assayed by measuring the levels of nitrite. Cell monolayers were treated with or without non-toxic doses of pulp or aril extracts or 25 µM ferulic acid (a well-known anti-inflammatory phenolic acid[24]) for 1 h prior to stimulation with LPS for 24 h. The spent media were collected to measure nitrite concentrations using the Griess reaction by adding 100 µL of Griess reagent (0.1% naphthyl-ethylene-diamide dihydrochloride in H_2O and 1% sulfanilamide in 5% concentrated phosphoric acid) to 100 µL of sample. Nitrite concentration was calculated by comparison with sodium nitrite standard.

Measurement of cytokines

TNF-α and IL-6 in monolayer-treated culture medium were determined by a quantitative "sandwich" ELISA using paired antibodies purchased from Peprotech Inc. (Rocky Hill, NJ, USA) and Biolegend Inc. (San Diego, CA, USA), respectively. In brief, high-binding plates (NUNC, Roskilde, Denmark) were coated with capture antibody for mouse TNF-α and IL-6 overnight at 25 °C. Excess antibodies were washed and blocked unbinding sites with 1% bovine serum albumin (BSA) in PBS for 1 h at 25 °C. After washing excess materials, culture medium or recombinant mouse TNF-α and IL-6 standards were added to bind with captured antibodies for 2 h at 25 °C prior to addition of biotinylated TNF-α and IL-6 antibodies to each well. After 1 h incubation at 25 °C, the immune complex was detected using a streptavidin HRP–tetramethylbenzidine detection system incubated for 30 min at 25 °C. Reactions were terminated with H_2SO_4 and absorbance at 450 nm was determined using a microtiter plate reader (BioTek® Instruments, Vermont, USA). Concentrations of TNF-α and IL-6 in samples were calculated by comparing absorbance with their standard curves.

Western blot analysis

Treated cell monolayers were washed twice with ice-cold PBS, and resuspended in ice-cold lysis buffer [50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetra-acetic acid, 1% Triton X-100, 0.1% sodium dodecylsulfate, 50 mL of sodium fluoride, 10 mM sodium pyrophosphate, 0.5% protease inhibitor cocktail (Sigma), 1% phosphatase inhibitor (Bio basic; Markham ON, Canada)] for 30 min at 4 °C. Cell lysate was collected after centrifugation at 13,500 × g at 4 °C for 5 min. Protein content was determined by a bicinchoninic acid assay using BSA as a standard. Samples (40-80 μg of protein/well) were separated by 8% (for detection of iNOS and COX-2 protein) or 10% (MAPKs and IκB-α protein) SDS-PAGE and transferred onto nitrocellulose membranes (Merck-Millipore). Membranes were washed 3 times using Tris-buffered saline containing 0.1% Tween 20 (TBST) and blocked with 5% non-fat dry milk in TBST for 1 h. After washing, membranes were incubated with specific primary antibody diluted with 5% BSA in TBST overnight at 4 °C. After washing, membranes were reacted with horseradish peroxidase (HRP)-conjugated secondary antibody for 2 h. After five washes, membranes were incubated with Super Signal solution (Endogen) for 5 min and exposed to X-ray film. The same membranes were then stripped of bound antibody and re-probed with anti–β-actin or

anti-total MAPK protein to assess the equal loading protein. The density of target bands was quantified by Image J software. Results are expressed as relative ratios of band density between the protein of interest and β-actin.

Statistical analysis

Statistical analysis was performed using SPSS 19.0 (SPSS Inc., Chicago, IL, USA). Data were presented as mean ± SD. The mean values were calculated from at least three separate experiments conducted on separate days. Statistical differences were determined by one-way analysis of variance followed by Tukey's Test for multiple comparisons of group means. Statistical significance was set at $P < 0.05$.

Results

As shown in Table 1, the ethanol extract of pulp contained 98 and 66 μg/g dry weight of β-carotene and lycopene, while those of aril were 2.6 times and 5 times greater than those of pulp extract, respectively. The hexane/acetone/ethanol extract of pulp contained 122 and 94 μg/g dry weight of β-carotene and lycopene, while those of aril extract had β-carotene and lycopene had 4.8 times and 23 times of pulp extract, respectively. Anti-inflammatory activity of ethanol and hexane/acetone/ethanol extract was compared in LPS-induced NO and TNF-α production by RAW264.7 cells. According to the IC_{50} of NO and TNF-α, the ethanol extract from pulp and aril exhibited much better anti-inflammatory activity than those of hexane/acetone/ethanol extract, although hexane/acetone/ethanol extract had higher β-carotene and lycopene than ethanol extract (Table 1). Consequently, only ethanol extracts of pulp and aril were used to assess their anti-inflammatory activity in the present study.

Pulp and aril extract decreases NO production and iNOS expression

Pretreatment of RAW264.7 cells with 0.5–2.0 mg/mL pulp or aril extracts dose dependently decreased LPS-induced NO production by 22%-70% and 33%-89%, respectively, without exerting cytotoxicity (data not shown), while 25 μM ferulic acid treated cell monolayer decreased NO production by 65% (Figure 1A-1B). NO is the product of enzyme inducible nitric oxide synthases (iNOS). As expected, the iNOS protein expression was significantly increased after stimulation by LPS (Figure 1C-1D). Prior treatment cell monolayer with pulp or aril extract inhibited LPS-induced iNOS protein expression by 7%-42% and 5%-57%, respectively, while ferulic acid treated cells decreased LPS-induced iNOS expression by 49%. These

results indicated that both pulp and aril extract decreased NO production via suppression of iNOS protein expression, but the aril extract showed a greater inhibitory effect than the pulp extract.

Table 1. Carotenoids, flavonoids, total phenolic content, IC_{50} NO and IC_{50} TNF-α of 90% ethanol (EtOH) and Hexane/acetone/EtOH extracts from pulp and aril

Parameter	Pulp		Aril	
	90% EtOH	Hexane/acetone/EtOH	90% EtOH	Hexane/acetone/EtOH
β-carotene (μg/g)[1]	98.64 ± 5.04	121.89 ± 5.10	256.13 ± 12.02	594.42 ± 3.73
Lycopene (μg/g)	65.72 ± 1.56	93.53 ± 1.82	331.88 ± 6.33	2153.02 ± 8.16
Total flavonoids (mg/g)[1]	0.21 ± 0.01	-	2.81 ± 0.14	-
Total phenolic (mg/g)	2.34 ± 0.14	-	4.61 ± 0.17	-
IC_{50} NO (mg/mL)[2]	1.43 ± 0.02	11.92 ± 0.04	0.86 ± 0.02	6.86 ± 0.02
IC_{50} TNF-α (mg/mL)	1.91 ± 0.01	10.93 ± 0.12	1.14 ± 0.02	5.78 ± 0.25

[1] microgram or milligram per gram dry weight (mean±SD; n=3)
[2] IC50 represents the concentration of the extract required to decrease 50% of LPS-induced NO and TNF-α production by RAW264.7 cell monolayer treated with LPS only.

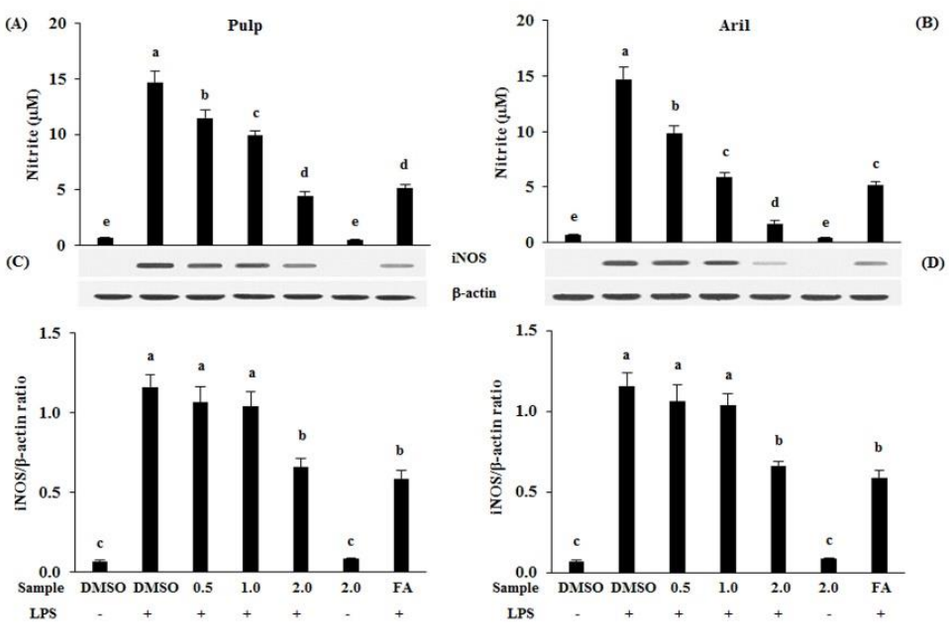

Figure 1. Pulp and aril extracts decrease NO production and iNOS expression in LPS-activated murine macrophages. Cell monolayers were pretreated with 0.5-2.0 mg/mL of pulp (A,C) or aril (B,D) extracts or 25 μM ferulic acid (FA) or DMSO prior to incubation with/without LPS for 24 h as described in materials and methods. Culture media were collected to determine nitrites (A and B). Lysates from treated cells were analyzed for iNOS and β-actin protein by immunoblot. Results are expressed as ratios of band intensity of iNOS to β-actin protein (C

and D). Data are means ± SD of three replicate experiments. Values with no common letters are significantly different from each other ($p < 0.05$).

Pulp and aril extract attenuates LPS-mediated increases in expression of COX-2, IL-6 and TNF-a

Treating RAW264.7 cell monolayer with pulp or aril extracts prior to co-incubation with LPS dose-dependently inhibited COX-2 protein expression by 9%-67% and 9%-83%, respectively, while ferulic acid strongly suppressed LPS-induced COX-2 expression (Figure 2). Likewise, LPS stimulated RAW264.7 cells to secrete IL-6 in culture medium from a basal level of 16 pg/mL up to more than 500 pg/mL (Figure 3). Pretreating RAW264.7 cells with the pulp or aril extract or ferulic acid significantly decreased IL-6 secretion by 65%-80% and 71%-94% and 72%, respectively, (Figure 3A-3B). As expected, TNF-α significantly increased in cells exposed to LPS (Figure 3C-3D). Pretreatment with the pulp or aril extract or ferulic acid significantly decreased TNF-α secretion by 13%-52% and 28%-70% and 68%, respectively. Similar to the effect on NO and iNOS, the aril extract had a greater potency to suppress COX-2, IL-6 and TNF-α than the pulp extract.

Pulp and aril extract inhibits phosphorylation and degradation of IκB-α

LPS significantly induced phosphorylation of IκB-α in RAW264.7 cells (Figure 4A-4B). Pretreatment of RAW264.7 cells with the pulp, aril extract and ferulic acid significantly attenuated LPS-mediated phosphorylation of IκB-α by 9%-53%, 17%-72% and 43%, respectively (Figure 4A-4B). LPS significantly induced IκB-α degradation (Figure 4C-4D). The pulp, aril extract and ferulic acid inhibited degradation of IκB-α in a dose dependent manner (Figure 4C-4D).

Pulp and aril extract suppresses MAPK signaling pathway activation

MAPKs, namely p38, ERK1/2 and JNK are activated by LPS in macrophages and many other cell types. Compared to the control cells, LPS increased the phosphorylation of p-38, ERK 1/2 and JNK, while total forms did not alter (Figure 5A-5F). Phosphorylation of p-38 was inhibited in RAW264.7 cells pretreated with the pulp and aril extracts and ferulic acid by 4%-37%, 17%-68% and 38%, respectively (Figure 5A-5B) As expected, the pulp and aril extracts and ferulic acid

suppressed LPS-induced phospho-JNK by 5%-24%, 11%-43% and 34%, respectively (Figure 5C-5D). In addition, LPS-mediated phosphorylation of ERK1/2 was suppressed by 12%-42%, 14%-80% and 46%, respectively (Figure 5E-5F). Similarly, the aril extract showed a better suppressive effect on MAPKs phosphorylation than the pulp extract. Consequently, the extract suppressed LPS-induced pro-inflammatory mediator production partly by blocking phosphorylation of the three MAPK proteins.

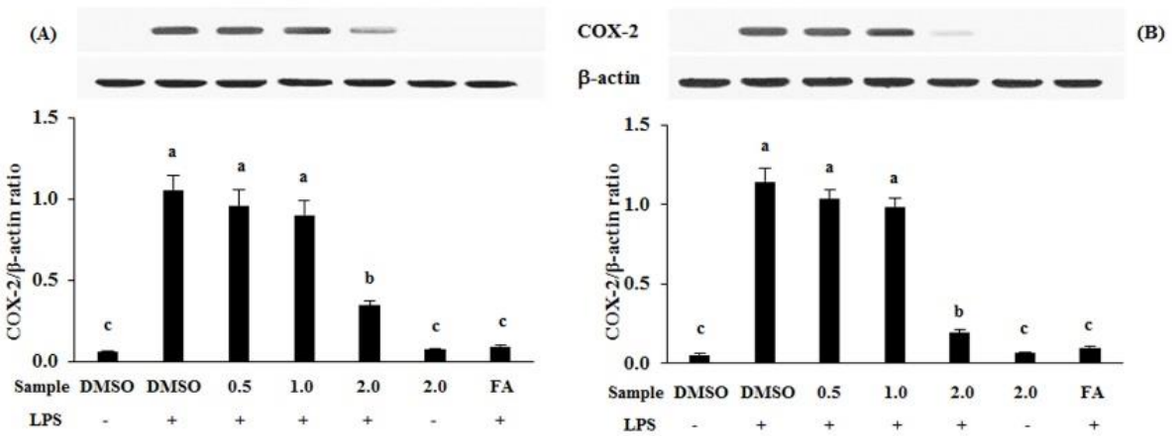

Figure 2. Pulp and aril extracts decrease COX-2 expression in LPS-activated murine macrophages. Cell monolayers were pretreated with 0.5 -2.0 mg/mL of pulp (A) or aril (B) extracts or 25 μM ferulic acid (FA) or DMSO prior to incubation with/without LPS for 24 h. Lysates from treated cells were analyzed for COX-2 and β-actin protein by immunoblot. Results are expressed as ratios of band intensity of COX-2 to β-actin protein. Data are means ± SD of three replicate experiments. Values with no common letters are significantly different from each other ($p < 0.05$).

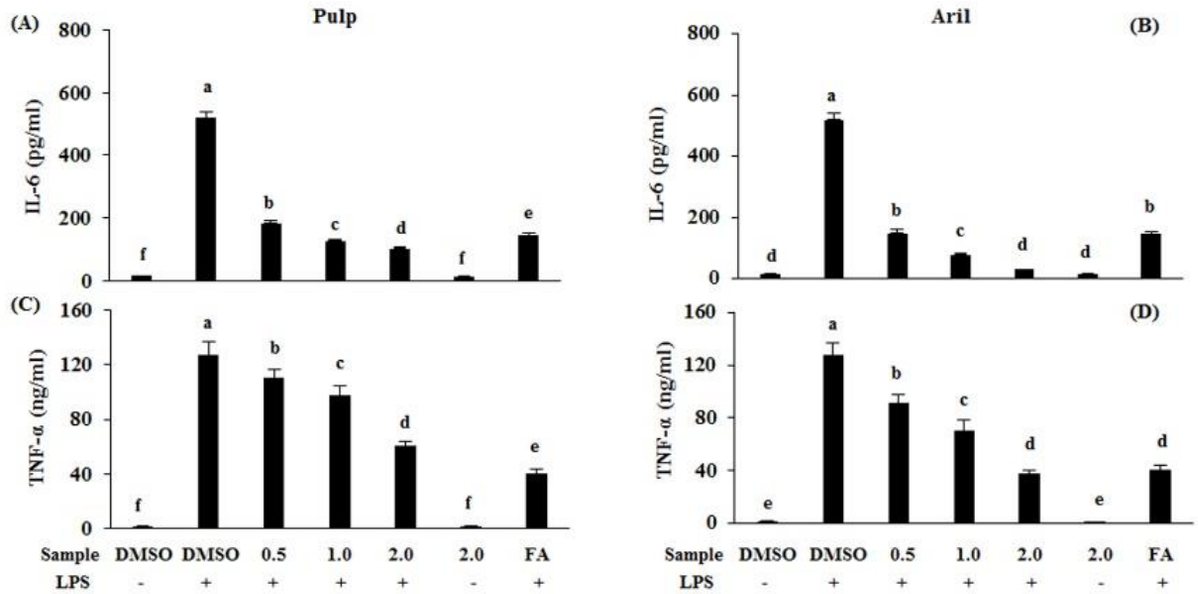

Figure 3. Pulp and aril extracts suppress IL-6 and TNF-α production by LPS-activated murine macrophages. Cell monolayers were pretreated with 0.5 -2.0 mg/mL of pulp (A,C) or aril (B,D) extracts or 25 μM ferulic acid (FA) or DMSO prior to incubation with/without LPS for 24 h. Culture media from treated cells were collected to measure (A,B), IL-6 (A,B) and TNF-α (C,D) by ELISA. Data are means ± SD of three replicate experiments. Values with no common letters are significantly different from each other ($p < 0.05$).

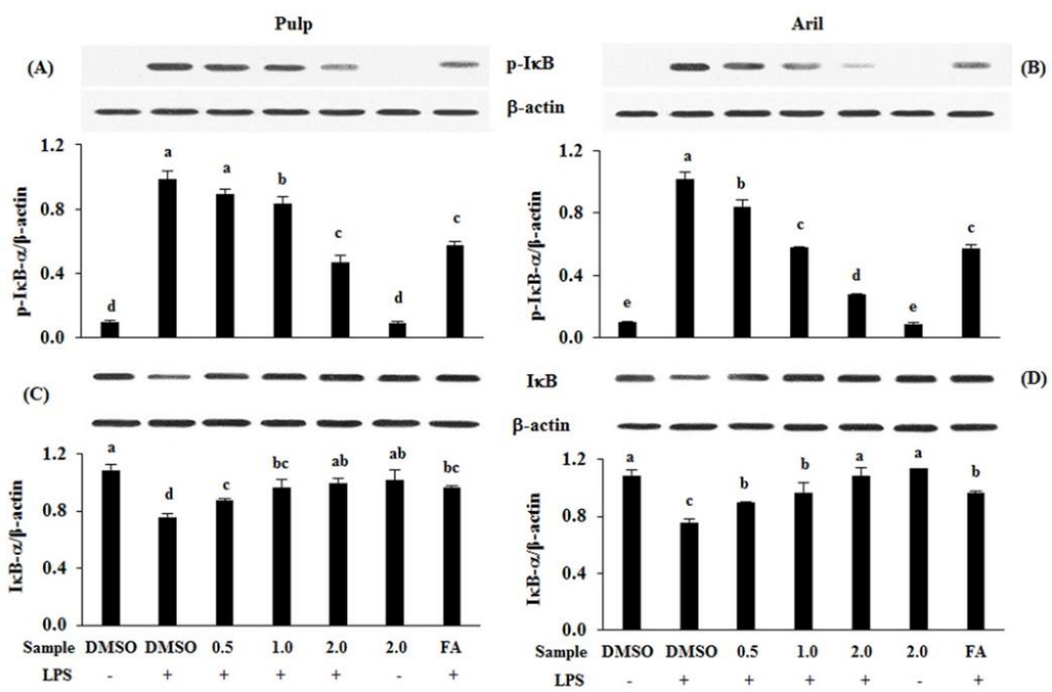

Figure 4. Pulp and aril extracts attenuate IκB-α activation in LPS-activated murine macrophages. Cell monolayers were pretreated with 0.5 -2.0 mg/mL of pulp (A,C) or aril (B,D)

extracts or 25 μM ferulic acid (FA) or DMSO prior to incubation with/without LPS for 24 h. Cell lysates from treated cells were analyzed for phospho-IκB-α and β-actin (A,B) or total IκB-α and β-actin (C,D) by immunoblot. Results are expressed as intensity bands of phospho-IκB-α/β-actin and total IκB-α/β-actin ratios. Data are means ± SD of three replicate experiments. Values with no common letters are significantly different from each other ($p < 0.05$).

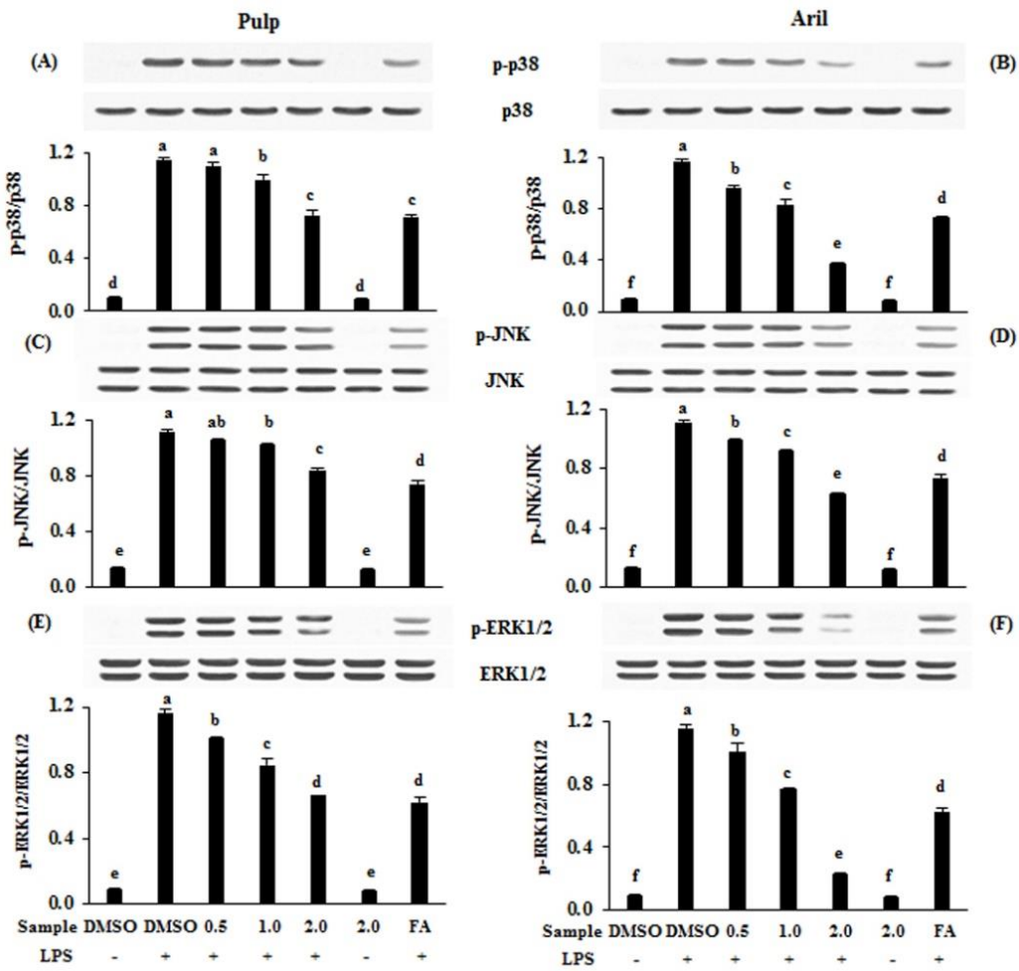

Figure 5. Pulp and aril extracts inhibit phosphorylation of p38, JNK and ERK expression by LPS-activated murine macrophages. Cell monolayers were pretreated with 0.5 -2.0 mg/mL of pulp (A,C,E) or aril (B,D,F) extracts or 25 μM ferulic acid (FA) or DMSO prior to incubation with/without LPS for 24 h. Cell lysates from treated cells were analyzed for phospho-p38 and total p-38 (A,B) or phospho-JNK and total JNK (C,D) or phospho-ERK1/2 and total ERK1/2 (E,F) by immunoblot. Results are expressed as intensity bands of phospho-p38/p-38, phospho-JNK/JNK and phospho-ERK/ERK ratios. Data are means ± SD of three replicate experiments. Values with no common letters are significantly different from each other ($p < 0.05$).

Discussion

The present study found hexane/acetone/ethanol extract from aril had a greater content than pulp which is consistent with those reported in other studies[2,3]. The hexane/acetone/ethanol extract had much higher contents of β-carotene and lycopene than ethanol extract, but the ethanol extract showed a greater anti-inflammatory activity in LPS-induced NO and TNF-α production than the hexane/acetone/ethanol extract (Table 1). This finding implies other hydrophilic compounds in 90% ethanol extract had a stronger anti-inflammatory effect than compounds in hexane/acetone/ethanol. Many previous *in vivo* and *in vitro* studies have reported health benefits of pulp and aril, such as provitamin A activity[25], antioxidant[26], anti-mutagenicity[27], anticancer activity[28], hypoglycemic effect[29] and lower blood pressure activity[30]. However, the anti-inflammatory effect of pulp and aril from gac fruit has never been reported. Hence, the present study assessed anti-inflammatory activity extract from pulp and aril in LPS induced murine macrophage cell lines (RAW 264.7 cells). The results demonstrated that ethanol extract of pulp and aril from gac fruits exhibited strong anti-inflammatory effect in LPS-stimulated macrophage cell line. However, the aril extract had a greater anti-inflammatory effect than the pulp extract which may be due to its higher content of carotenoids, total flavonoids and total phenolic content (Table 1). Both pulp and aril extracts significantly suppressed LPS-induced NO production via attenuate protein expression of iNOS (Figure 1). In addition, both extracts significantly suppressed LPS-induced COX-2, TNF-α and IL-6 production in a dose dependent manner (Figure 2-3).

Excess NO level can cause deleterious indirect effects through the formation of reactive nitrogen species (RNS), in particular peroxynitrite anion (OONO-) during oxidative stress status[31]. Peroxynitrite has a highly oxidative potential to trigger cytotoxic processes, such as lipid peroxidation, and DNA damage leading to tissue damage and inflammation[32]. Upregulate COX-2 expression has been reported to increase risk of tumor recurrence, advanced cancer stage, and/or poor prognosis of several types of cancers including breast cancer and colon cancer[33]. Collective data from 18 independent laboratories analysis reported that 44% of atypical hyperplasia, 65% of DCIS, 57% of invasive cancer and 87% of metastatic cancer had high expression of COX-2[34]. Suppression of NO level and COX-2 expression may delay progression of carcinogenesis. A previous study found that prolonged exposure of normal human

ovarian epithelial cells with TNF-α demonstrated a precancerous-like phenotype with structural and functional changes, such as tissue disorganization, epithelial polarity loss, cell invasion, and upregulate of cancer markers expression[35]. IL-6 plays a key role in promoting proliferation and inhibition of apoptosis of malignant cells by activating the JAK/STAT signaling pathway of the Janus kinases (JAK) and signal transducers and activators of transcription (STATs) STAT1 and STAT3[36]. The prevention of inflammatory disorders by blocking TNF-α and IL-6 may be an effective strategy for the treatment of cancer expression.

NF-κB is highly activated at sites of inflammation in diverse diseases such as rheumatoid arthritis, inflammatory bowel diseases, multiple sclerosis, psoriasis, and asthma[37]. The present study showed that both pulp and aril extract were able to inhibit the LPS-induced phosphorylation and degradation of IκB-α resulting in lower transactivation of NF-κB (Figure 4). Hence, the inhibition of the NF-κB pathway activation partly explains the potent activity of pulp and aril extracts as suppressors of inflammatory mediators and cytokines in the present study. In addition to the NF-κB pathway, MAPKs is another pathway playing a critical role in regulating expression of inflammatory mediators and cytokines induced by inducers including LPS which modulate severity of inflammatory diseases[15]. Pulp and aril extract significantly suppressed the LPS-induced phosphorylation of the three MAPKs, suggesting that suppression of the MAPK signal pathway might contribute to the anti-inflammatory effects of pulp and aril extract in the LPS-induced inflammatory response of RAW 264.7 cells.

β-carotene and lycopene have been shown to suppress pro-inflammatory mediator production *in vivo* and *in vitro*. A previous study found that β-carotene significantly inhibited NO, IL-1β, IL-6, and MCP-1 production via suppression on MAPKs and NF-κB activation in *Pseudorabies* virus infected RAW264.7 cells[38]. Lycopene inhibited LPS-induced production of NO, iNOS and IL-6 expression by inhibited LPS-induced IκB degradation and phosphorylation and NF-κB translocation[39]. Another study confirmed that pretreated pure lycopene significantly inhibited LPS-induced mRNA expression of TNF-α, IL-1β, IL-6, iNOS, and COX-2 in SW480 human colorectal cancer cells. In addition to β-carotene and lycopene, several studies have demonstrated the anti-inflammatory activities of dietary flavonoids and polyphenolic compounds *in vivo* and *in*

vitro[18, 40]. Some phenolic compounds can downregulate NF-κB in inflammatory pathway or up-regulate Nrf-2 of antioxidant pathways[41]. Several anti-inflammatory mechanisms of flavonoid have been proposed, such as antioxidant activity, inhibition of eicosanoid generating enzymes or the modulation of the production of pro-inflammatory gene expression leading to attenuation of the inflammatory response[42]. Anti-inflammatory effects in the present study may derive from β-carotene, lycopene, phenolic compounds and flavonoids in the pulp and aril extract.

Conclusion

The present data indicate that pulp and aril of gac fruits had anti-inflammatory activity by modulate via inhibition of the NO/iNOS pathway, as well as via inhibition of the production of pro-inflammatory cytokines, including TNF-α and IL-6. These effects were mediated partly through the inhibition of IκB phosphorylation and degradation through blocking of the MAPKs signaling pathway in LPS-induced murine macrophages. Due to their multiple healthy benefits including anti-inflammation, gac fruit pulp and aril are suitable for addition to dietary products to develop healthy products. Regular consumption of gac fruits may be useful for preventing inflammatory diseases. Further studies in animals and humans are also warranted in this regard.

Conflict of interest

No potential conflicts of interest were disclosed

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