ORIGINAL ARTICLE

Antioxidant and Anti-inflammatory Properties of *Punica granatum* Peel Methanolic Extract in BV2 Microglial Cell Line

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ABSTRACT

Introduction: *Punica granatum* is a fruit-bearing deciduous shrub or a small tree belonging to the family of *L. Punicaceae*. Pharmacological activities of *P. granatum* include antioxidant, anticarcinogenic, and anti-inflammatory properties. The purpose of this study is to investigate the antioxidant and anti-inflammatory properties of *P. granatum* methanolic extract (PGME) in the BV2 microglial cell line. **Method:** The bioactive component from *P. granatum* peel was extracted by a standard methanolic extraction protocol. The antioxidant properties of PGME were analyzed by total phenolic content (TPC) assay, 1,1-dipheyl-2-pycrylhydrazyl (DPPH) free radical scavenging assay, and Ferric Reducing Antioxidant Power (FRAP). The cytotoxicity activity of PGME on BV2 cells was determined by a viability test using MTS reagent. Analyses of the production of inflammatory mediators, including nitric oxide (NO) and cytokines (tumor necrosis factor-alpha [TNF-α], Interleukin [IL]-6 and monocyte chemoattractant protein [MCP]-1) have been carried out to evaluate the anti-inflammatory activity of PGME in LPS-stimulated BV2 cell line. **Result:** The TPC and radical scavenging activity of PGME increased in a concentration-dependent manner. However, the ferric reducing ability of PGME was slightly lower and no significant difference compared to the reference standard. PGME treatment on the LPS-stimulated BV2 cells significantly inhibits the production of NO and TNF-α and slightly reduced IL-6 level. **Conclusion:** PGME possesses antioxidative and anti-inflammatory properties. The PGME demonstrated anti-inflammatory activity through inhibition of pro-inflammatory immune mediators on the BV2 microglial cell line. Therefore, we suggest that PGME potentially inhibits oxidative and inflammatory processes.

Keywords: *P. granatum* methanolic extract (PGME), Microglia, Antioxidant, Anti-inflammatory, Nitric oxide (NO)

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INTRODUCTION

The history of *Punica granatum* goes back to ancient times as it had been used extensively in folk and Ayurvedic medicine (1). In Ayurvedic medicine, "dalima" (fruit) is a given name for *P. granatum* in Sanskrit, and described as 'a pharmacy unto itself' (2). *P. granatum* contains a substantial amount of phenolics and flavonoids (3). *P. granatum* exhibits neuroprotective effects in Alzheimer's disease (4) and the peel extract demonstrated a significantly higher antioxidant content in comparison to the pulp, juice, and seed (5).

Antioxidants are compounds with the ability to eliminate reactive free radicals. It is also described as a substance that significantly prevents or eliminates the oxidative source that could damage the target molecules (6). Plants are a good example of a source of antioxidants because they contains phenolic compounds (7). Many studies have shown that the growth and progression of cancer, neurodegenerative diseases and cardiovascular diseases are significantly promoted by oxidative stress (8). Low levels of antioxidants in plasma have been found to be associated with oxidative stress in patients, including patients with Alzheimer's disease (AD) (9). Antioxidants possess several essential biological properties, including anti-aging and anti-inflammatory effects. They also protect against some diseases such as atherogenesis, cancer, and neurodegenerative diseases (10). In addition, the long-term progression of atherosclerotic lesion can be influenced by antioxidant intervention (11).

Neurodegenerative disorder is a group of chronic and progressive neuronal dysfunctions characterized by deprivation of neurons and axon in the central nervous system (CNS) (12). Several neurodegenerative diseases include AD, Huntington's disease, Parkinson's disease (PD), and traumatic brain injury (12,13). AD is the most prevalent neurodegenerative disorder characterized by a progressive impairment of cognition and function as well as memory loss (14). The primary neuropathological

hallmarks of AD include an abnormality in the production of cerebral or agglomeration of β-amyloid and neurofibrillary tangles deposition (14). Several lines of hypothesis proved that agglomerated or aggregated proteins stimulate the activation of microglial cells and lead to a constant pro-inflammatory chemokines and cytokines expression (14). PD is the second most common neurodegeneration which could be characterized by the presence of abnormal aggregation as well as accumulation of α-synuclein (14). Chronic inflammation and microglial activation are the main pathologies of PD (15-17). An increase in the number of activated microglia was found in several parts of brain including the hippocampus, putamen, cingulate cortex, temporal cortex, and transentorhinal cortex in patients with PD (18). Although the activation of microglia could initiate a neuroprotective environment through cytokine production, excessive expression of pro-inflammatory cytokines can subsequently become neurotoxic especially during the progression of PD and other neurodegenerative diseases (19).

The aim of this study was to investigate the antioxidant and *in vitro* anti-inflammatory properties of PGME on the BV2 microglial cell line challenged by a gram negative endotoxin, lipopolysaccharide (LPS). We hypothesized that PGME contains strong antioxidant and anti-inflammatory activities through inhibition of nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, and monocyte chemoattractant protein (MCP-1).

MATERIALS AND METHODS

P. granatum

Samples of commercial *P. granatum* were purchased at Pasar Borong Seri Kembangan, Selangor. Authentication and verification was done by Dr. Mohd Firdaus Ismail from Institute of Bioscience, Universiti Putra Malaysia (UPM). Sample was deposited at Herbarium, Institute of Bioscience.

Methanol extraction of *P. granatum*

Extraction of the bioactive compound from *P. granatum* was done by the cold methanol extraction method. The small pieces of peels from *P. granatum* were dried by liquid nitrogen and grounded into powder by using pestle and mortar following the addition of methanol with 1:1 ratio (powder:solvent) which weighed in grams (g). The extract then was placed in an incubator shaker at 100 RPM for 1 hour (hr) at 27 ± 2°C. Afterward, the mixture was filtered and solvent was removed by rotatory evaporator at 45°C. The extract were placed into small vials and stored in the pharmaceutical refrigerator at 4°C. This extract is referred to as *P. granatum* methanolic extract (PGME).

Dilution of PGME

A 10000 µg PGME was vigorously mixed with 1 mL of dimethyl sulfoxide (DMSO) by using vortex to make a stock concentration of 10 mg/mL. The stock solution (10000 µg/mL PGME) was further diluted into a serial concentration (1000 µg/mL, 100 µg/mL, 10 µg/mL, 1 µg/mL, 0.1 µg/mL, 0.01 µg/mL) in phosphate buffer saline (PBS) for subsequent experiment.

1, 1-Diphenyl-2-picrylhydrazyl (DPPH) assay

The 1,1-Diphenyl-2-picrylhydrazyl (DPPH) solution was prepared in methanol. One part of PGME or ascorbic acid (positive control) was combined with two parts of DPPH followed by 30 minutes (min) incubation at room temperature. The absorbance of each PGME sample was measured at 517 nm by using UV-VIS spectrophotometer and the percentage of scavenging activity was calculated with the given formula below. The methanol alone was used as a negative control.

Scavenging activity (%) = [Control (Ac) – Sample (As) / Control (Ac) – Sample (As)] x 100

Where, Ac is the control (DPPH and methanol) and As is the sample (PGME or ascorbic acid as standard) absorbance value.

Total Phenolic Content (TPC)

A 0.5 mL of PGME sample from each different concentration was combined with 2.5 mL of 10-fold-diluted Folin-Ciocalteu solution. The mixture was left to stand for 2 to 5 min followed by adding 2 mL of 7.5% w/v sodium carbonate, and room temperature incubation in dark for 30 min. UV-Vis spectrophotometer was used to measure the absorbance at 765 nm. The total phenolic content was measured as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g) of the extract.

Ferric Reducing Antioxidant Power Assay (FRAP)

Freshly prepared FRAP reagent was used in a proportion of 10:1:1 (v/v) containing 500 mL of 300 mM acetate buffer (pH 3.6), 50 mL of 10 mM 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) in hydrochloric acid (HCl), and 50 mL of 20 mM ferric chloride hexahydrate ($FeCl_3.6H_2O$) solution. A serial dilution of PGME was combined with FRAP reagent and pre-incubated for 30 min in 37°C of water bath. The absorbance was immediately measured at 593 nm. The result was analyzed based on a standard curve generated from the absorbance of ferrous II ion solution that was prepared from ferrous sulfate heptahydrate ($FeSO_4.7H_2O$).

Cell culture and LPS stimulation

Mouse-derived BV2 microglial cell line (originally from ATCC) was cultured in a 96-well plate and DMEM media supplemented with 10% fetal bovine serum, Penicillin and Streptomycin, and MEM non-Essential amino acid. The cells were stimulated with 1µg/mL of a gram-negative *Escherichia coli* (O111:B4) endotoxin, LPS (Sigma-Aldrich) for 1 hr before treatment with various concentrations of PGME or pterostilbene (Aegle

Pharma, Malaysia) as a positive control. The cells were then incubated for 24 hrs in a 5% CO_2, 37°C incubator. The cell culture supernatant was collected for the subsequent experiment.

Cell viability assay

The viability of PGME-treated BV2 cells was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) of CellTiter 96® AQueous One Solution (Promega, USA). A 20 µL of MTS reagent was added into each well of 100 µL DMEM cell-containing medium in the 96-well assay plate following incubation in a 5% CO_2 incubator for 2 hrs. Finally, the absorbance was recorded at 490 nm using a 96-well DYNEX MRX version 2.02 microplate reader.

Nitric oxide production by Griess assay

A 50 µL of BV2 culture supernatant from previously LPS-stimulated PGE-treated BV2 cells was aliquoted into a new 96-well plate. An equal volume of freshly prepared Griess reagent (0.1 % N-1-naphthyl-ethylenediamine hydrochloride and 1 % sulphanilamide in 4 mL of 2.5% of phosphoric acid) was added to the plate followed by 10 min of incubation in the dark environment. Sodium nitrate (NaNO3) was used as a standard in which preparation of standard curve was conducted by diluting sodium nitrate using distilled water in1:1 ratio (concentration ranging from 0 to 100 µM). Finally, absorbance values were recorded at 530 nm by using a Dynex MRX version 2.02 microplate reader.

Cytokine profile

Cytometric Bead Array Mouse Inflammation Kit was used to measure the production of secreted cytokines in the cell culture supernatant from previously cultured BV2 cells with LPS and PGME. The protocol was performed according to the manufacturer's instructions and the samples were acquired on the BD FacsDIVATM software (BD Biosciences, USA). The results were analyzed by FCAP Array software (BD Biosciences, USA).

Statistical analysis

Results were presented as mean ± SEM and the statistical comparisons between groups which analyzed using the Statistic Package for Social Sciences program version 23 (IBM SPSS 23.0, 2017) and Microsoft Excel 2013. One-way analysis of variance (ANOVA) was performed to identify any significant difference between groups, whereas Post-Hoc 'Tukey's HSD test was conducted to determine the significant difference between groups. Data with a P-value less than 0.05 was considered statistically significant.

RESULTS

Free radical scavenging activity of PGME

Table 1 shows the comparison of DPPH radical scavenging acivity between PGME and ascorbic acid.

Table I: DPPH radical scavenging activity of PGME and ascorbic acid.

Concentration of sample (µg/mL)	Percentage Scavenging Activity (%)	
	PGME	Ascorbic acid
	Mean (%) ± SEM	
0.0001	17.07 ± 11.22	4.78 ± 0.13
0.001	30.52 ± 00.04	23.83 ± 0.13
0.01	34.41 ± 09.61	26.12 ± 0.00
0.1	48.42 ± 04.15	29.72 ± 1.53
10	69.21 ± 09.83	71.69 ± 0.27
100	95.11 ± 00.26	89.71 ± 5.17
1000	96.29 ± 00.48	94.74 ± 0.04
10000	95.11 ± 00.70	96.54 ± 0.04

Data are expressed as mean ± SEM from two independent experiments.

The percentage scavenging activity of both PGME and ascorbic acid were constructed into a dose-response logarithmic function which depicted in Figure 1 and equation obtained was used to calculate the EC50 value. Figure 1 shows the percentage of DPPH scavenging activity of PGME and ascorbic acid on radicals in a concentration-dependent manner which increased from 17.07% to 96.29% and 4.78% to 96.54%, respectively. It is shown that the dose-response of scavenging activities of PGME and ascorbic acid is proportionate to the treated concentrations (20). The EC50 value of ascorbic acid (0.425 µg/mL) was slightly higher than PGME (0.102 µg/mL), and no significant difference was shown between these two treatments.

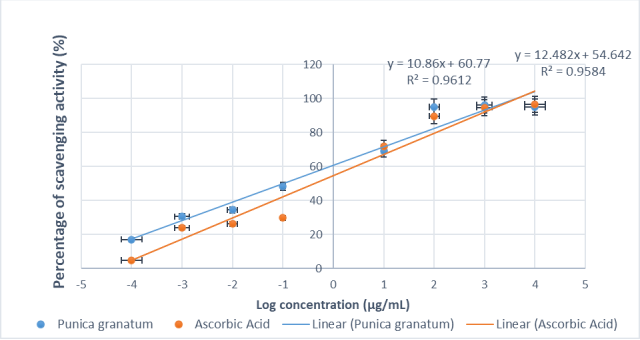

Figure 1: Percentage of free radical scavenging activity of PGME in comparison to ascorbic acid measured at various concentration (0.0001-10000 µg/mL). The percentage scavenging activity of both PGME and ascorbic acid were constructed into a dose-response logarithmic function and the equation obtained was used to calculate the EC50 value. Concentration of -4, -3, -2, -1,1,2,3 and 4 represent concentration of 0.0001, 0.001, 0.01, 0.1, 10,100,1000 and 10000 µg/mL, respectively. Each value was the result of two independent experiments (n = 2) and the data are expressed as mean ± SEM

Ferric Reducing Antioxidant Power (FRAP) activity of PGME

The PGME and ascorbic acid FRAP data were expressed as mM Fe2+/g of extract. Figure 2 shows the FRAP value of PGME was slightly lower compared to ascorbic acid. Independent t-test shows that there is no significant difference between these two samples. This indicates

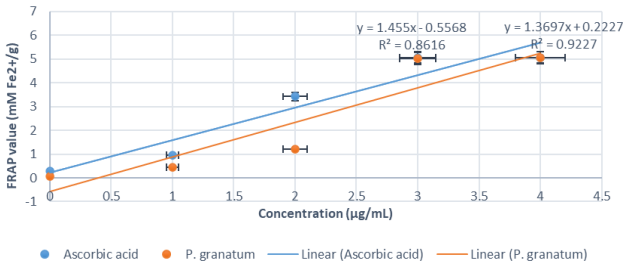

Figure 2: FRAP value of PGME and ascorbic acid expressed as mM Fe2+ /g of extract. Concentration of 0,1,2,3 and 4 represent concentration of 1,10,100,1000 and 10000 µg/mL, respectively. Each value was the result of two independent experiments (n = 2) and the data are expressed as mean ± SEM.

that PGME has similar antioxidant activity to ascorbic acid and has a strong ferric reducing power.

Effect of PGME on cell viability of BV2 Microglial cells

All concentrations of PGME (0.01 to 1000 mg/ml) retained the survival of the cells at high percentage (Figure 3). This finding indicates that the PGME extract (up to 1000 µg/ml) has no cytotoxicity effects on the viability of BV2 cells.

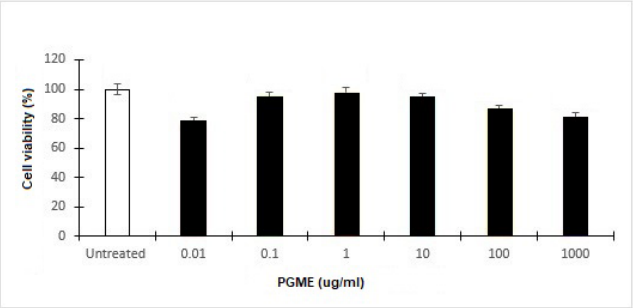

Figure 3: Effect of PGME at different concentration on cell viability of BV2 cells. The cells were treated with various concentration of PGME for 24 hours followed by a viability test using MTS reagent. Each value was the result of at least three independent experiments (n = 3) and the data are expressed as mean ± SEM.

Determination of NO levels in PGME-treated LPS-stimulated BV2 cells

The levels NO in PGME- and pterostilbene-treated LPS-induced BV2 cells were determined by the level of nitrite (Figure 4). The PGME was evaluated at a concentration range from 0.01 to 10 µg/mL as the concentrations exhibited high percentage cell viability in MTS assay. There was a significant increased ($p<0.05$) in the production of NO in LPS-stimulated BV2 cells when compared to the untreated control. However, no substantial reduction was observed on NO levels when the cells were treated with PGME or pterostilbene.

Effects of PGME on pro-inflammatory cytokine production

The production of TNF-α in LPS-stimulated BV2 microglial cells was significantly suppressed by pterostilbene and PGME in a concentration-dependent

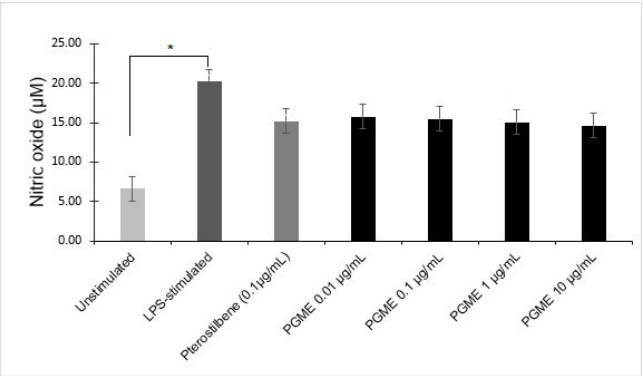

Figure 4: Production of NO in LPS-induced BV2 cells upon treatment of PGME. Griess assay was performed to determine the production of NO in PGME or pterostilbene treated LPS-stimulated BV2 cell supernatant. Data are expressed as mean ± SEM of three independent experiment with *$p<0.05$ indicates significant increase when compared to unstimulated control analyzed by one-way ANOVA post hoc LSD.

manner (Figure 5). However, no significant difference was observed for MCP-1 and IL-6 production in the PGME-treated LPS-stimulated BV2 cells.

DISCUSSION

P. granatum peel has an irregular shape and characterized by an interior network of membranes that contain high levels of phenolic compounds, including flavonoids and hydrolyzable tannins (3,21,22). Also known as pomegranate, *P. granatum* is a polyphenol-rich fruit with various potential agents including anti-inflammatory, anti-oxidative and anti-cancer in many experimental models (23). Our study aimed to use different antioxidant assays to investigate the antioxidant properties of PGME, the Griess assay and Cytokine Bead Array (CBA) Mouse Inflammation Kit to investigate the inhibitory effect of PGME on pro-inflammatory mediators produced by LPS-stimulated BV2 microglial cells.

As shown in DPPH data analysis, PGME may have a similar activity to ascorbic acid, which is known as among the strongest antioxidant. This finding may indicate that PGME is likely have a strong antioxidant power as ascorbic acid. The EC50 value of ascorbic acid was 0.425 µg/mL, which was slightly higher than PGME (0.102 µg/mL). It may indicate that PGME has a higher antioxidant activity than ascorbic acid as it required a low concentration to scavenge 50% of DPPH radical. The low value of EC50 indicates that the plant extract contains high antioxidant activity (24). A previous study has reported that PGME peel has a lower EC50 value (4.55 µg/ml) compared to *P. granatum* that extracted from seed and leaf (25). However, both EC50 values were slightly higher compared to our finding (0.102 µg/ml). Nevertheless, the radical scavenging activities of various phenolic compounds react distinctly in which it depends on the location and number of hydroxyl groups of phenolic (26).

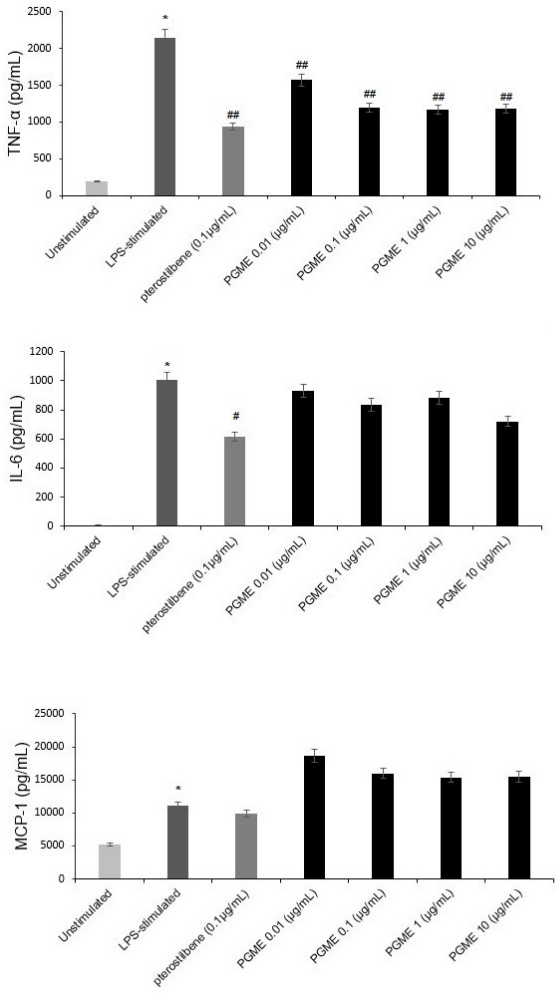

Figure 5: Inhibitory effects of PGME and pterostilbene on TNF-α, IL-6 and MCP-1 in LPS-induced BV2 cells. The cytokine levels in PGME or pterostilbene treated LPS-stimulated BV2 cell supernatant were measured by CBA inflammatory kit on flow cytometry. Data are expressed as mean ± SEM of three independent experiment with *p<0.05 indicates significant increase when compared to unstimulated control and #p<0.01 or ##p<0.05 indicates significant decrease when compared with LPS-stimulate cells, analyzed by one-way ANOVA post hoc LSD

The antioxidant activity of pomegranate extract has a strong correlation with phenolic composition (27). This finding was supported by other studies (28,29). In addition, total phenolic content corresponds to the ferric reducing capacity proportionally. It was demonstrated that breaking the free radical chain through donating a hydrogen atom denoted positive reducing potential which is associated with the presence of phenolic compounds (20). The phenolic compound of PGME peel extracts is responsible for free radical scavenging activity which involves in a donation of an electron to free radicals that converts them relatively to more stable compounds (30).

Our study has found that there is no difference in the cytotoxicity of PGME extract in all concentrations tested in BV2 cells. A similar finding was observed in a previous study of phytochemical punicalagin isolated from *P. granatum* on the viability of RAW 264.7 macrophages cell line which indicated no significant differences between the sample groups (p>0.05) (31). Our finding demonstrated that the viability of BV2 microglial cells maintained at a high percentage (>70%) in all concentrations tested indicating that PGME has a non-toxicity effect on BV2 cells. A similar finding was also reported in the study of galangin, an active polyphenol, on BV2 cells which showed no cytotoxicity effects (32). Moreover, low concentrations of *P. granatum* flower extract (0 to 100 µg/mL) has also no cytotoxicity effect on the viability of RAW 264.7 cells (33).

Phenolic compounds are a secondary metabolite of a plant whose anti-oxidative and anti-inflammatory properties were widely identified and well-known in most of the studies (34). Quercetin, a plant phenolic compound exerts anti-inflammatory activity through regulation of NO production by the nuclear factor-kappaB (NF-kB) pathway in LPS-stimulated macrophages (35). Another natural flavonoid namely chrysin has also been reported to exhibit anti-inflammatory and antioxidant effects and potentially decreasing the production of NO in LPS-stimulated microglia (36,37). In addition, flavonoids content in quercetin may effectively suppress LPS-induced NO generation in microglial cells (38,39). These phenolic compounds may exhibit in PGME which was identified previously in a similar study (40). Therefore, we suggest that these compounds may responsible for the anti-inflammatory effects showed by PGME. The study also found that ellagic acid, gallic acid, and punicalagin treatments can reduce nitrite generation in LPS-induced RAW 264.7 macrophage cells in a concentration-dependent manner (40). This finding is in line with our data which show that PGME is ably decreased NO production in LPS-stimulated BV2 cells, although no significant change was observed in all concentrations tested.

NF-κB is a transcription factor that control the regulation of inflammatory gene expression including cytokine genes (41). Increase production of proinflammatory cytokines often associated with various inflammatory diseases. Antioxidants may have an anti-inflammatory property by which can inhibit the production of proinflammatory mediators. In a recent study, punicalagin (2,3 hexahydroxydiphenoylgallagyl -D-glucose), a major polyphenol isolated from *P. granatum*, inhibits inflammatory mediators and cytokine overproduction in LPS-induced RAW 264.7 cells including NO, PGE2, IL-1β, IL-6, and TNF-α via suppression of MAPKs and NF-κB activation, which may contribute to the anti-inflammatory effect of PGME (42). PGME or its polyphenols have been shown in several studies to regulate inflammatory response by inhibiting proinflammatory cytokine production and providing antioxidant protection. TNF-α is a key inflammatory cytokine and could be a target in many inflammatory

diseases. Our findings revealed that PGME can reduce the production of TNF-α in LPS-stimulated BV2 cells. This data suggests that PGME potentially inhibit inflammatory response mediated by LPS in BV2 cells through inhibition of TNF-α.

CONCLUSION

This study revealed the potential of PGME in inhibiting several neuroinflammatory mediators and free radicals through anti-inflammatory and scavenging activities. The PGME has shown good antioxidative effects in both DPPH assay and FRAP assay in a concentration-dependent manner. This study also has demonstrated the effects of PGME on some inflammatory mediators of neuroinflammation in comparison with pterostilbene. The ability of PGME to inhibit the production of NO, TNF-α, and IL-6 production in BV2 cells suggests the anti-inflammatory property of PGME. The findings of this study might be useful for future investigation of identification, characterization, and isolation of the bioactive compound in *P. granatum* which potentially modulate neuroinflammation.

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