Research article

Gac Fruit Extract Suppresses Oxidative Stress in LPS-induced RAW264.7 Cells

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ABSTRACT

Gac fruit (*Momordica cochinchinensis*) contains various bioactive compounds that possess several health benefits, including antioxidant activity. The present study assessed the antioxidant effects of ethanol-extract from ripe Gac fruit on LPS-induced murine macrophage cell line (RAW 264.7 cell). Pulp and aril from ripe Gac fruit were collected separately, homogenized, and lyophilized. An equal weight of dry powder pulp and aril were homogeneously mixed (Gac fruit powder). The ethanol extract of Gac fruit powder had β-carotene and lycopene of 206 and 231 μg/g dry weight (DW), respectively; total flavonoids of 1.5 mg Quercetin Equivalent (QE)/g DW; and phenolic compounds of 3.0 mg Gallic Acid Equivalent (GAE)/g DW. RAW264.7 cells significantly generated reactive oxygen species (ROS) once exposed to Lipopolysaccharides (LPS). Treatment RAW264.7 cells with 0.5 – 2.0 mg/mL of Gac fruit powder ethanol-extract significantly suppressed LPS-induced intracellular ROS formation in a concentration-dependent manner. Treatment with the extract protected the LPS activated stressed cells by significantly restoring total glutathione content, superoxide dismutase, and catalase activities in LPS-induced RAW264.7 cells. These results indicate that Gac fruit ethanol-extract exhibited moderate antioxidant effect in this cell model.

Key words: Gac fruit, Antioxidant enzyme, Oxidative stress

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บทความวิจัย

สารสกัดจากผลฟักข้าวสุกยับยั้งการเกิดภาวะเครียดออกซิเดชันในเซลล์เม็ดเลือดขาวหนูที่เหนี่ยวนำด้วยไลโปโพลิแซคคาไรด์

เกมิกา แพรงาม, พรพรรณ สุขบุญ, ศิริพร ตันติโพธิ์พิพัฒน์*

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บทคัดย่อ

ผลฟักข้าวสุก (*Momordica cochinchinensis*) ประกอบด้วยสารออกฤทธิ์ทางชีวภาพที่มีประโยชน์ต่อสุขภาพมากมายหลายชนิดรวมทั้งฤทธิ์ต้านอนุมูลอิสระ ในการศึกษานี้ประเมินประสิทธิภาพการต้านอนุมูลอิสระของสารสกัดจากฟักข้าวสุกด้วยเอทานอลในเซลล์แมคโครฟาจน์ของหนู (RAW 264.7 cell) ซึ่งเหนี่ยวนำให้เกิดภาวะเครียดออกซิเดชันด้วยไลโปโพลิแซคคาไรด์ แยกส่วนที่กินได้คือเนื้อและเยื่อหุ้มเมล็ดของฟักข้าวสุก นำไปปั่นและทำให้แห้งด้วยวิธี Lyophilization ชั่งน้ำหนักของผงเนื้อและผงเยื่อหุ้มเมล็ดในอัตราส่วนที่เท่ากันผสมให้เข้ากันซึ่งเรียกส่วนนี้ว่า “ผงฟักข้าว” นำไปสกัดด้วยเอทานอลวิเคราะห์ด้วย HPLC พบว่าสารสกัดประกอบด้วยเบต้าแคโรทีน และ ไลโคปีน 206 และ 231 ไมโครกรัมต่อกรัมน้ำหนักแห้ง ตามลำดับ ใน 1 กรัมของผงฟักข้าวแห้ง มีฟลาโวนอยด์รวมเทียบเท่ากับเควอซิทิน 1.5 มิลลิกรัม และ ฟีนอลิกรวมเทียบเท่ากับกรดแกลลิก 3 มิลลิกรัม เมื่อเหนี่ยวนำเซลล์ RAW 264.7 ด้วยลิโพพอลิแซ็กคาร์ไรด์ (LPS) เซลล์ผลิตอนุมูลอิสระชนิดที่มีออกซิเจนเป็นองค์ประกอบ (ROS) เพิ่มขึ้น อย่างมีนัยสำคัญ ($p<0.05$) สารสกัดผงฟักข้าวที่ความเข้มข้น 0.5-2.0 มิลลิกรัมต่อมิลลิลิตร ทำให้เซลล์มีการผลิต ROS ลดลงอย่างมีนัยสำคัญ ($p<0.05$) นอกจากนี้สารสกัดผงฟักข้าวทำให้เซลล์ RAW264.7 ผลิตกลูตาไทโอนเพิ่มขึ้นอย่างมีนัยสำคัญ ($p<0.05$) และสามารถเพิ่มการทำงานของเอนไซม์ต้านอนุมูลอิสระชนิด Superoxide dismutase และ Catalase ในเซลล์ RAW264.7 ที่เหนี่ยวนำด้วย LPS ผลจากการศึกษาในเซลล์ต้นแบบนี้พบว่าสารสกัดจากผลฟักข้าวสุกมีประสิทธิภาพต้านการเกิดอนุมูลอิสระได้ปานกลาง

คำสำคัญ: ผลฟักข้าว เอนไซม์ต้านอนุมูลอิสระ ภาวะเครียดออกซิเดชัน

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Introduction

Gac fruit is widely cultivated in South and South-East Asia and is used as food and traditional medicine in Southeast Asia[1]. Gac fruits are usually harvested when the skin becomes dark orange or red (ripe). The edible red membrane covering the black seed or aril of Gac fruit is reported to be the most nutritious part containing high amounts of fatty acid and carotenoids, including β-carotene and lycopene[2]. The pulp (yellow colored layer) is another edible portion containing lutein, β-cryptoxanthin, zeaxanthin, α-carotene, β-carotene, cis-lycopene, trans-lycopene, vitamin C, vitamin E, and some fatty acids[3,4]. Regarding polyphenolic content, ferulic acid and p-hydroxybenzoic acid are predominant in pulp. Apigenin is the most predominant flavonoid in ripe pulp, whereas the highest contents of rutin and luteolin are found in aril. Myricetin, a member of the flavonoid group, and gallic acid, a phenolic compound[1], are found in all parts of Gac fruit. The aforementioned bioactive compounds in pulp and aril have been reported to play vital bio-functional roles, including anti-inflammatory and anti-oxidant effects[1,5]. These phytonutrients have been shown to suppress pro-inflammatory mediators and reactive oxygen species (ROS) from activated macrophages[6-8].

Inflammation is a physiological immune response that defends against foreign pathogens including lipopolysaccharide (LPS) or damaged host tissue. Activated inflammatory immune cells, such as macrophages and neutrophils, secrete various pro-inflammatory mediators and ROS via NADPH oxidases in order to eliminate invading microorganisms or damaged tissue during inflammatory response[9]. In addition to the inflammatory process, ROS can be generated as byproducts of cellular metabolism via the electron transport chain (ETC) in mitochondria and via the cytochrome P450. At physiological concentrations, ROS function as signaling molecules that regulate cell growth, the adhesion of cells to other cells, differentiation, senescence, and apoptosis[10,11]. If the inflammatory process is prolonged, overproduction of ROS adversely results in progression of inflammatory associated diseases[12]. An imbalance due to higher ROS generation than the presence of antioxidant substances in the body causes oxidative stress, which then leads to damage of cellular macromolecules, including lipids, proteins, and DNA. These oxidized macromolecules appear to be causative factors in age-related diseases[13]. The major ROS molecules include superoxide anion (O_2•-), hydrogen peroxide (H_2O_2), hydroxyl anions (OH-), hydroxyl radical (OH•), and hypochlorous acid (HOCl)[10]. Such ROS molecules could be neutralized by endogenous and exogenous antioxidant substances.

Antioxidants can be classified into enzymatic and non-enzymatic agents. Superoxide anion is rapidly dismutated to H_2O_2 by superoxide dismutase (SOD). Moreover, activated macrophages also produce nitric oxide (NO) during an inflammatory response. Superoxide anion rapidly reacts with NO to produce a highly reactive molecule called peroxynitrite (ONOO-). This reaction is three to four times faster than the dismutation of superoxide to H_2O_2. Hydrogen peroxide can be converted to highly toxic hydroxyl radicals in the presence of Fe^{2+} via Fenton's reaction. The hydroxyl radical is the most potent oxidizing

species that interacts with biological membrane proteins and lipids. However, H_2O_2 can be converted to H_2O and O_2 by enzyme catalase, glutathione peroxidase (GPX), or peroxiredoxins (Prx). Peroxiredoxins use thioredoxin (Trx) to detoxify H_2O_2[14]. Glutathione, vitamin C, vitamin E (α-tocopherol), vitamin D, N-acetyl cysteine (NAC), alpha lipoic acid, carotenoids, bilirubin, and uric acid all belong to non-enzymatic antioxidant molecules. Carotenoids are one of the most efficient singlet oxygen quenchers and ROS scavengers operating in the lipid bilayers of cell membranes[15]. Phenolic compounds and flavonoids are natural antioxidants[16] that are very potent scavengers of hydroxyl and superoxide radicals, as well as active chelators of transient elements[17,18].

A previous study found that ethanol extract of pulp and aril from ripe Gac fruit exhibited potent anti-inflammatory activity in the LPS-induced murine macrophage cell line by suppressing pro-inflammatory mediators' production[4]. In addition, an *in vivo* study showed that ethanol extract of aril from Gac fruit prevented reduction of blood glutathione and restored redox status in L-NAME induced hypertensive rats[19]. Although aril and pulp of ripe Gac fruit are rich in carotenoids and several phenolic compounds, the antioxidant activities in a cell model have never been reported. Currently, Gac fruits are consumed as a whole fruit drink. Consequently, potential health benefits should be assessed using the whole fruit. The present study aims to assess the antioxidant effect of a whole Gac fruit extract using LPS-induced murine macrophage cell line (RAW264.7 cells).

Material and Method

Chemicals

Fetal bovine serum was purchased from Millipore (US Origin, EmbryoMax® ES Cell Qualified FBS), while penicillin and streptomycin were purchased from Invitrogen (Grand Island, NY, USA). Dulbecco's Modified Eagle's Medium (DMEM), LPS (Escherichia coli O11:B4) and β-carotene, α-carotene, lycopene, lutein, β-cryptoxanthin, zeaxanthin standard were obtained from Sigma (St. Louis, MO, USA). Chemical and reagents were analytical and HPLC grade.

Gac fruit preparation and extraction

Fully ripe Gac fruits (deep red colored peel) were purchased during May-July in 2017 from three commercial farms in Nakhonpathom province, Thailand. The edible pulp and aril (seed membrane) were collected and homogenized separately using a kitchen blender. An equal amount of aril or pulp from each farm was pooled and boiled in deionized water (DI) at a ratio of 1:1 (w/v) for 20 min, allowed to cool to room temperature (25 °C), and then lyophilized. The moisture contents in aril and pulp were 96.1 ± 0.1% and 90.1 ± 0.3%, respectively. A dry sample was ground using a kitchen blender. An equal weight of pulp and aril at a ratio of 1:1 (w/w) were thoroughly mixed (Gac fruit powder), packed in vacuum-aluminum foil, and then stored at -20 °C until analysis.

The dried Gac fruit powder (0.02 ± 0.002 g) was extracted 3 times with 10 mL of 90% ethanol. The extracted solution was then pooled and evaporated using a rotary evaporator (Buchi Rotavapor-Re-124, Flawil, Switzerland) under vacuum at 38-40 °C until dry[4].

Determination of carotenoids content by HPLC

The dried film from Gac fruit ethanol-extract was dissolved in 2 mL of mobile phase [methyl-tert-butyl-ether (MtBE): methanol (MeOH) (3:1)] and filtered through a 0.22 μm (polytetrafluoroethylene; PTFE) membrane followed by HPLC analysis (Waters 600 controller module with a photodiode array detector; Agilent 1100 series, Agilent Technologies, Santa Clara, CA, USA). Twenty microliters of the extracts were injected into the HPLC system[20] consisting of a reverse-phase column C30 (YMC carotenoid 150 x 4.6 mm ID, 5μm, serial no., 114FA70081, Japan) directly connected to a C18 cartridges guard column (Phenomenex C18, 4 x 3 mm Torrance, USA). Carotenoids were separated by gradient elution programs at a flow rate of 0.6 mL/min of mobile phase solvent (98% methanol containing 2% ammonium acetate pH 4.6 and 100% methyl tertiary-butyl ether). Individual carotenoid was identified by retention time and spectral characteristic compared with known standards. Final concentration of carotenoid was obtained from area under the curve using a calibration standard curve with known concentrations of pure β-carotene, α-carotene, lutein, β-cryptoxanthin and zeaxanthin at 450 nm, and lycopene at 470 nm.

Analysis of total phenolics and total flavonoids content

The extract protocol for measuring total phenolics and total flavonoid contents was according to the method of Kubola and Siriamornpun (2011)[1]. Total phenolics content was determined by Folin-Ciocalteu reagent[21]. Optical density was measured at 760 nm by Microplate Reader (BioTek® Instruments, Vermont, and USA). Results were calculated and expressed as gallic acid equivalents per gram of dry weight (mg GAE/gDW). Total flavonoid content was determined using aluminium chloride colorimetric method as previously described[22]. Optical density was immediately measured at 415 nm using a Microplate Reader. Results were expressed as mg quercetin equivalents in 1 g of dried sample (mg QE/gDW).

Cell culture

Murine macrophage RAW264.7 cell line (TIB71) was purchased from American Type Culture Collection (Bethesda, MD, USA). Cells were cultured in a complete medium composed of DMEM containing 10% fetal bovine serum, 100 units/mL of penicillin, and 100 μg/mL of streptomycin, and incubated in a humidified atmosphere of 5% CO_2/ 95% air at 37 °C. Cells were used for the experiment at 80% confluence between passages no. 10 - 20.

Sample extraction for cell culture treatment

The dried Gac fruit powder (0.5 ± 0.002 g) was extracted 3 times with 20 mL of 90% ethanol according to the method of Sukboon et al. (2019)[4]. The extracted solution was pooled and evaporated with a rotary evaporator under a vacuum system until dry. The dried extract film was re-solubilized with 0.2% dimethyl sulfoxide (DMSO) in phenol red and serum-free medium, filtered through a sterile 0.2 μm filter (cellulose acetate), and then diluted to concentrations ranging from 0.5 to 2.0 mg/mL with 0.2% DMSO in phenol red and serum-free medium before adding to the RAW264.7 cell monolayer.

Cytotoxicity test

The sulforhodamine B (SRB) assay[23] was used to determine the non-toxic concentration of Gac fruit ethanol-extract on the cell viability of RAW 264.7 cells to investigate various parameters. In brief, RAW264.7 cell monolayers were incubated with 0.5 – 2.0 mg/mL of extracts or 25 μM ferulic acid [a well-known antioxidant phenolic acid[24]] for 1 h prior to incubation with 2 ng/mL of LPS in 0.2% DMSO (positive control) or 0.2% DMSO in basal medium (negative control group) for another 24 h. The stimulated cells were measured for stoichiometric binding of SRB dye to proteins under mildly acidic conditions and its subsequent extraction under basic conditions according to Vichai and Kirtikara (2006)[23]. Optical density of the positive control group was defined as 100% viability. An acceptable viability of treatment groups should be more than 80%.

Intracellular reactive oxygen species (ROS)

Intracellular reactive oxygen species (ROS) levels were quantified by measuring the oxidation level of 2′, 7′-dichlorodihydrofluorescein diacetate (DCF-DA). After incubation with DCF-DA 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 to immediately measure fluorescent signal using a microplate reader at excitation wavelength 485 nm and emission wavelength 530 nm. The fluorescent intensity was proportionate to the ROS content[25].

Determination of Glutathione level, Superoxide dismutase and catalase (CAT) activity

After LPS-activation, cell monolayers were washed with ice-cold PBS, collected and centrifuged (Hettich, Rotina 38R, Tuttlingen, Germany) at 4,140 x g at 4 °C for 10 min. Cell pellets were resuspended in ice-cold PBS, then lysed on ice using an ultrasonic processor (130 Watt, 20 kHz; Sonics & Materials, Inc., Newton, USA) for three cycles of 30 sec on/off pulsing, and centrifuged at 4,140 g at 4°C for another 10 min. Supernatants from cell lysis were used to measure total glutathione (GSH), superoxide dismutase, and catalase activities. The concentration of protein was measured by the Bradford assay using bovine serum albumin as a standard. Measurement of total GSH level involves oxidation of GSH by the sulfhydryl reagent 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) to form the yellow derivative 5′-thio-2-nitrobenzoic acid (TNB). Total GSH concentration in the samples was calculated using linear regression of the known concentration of the GSH standard curve (0.4-26 nM/ml as GSH equivalent). The level of total GSH concentration in the treated cell was expressed as nM/mg protein[26,27]. Results were expressed as a percentage of the negative control group (0.2% DMSO in basal medium).

Intracellular superoxide dismutase (SOD) and catalase (CAT) activities were measured according to manufacturer protocol (Cayman Chemical, Ann Arbor, Michigan, USA). The SOD and CAT activities were calculated using the standard curves of the manufacturer (the kit booklet, Item No. 706002 and 707002) and expressed as U/mg protein and

nmol/min/mg protein, respectively. These activities were expressed as a percentage of vehicle control without LPS addition.

Data analyses

SPSS (version 19.0, SPSS Inc., Chicago, IL) was used to analyze the data. All data were presented as mean ± standard deviation (SD) for at least a duplicate value for each experiment and conducted at least two times on different days. 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 & Discussion

Carotenoids, phenolic and flavonoid content in Gac fruit ethanol-extract

Dietary carotenoids, phenolic compounds, and flavonoids exhibit several biological activities, including antioxidant activity. Consequently, carotenoid, total phenolic, and flavonoid contents in Gac fruit extracts were measured. Our preliminary study of ethanol extract exhibited a better suppressive effect on LPS-induced ROS production in RAW264.7 cells than the extraction from the less polar mix solvent (hexane: acetone: ethanol). In this study, the bioactive compounds in Gac fruit powder were extracted by 90% ethanol. Gac fruit consisted of 47% of pulp and 20% of aril. The moisture contents of fresh pulp and aril were 91.8 ± 0.2 % and 80.1 ± 0.4%, while those of the boiled pulp and aril were 96.1 ± 0.1 and 90.1 ± 0.3, respectively. The concentrations of β-carotene and lycopene from the ethanol extract of Gac fruit powder were 206 ± 6.3 and 231 ± 9.3 mg/g dry weight (DW), which showed two main peaks of β-carotene and lycopene (**Figure 1**). Total flavonoid and total phenolic contents in the Gac fruit ethanol-extract were 1.5 ± 0.08 mg QE/g DW and 3.0 ± 0.07 mg GAE/g DW. β-carotene and lycopene in pulp and aril in the present study extracted using ethanol was lower than those reported in another study using a different extraction solvent (methanol: tetrahydrofuran: hexane, 1: 1: 1)[28]. Different organic solvents significantly affect the extractable amounts of β-carotene and lycopene in Gac fruit[29]. A recent study has reported ethanol extract from pulp and aril containing 99 and 256 mg/g DW of β-carotene and 66 and 332 mg/g DW of lycopene, respectively. The same study found total flavonoids of 0.21 mg QE/g DW and total phenolic of 2.34 mg GAE/g DW in pulp extract, while aril extract contained 2.81 mg QE/g DW and 4.61 mg GAE/g DW, respectively.

Gac fruit is reported to be a good source of phytochemicals that possess antioxidant activities[30]. Most previous research studies have focused on phytochemicals and antioxidant activity of different Gac fruit fractions (peel, pulp, aril, and seed). Currently, several commercial products, such as fruit juice, functional drinks, sauce, and snacks, are made of a mixture of pulp and aril. Consequently, a mixture of aril and pulp powder at 1:1 (w/w) was used to assess antioxidant effect in the present study.

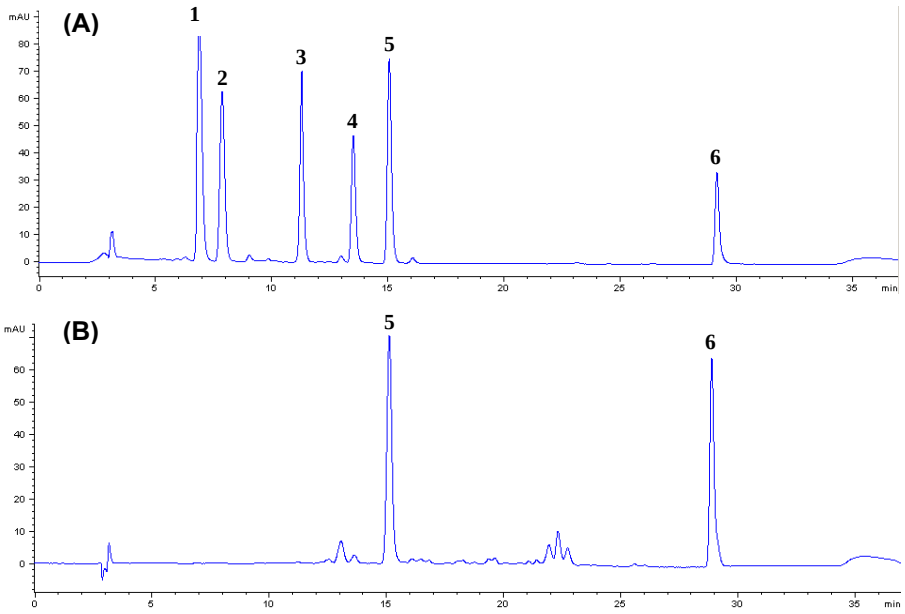

Figure 1. HPLC chromatograms of carotenoids. (A) Six peaks of carotenoid standards [(1) Lutein, (2) Zeaxanthin, (3) β-cryptoxanthin, (4) α-carotene, (5) β-carotene, and (6) Lycopene]. (B) Carotenoid profile in the Gac fruit ethanol-extract.

Gac fruit extract suppresses LPS-induced intracellular ROS formation

Although an inflammatory response is a normal physiological immune defense, it is important to note that an unabated longer duration pro-inflammatory cascade may lead to cellular injury through the excessive generation of ROS[31]. Excess ROS can nonspecifically damage almost all classes of bio-macromolecules including lipid, protein and DNA in living cells. Many diseases, including atherosclerosis, diabetes, cancer, and Alzheimer's disease, as well as ageing, are attributable to oxidative damage caused by ROS[32]. The present study's results indicated that stimulated RAW264.7 cells with LPS significantly produced intracellular ROS up to 6 fold (3,140 arbitrarily units: AU) above the baseline (486 AU) (**Figure 2**) without cytotoxicity. Prior treatment of RAW264.7 cells with the Gac fruit ethanol-extract significantly attenuated LPS-induced ROS formation in a concentration-dependent manner (18%-49%) compared to those from cells treated with LPS alone. Moreover, the cell monolayer treated with 2 mg/mL Gac fruit extract alone did not have a significant effect on ROS formation (**Figure 2**). RAW264.7 cells treated with 25 μM ferulic acid also significantly reduced LPS-induced ROS accumulation by 63% (**Figure 2**). Previous studies have demonstrated that bioactive compounds in the extract, such as, β-carotene[33], lycopene[34], phenolic compounds[35,36] and flavonoids (myricetin[37] and apigenin[38]), decreased LPS-induced intracellular ROS production. Hence, the suppressive effect of LPS-induced ROS formation found in this study may arise from such bioactive substances in the Gac fruit powder.

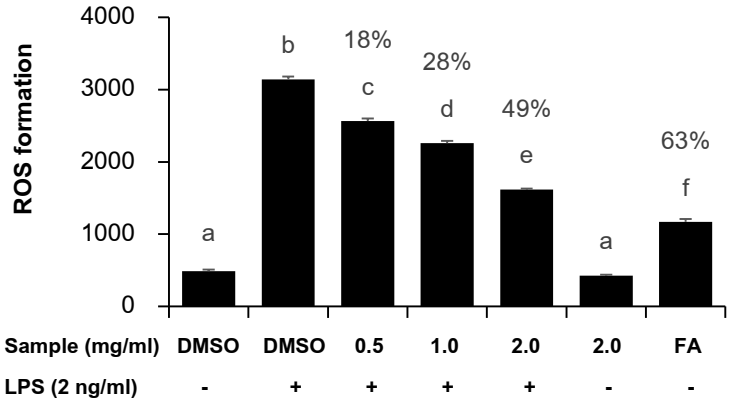

Figure 2. Gac fruit extract reduces intracellular LPS-induced ROS accumulation. Cells were pretreated with or without Gac fruit extract at 0.5-2.0 mg/mL and ferulic acid (FA) at 25 μM for 1 h before stimulating with or without LPS (2 ng/mL) for 24 h. After stimulation with LPS, cells were collected to measure ROS formation. Results are presented as mean ± SD (n=6). Different superscripts indicate significant differences ($p<0.05$) among treatments. The error bars represent standard deviations.

Effect of Gac fruit extract on glutathione level, SOD and CAT activity

Oxidative stress biomarkers including glutathione (GSH) content, SOD and CAT activities were used to assess the defensive function of Gac fruit extract against LPS-induced oxidative stress. Resting RAW264.7 cells treated with 1 and 2 mg/mL of Gac fruit extract or 25 μM ferulic acid significantly increased GSH content by 116%, 129%, and 117%, respectively, compared to the untreated group (negative control = 100%) (**Figure 3**). RAW264.7 cells exposed to LPS significantly reduced GSH level by 20% (80% compared to the negative control). Pre-treatment with 1 and 2 mg/mL of Gac fruit extract or 25 μM ferulic acid protected LPS induced stressed cells by significantly increasing the GSH level up to 108%, 115%, and 105%, respectively, compared to that of cells treated with LPS alone (80%) (**Figure 3**). This result is consistent with a previous study that found that intragastric administration of *M. cochinchinensis* aril extract prevented the reduction of blood GSH and restored the redox status in L-Nω-nitro-L-arginine methyl ester (L-NAME) induced hypertensive rats[19]. Another report found that 5 mg/kg β-carotene supplementation prevented ethanol-induced liver damage by the increased GSH concentrations in rats[39].

Resting RAW264.7 cells exposed to Gac fruit ethanol-extract or 25 μM ferulic acid did not significantly affect SOD activity. RAW264.7 cells treated with LPS significantly decreased SOD activity by 27 % ($p<0.05$) (**Figure 4A**). This result agrees with a previous study that found SOD activity in LPS treated macrophages was lower than that observed in resting macrophages[40]. However, pretreatment RAW 264.7 cells with Gac fruit extract 1 and 2 mg/mL significantly increased SOD activity relative to cells treated with LPS alone. Treated RAW264.7 cells with 25 μM ferulic acid prior to stimulation with LPS also significantly restored SOD activity relative to that of cells treated with LPS only (**Figure 4A**).

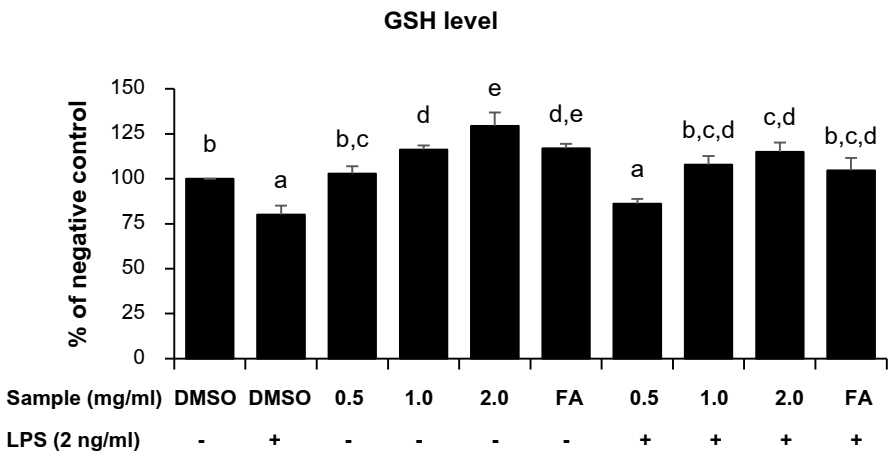

Figure 3. Gac fruit extract increased glutathione (GSH) content in LPS-induced RAW264.7 cells. The cells were pretreated with or without Gac fruit extract at 0.5-2.0 mg/mL and ferulic acid (FA) at 25 μM for 1 h before stimulating with or without LPS (2 ng/mL) for 24 h. Cell lysates were analyzed GSH level using spectrophotometric assays. Results were expressed as the fold increase relative to the control and are presented as mean ± SD (n=6). Different superscripts indicate significant differences ($p<0.05$) among treatments. The error bars represent standard deviations.

Gac fruit ethanol-extract at 2.0 mg/mL significantly enhanced CAT activity in resting RAW264.7 cells by 70% (**Figure 4B**) compared to the negative control. However, RAW264.7 cells treated with LPS did not significantly affect CAT activity, while pre-treatment cells with 2.0 mg/mL of the Gac fruit ethanol-extract significantly increased CAT activity compared to that of cells treated with LPS alone. Treated cells with ferulic acid prior to stimulation with LPS did not significantly affect CAT activity (**Figure 4B**).

Modulation of CAT and SOD activities observed in the present study may be the effect of lycopene and β-carotene. An *in vivo* study has demonstrated that the administration of 20 mg/kg lycopene could restore CAT, SOD, and GSH in colistin-induced nephrotoxicity in mice to the normal range observed in the control group[41]. Another previous study showed that administration of 10 mg/kg of β-carotene significantly increased the anti-oxidative activities of SOD, CAT and enhanced GSH content in acetaminophen-induced liver damage in rats[42]. Another study also showed that 30 mg/kg of β-carotene administration protected renal tissue against ischemia/reperfusion-induced oxidative damage in rats through improved renal antioxidant activities of SOD, CAT, and GSH[43]. Previous studies also have shown that pretreatment with 10 mg/kg lycopene for 6 days before the induction of liver injury using D-GalN/LPS in rats could restore antioxidant enzyme activity including SOD and CAT[44]. In agreement with these *in vitro* research studies, a dietary supplementation study using 200 g of ripe tomatoes (cooked) daily for a period of 60 days resulted in significant improvements in GSH and SOD levels in the plasma of hypertension[45] and serum of coronary heart disease patients[46]. However, other phytochemicals in Gac fruits may also exert these activities together with lycopene and β-carotene.

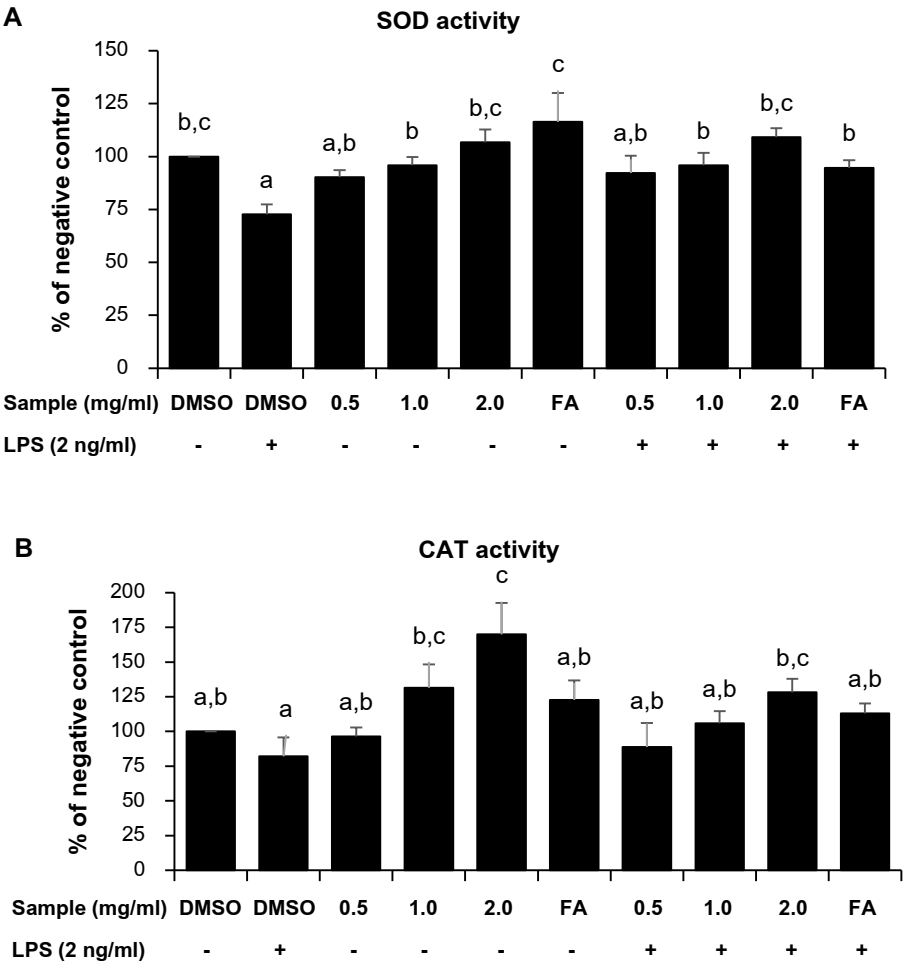

Figure 4. Gac fruit extract activated activity of superoxide dismutase (SOD) (A), and catalase (CAT) (B) in LPS-induced RAW264.7 cells. Cells were pretreated with or without Gac fruit extract at 0.5-2.0 mg/mL and ferulic acid (FA) at 25 μM for 1 h before stimulated with or without LPS (2 ng/mL) for 24 h. Cell lysates were analyzed for anti-oxidant enzyme using spectrophotometric assays. Results were expressed as the fold increase respect to control and are presented as mean ± SD (n=6). Different superscripts indicate significant differences ($p<0.05$) among treatments. The error bars represent standard deviations.

Conclusions

These results indicate that Gac fruit is a good source of phytochemicals. It modulates antioxidant effect by decreasing ROS production via enhancing glutathione content and restoring superoxide dismutase and catalase activities. However, the mechanism to modulate antioxidant activity of Gac fruit extracts warrant further exploration in the future.

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Conflict of interests

No potential conflicts of interest were disclosed.

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