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Original Research Article

Antioxidant and anti-inflammatory effects of *Aegle marmelos* fruit and *Moringa oleifera* leaf extracts on lipopolysaccharide-stimulated BV2 microglial cells

Nootchanat Mairuae[1]*, Benjaporn Buranrat[1], Supataechasit Yannasithinon[1], Poonlarp Cheepsunthorn[2]

[1]Faculty of Medicine, Mahasarakham University, 296 Nakhornsawan Road, Talad Subdistrict Muang District, Maha Sarakham 44000, [2]Department of Anatomy, Faculty of Medicine, Chulalongkorn University, Bangkok 10300, Thailand

For correspondence:* ***Email:* *mairuae.n@gmail.com; nootchanat.m@msu.ac.th;* ***Tel:*** *6643-712-992*

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Abstract

Purpose: *To determine the antioxidant and anti-inflammatory effects of Aegle marmelos fruit and Moringa oleifera (M. oleifera) Lam leaf extracts on LPS-stimulated BV2 microglial cells.*
Methods: *BV2 cells were incubated with LPS for 24 h in the presence or absence of A. marmelos fruit extract or M. oleifera leaf extract. Subsequently, the levels of reactive oxygen species (ROS), nitric oxide (NO), tumor necrosis factor α (TNF α), and interleukins (IL) 6 were assessed. Thereafter, 2,2-diphenyl-1-picrylhydraz (DPPH), 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) antioxidant assays were performed.*
Results: *The levels of ROS, NO, TNF α, and IL 6 in LPS-treated BV2 cells were significantly higher when compared to those in control group ($p < 0.05$). However, exposure of LPS-treated BV2 cells to either A. marmelos or M. oleifera leaf extract led to significant decrease in the levels of ROS and inflammatory mediators, when compared with LPS-treated group ($p < 0.05$). Decrease in the levels of ROS and inflammatory mediators in M. oleifera leaf extract-treated cells was also significantly larger when compared with A. marmelos-treated group ($p < 0.05$). The DPPH, ABTS, and FRAP assays revealed that M. oleifera leaf extracts had greater antioxidant capacity than A. marmelos extracts.*
Conclusion: *Aegle marmelos fruit and M. oleifera leaf extracts exert antioxidant and anti-inflammatory effects on LPS activated BV2 cells in an in vitro model. Therefore, A. marmelos fruit and M. oleifera leaf extracts may be agents in the development of novel anti oxidative and anti-neuroinflammatory herbal medicines, or as food supplements.*

Keywords: *Aegle marmelos fruit, BV2 microglia, Moringa oleifera, Neuroinflammation, Reactive oxygen species (ROS)*

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INTRODUCTION

Neuroinflammation and oxidative stress mediated by activated microglia have been implicated in the onset and progression of numerous neurodegenerative disorders [1]. Accumulating evidence point towards activated microglia as being a chronic source of multiple

neurotoxic factors, including reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α), interleukins (IL)-6 and IL-1β, and nitric oxide (NO) [2]. The overproduction of these neurotoxic factors drives progressive neuronal damage. Therefore, controlling microglial activation should theoretically provide a beneficial approach for the treatment and prevention of neuroinflammatory and neurodegenerative disorders. Many medicinal plants have been reported to have anti-inflammatory properties [3]. *Aegle marmelos* (L.) Corr. Serr (*A. marmelos*), commonly known as *Bael* or *Bilva*, belongs to the family Rutaceae, and it has been widely used in traditional medicine systems of Asian countries due to its various medicinal properties such as anti-diarrhoeal, anti-proliferative, anti-inflammatory, antipyretic, hypoglycemic, antioxidant and anti-fungal activity [4,5]. Although several medicinal properties of *Aegle marmelos* have been reported, the scientific data on the effect of *Aegle marmelos* extract on microglial activation and neuroinflammation has not yet been reported. *Moringa oleifera* (*M. oleifera*) Lam (Moringaceae family) is a plant native to northwestern India, and it is widely cultivated in sub-tropical as well as tropical regions [6]. The plant *has* numerous common names across *regions* where it is cultivated, *with* horseradish tree, *drumstick tree,* or simply moringa *used* as English names. Every part of the moringa plant is edible [7]. *Moringa oleifera* has been used worldwide in traditional medicines for the treatment of disorders associated with inflammation, including glandular swelling, bronchitis and articular pain [8]. A major flavonoid found in *M. oleifera* leaves is astragalin, which is one of the bioactive constituents associated with anti-inflammatory and antioxidant properties [9]. Even though *M. oleifera* has several claimed medical benefits, there is currently a dearth of research on how its leaf extract affects neuroinflammation and microglial activation. Thus, the objective of this study was to investigate the anti-inflammatory and antioxidant effects of *A. marmelos* fruit and *M. oleifera* leaf extracts on BV2 microglial cells stimulated with lipopolysaccharide (LPS). The levels of the inflammatory mediators IL-16, TNF-α, NO and ROS released from the cells were determined.

EXPERIMENTAL

Preparation of *A. marmelos* fruit and *M. oleifera* leaf extracts

Extracts of *A. marmelos* fruit and *M. oleifera* leaves were prepared as described previously [10].

Culture and treatments of murine BV2 cells

The murine BV2 microglial cell line was kindly provided by Dr James R. Connor of the Department of Neurosurgery, College of Medicine, Pennsylvania State University (Hershey, PA, USA).

The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin/streptomycin in 5 % fetal bovine serum at 37 ^{o}C in a humidified atmosphere containing 5 % CO_2 as previously described [15]. All cell culture reagents were obtained from HyClone (now a brand of Cytiva; Logan, UT, USA).

Evaluation of cell viability

After 24 h of treatment with either *A. marmelos* fruit or *M. oleifera* leaf extract at concentrations of 25 - 100 µg/mL with or without LPS, the incubation medium was discarded, and MTT reagent at 0.4 mg/mL (MilliporeSigma, St. Louis, MO, USA) in serum-free DMEM was added to each well. After a further incubation at 37 ^{o}C in a humidified atmosphere containing 5 % CO_2 for 2 h, the MTT medium was removed, and purple formazan crystals formed were solubilized in dimethyl sulfoxide (DMSO).

The absorbance of each well was read at 570 nm in a plate reader (Spectramax 340 PC; Molecular Devices, LLC, Sunnyvale, CA, USA).

NO assay

To quantify the accumulation of nitrite, the NO levels in cell culture supernatants were estimated using Griess reagent (which comprises 1% sulfanilamide + 0.1 % *N*-(1-naphthyl)-ethyl-enediamine dihydrochloride + 2.5 % phosphoric acid) as described in a prior work [10].

Intracellular ROS assay

The levels of ROS within cells were measured using the technique of assessing the oxidation of 2',7'-dichlorofluorescein diacetate (DCFH-DA) according to a prior study [10].

Assay of IL-6 and TNF-α production

The concentrations of IL-6 and TNF in the culture supernatant were determined using enzyme-linked immunosorbent assay (ELISA) kits, as described in an earlier study [10].

Determination of the DPPH, ABTS and FRAP radical-scavenging activities

The 2,2-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging capacities of extracts were calculated using the reduction of the reaction color between the DPPH solution and the sample extracts, as previously indicated by the prior approach [11]. Ascorbic acid and Trolox® were employed as reference materials in this investigation. In each experiment, blanks were used. The percentage DPPH radical ability (D) was expressed as IC_{50} mg/mL and the inhibition percentage was calculated using Eq 1.

$$D\ (\%) = \{(A_0-A_1)/A_0\}100 \quad \ldots\ldots\ldots\ldots\ldots\ (1)$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

The principle of ABTS assay measures the relative ability of antioxidants to scavenge the ABTS generated in aqueous phase, as compared with a Trolox (water soluble vitamin E analogue) standard [11]. The ABTS scavenging ability (A) was expressed as IC_{50} (mg/mL) and the inhibition percentage was calculated using Eq 2.

$$A\% = \{(A_0-A_1)/A_0\}100 \quad \ldots\ldots\ldots\ldots\ldots\ (2)$$

Where A_0 is the absorbance of the control and A_1 is the absorbance of the sample.

The ferric reducing antioxidant power (FRAP) assay is a typical method that measures the reduction of ferric ion (Fe^{3+})-ligand complex to the intensely blue-colored ferrous (Fe^{2+}) complex by antioxidants in an acidic medium. Antioxidant activity is determined as increase of absorbance at 593 nm, and results are expressed as relative to an antioxidant standard. In this study, the antioxidant activity of *A. marmelos* fruit or *M. oleifera* leaf extract was determined in accordance with the method outlined by Puangpornpitag *et al* [11].

Determination of total phenol and flavonoid contents

Total phenolic content was determined according to a modified procedure [11]. The sample (100 µL) was oxidized with 500 µL of 0.2 N Folin-Ciocalteu's reagent and neutralized by adding 400 µL of 7.5 % Na_2CO_3. After mixing and incubating at room temperature for 30 minutes, the absorbance was measured at 765 nm. The results were expressed as gallic acid equivalents (mgGE/gExt)

Flavonoid content was estimated using the aluminum chloride colorimetric method. The plant extract was mixed with 100 µL of 5 % aluminum chloride (w/v), 400 µL of 2.5 % Na_2NO_3. After 5 min, 500 µL of 5 % $AlCl_3$ was added. The mixture was allowed to stand at room temperature for 10 min. Two milliliters were added to the solution. The absorbance was measured at 415 nm. The TFC was calculated from a standard quercetin equivalent (mgQE/gExt) [11].

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). One-way ANOVA and Bonferroni's post-hoc tests were used for analysis of statistically significant differences amongst multiple groups. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Effect of *A. marmelos* fruit and *M. oleifera* leaf extracts treated with LPS on BV2 cell viability

First, it was necessary to evaluate the effect of *A. marmelos* and *M. oleifera* leaf extracts on the viability of the BV2 cells prior to the analyses of their potential anti-inflammatory and antioxidant properties. The results from the MTT assay demonstrated that, after 24 h of treatment with *A. marmelos* (Figure 1 A) or *M. oleifera* leaf (Figure 1 B) extract at concentrations ranging from 25 - 50 µg/mL, the cell viability was not affected when compared with that of the control (untreated) cells. Therefore, extracts at concentrations of 25 and 50 µg/mL were chosen for subsequent anti-oxidative stress and anti-inflammatory studies.

Attenuation of ROS production in LPS-treated microglial cells

To determine the antioxidant capacities of the leaf extracts *A. marmelos* and *M. oleifera* in LPS-treated BV2 cells, the intracellular level of ROS was determined. There was a significant increase (by 2.3-folds) in the level of ROS in LPS-treated cells, when compared with the control cells. However, the ROS level was significantly decreased in a concentration-dependent manner in *A. marmelos* and *M. oleifera* leaf extract-treated BV-2 cells, when compared with cells treated with LPS alone ($p < 0.01$; Figure 2). Treatment of cells with *M. oleifera* leaf extract reduced ROS level more than treatment with A. marmelos.

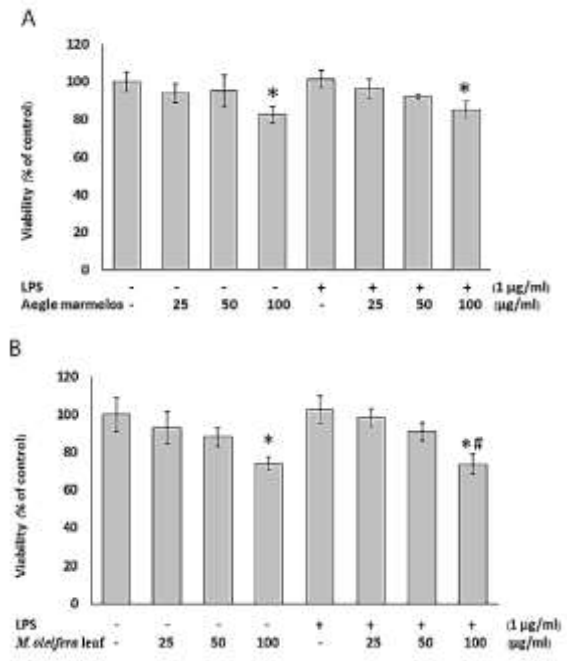

Figure 1: Effects of *A. marmelos* fruit (A) and *M. oleifera* leaf extract (B) on BV2 cell viability using MTT assay. $P < 0.05$ vs. the control group, $^{\#}p < 0.05$, vs. control group. (LPS: lipopolysaccharide)

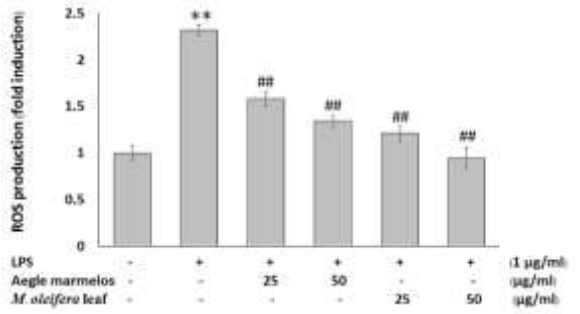

Figure 2: Effect of *A. marmelos* fruit and *M. oleifera* leaf extracts on ROS production. $^{**}P < 0.01$ vs. the control group; $^{\#\#}p < 0.01$ vs. the LPS-treated group

Attenuation of NO production in LPS-induced microglial cells

To investigate the anti-inflammatory properties of *A. marmelos* and *M. oleifera* leaf extracts, the effect of the extract on LPS-induced NO production was indirectly measured using determining the nitrite content of the BV2 cells after 24 h of treatment. The results demonstrated that LPS treatment caused a significant increase (by 8.4 folds) in the NO level in the culture medium of BV2 microglial cells when compared with the untreated control cells. However, treatment with *A. marmelos* and *M. oleifera* leaf extracts led to significant and concentration-dependent decreases in the NO levels compared with cells treated with LPS alone ($p < 0.05$, Figure 3). However, *M. oleifera* leaf extract at the same concentration resulted in lower NO production when compared with *A. marmelos* extract.

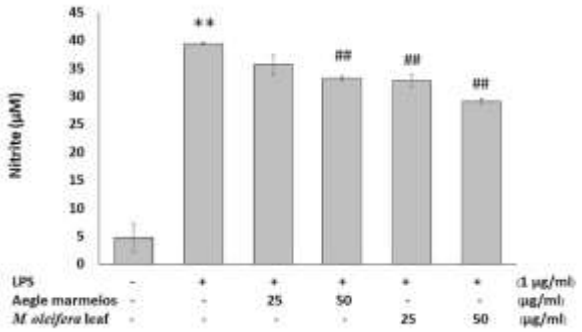

Figure 3: Effect of *A. marmelos* fruit and *M. oleifera* leaf extract on NO levels. The BV2 cells were treated as described in the material and method. After 24 h of treatment, the NO level in the culture medium was determined using Griess assay. Data are presented as mean ± S.E.M., and the data are shown as the mean of three independent experiments. $^{**}P < 0.01$ vs. control group; $^{\#}p < 0.05$ vs. the LPS-treated group. (LPS= lipopolysaccharide; NO = nitric oxide)

Attenuation of TNF-α levels in LPS-induced microglial cells

To further determine the anti-inflammatory properties of *A. marmelos* and *M. oleifera* leaf extracts in LPS-activated BV2 microglial cells, the TNF-α level in the culture media of BV2 cells was determined using ELISA. As shown in Figure 4, the TNF-α level in the culture media of BV-2 cells was significantly increased (by 43 folds) following treatment with LPS compared with the control (untreated) cells. However, when the cells were co-treated with LPS and either *A. marmelos* or *M. oleifera* leaf extract, the level of TNF-α was significantly decreased in a concentration-dependent manner when compared with cells treated with LPS alone ($p < 0.01$; Figure 4). However, *M. oleifera* leaf extract at the same concentration resulted in lower TNF-α production than with *A. marmelos* extract.

Attenuation of IL-6 levels in LPS-induced microglial cells

To further determine the anti-inflammatory properties of *A. marmelos* and *M. oleifera* leaf extracts in LPS-activated BV2 microglial cells, the IL-6 level was determined using ELISA. As shown in Figure 5, the IL-6 level in the culture media of BV-2 cells was significantly increased (by 21 folds) following treatment with LPS when compared with the control (untreated) cells. However, when the cells were co-treated with LPS and the *M. oleifera* leaf extract, the levels of

IL-6 were significantly decreased in a concentration-dependent manner when compared with treatment of the cells with LPS alone ($p < 0.01$; Figure 5). Co-treatment with LPS and *A. marmelos* extract also led to a decrease in IL-6 production, although this decrease was not statistically significant. Therefore, it appeared that the leaf extract of *M. oleifera* had a greater anti-inflammatory potential than *A. marmelos* extract.

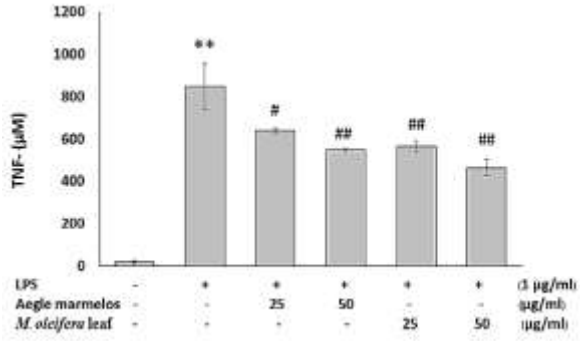

Figure 4: Effect of *A. marmelos* fruit and *M. oleifera* leaf extracts on TNF-α levels. $^{**}P < 0.01$ vs. control group, $^{\#}p < 0.05$ vs. LPS-treated group, $^{\#\#}p < 0.01$ vs. LPS-treated group

In vitro antioxidant properties of *A. marmelos* fruit and M. oleifera leaf extracts

The results of the antioxidative potential of *A. marmelos* fruit and *M. oleifera* leaf extracts is shown in Table 1.

Total phenolic and flavonoid contents of *A. marmelos* fruit and *M. oleifera* leaf extracts

The phenolic and flavonoid contents of the extracts are shown in Table 2.

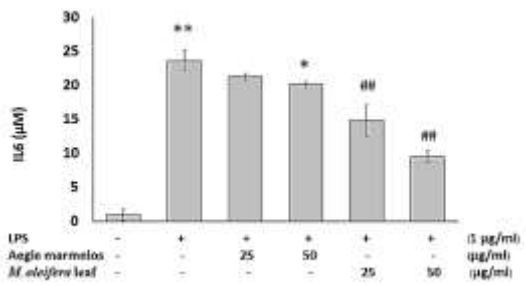

Figure 5: Effects of *A. marmelos* fruit and *M. oleifera* leaf extracts on IL-6 levels. $^{**}P < 0.01$ vs. control group, $^{\#}p < 0.05$ vs. LPS-treated group, $^{\#\#}p < 0.01$ vs. LPS-treated group

DISCUSSION

Progressive neuronal cell death is a characteristic feature of neurodegenerative diseases that result in dementia, cognitive impairment, loss of motor control, impairment of functional abilities and ultimately, death. Owing to the lack of effective treatments, neurodegenerative diseases are becoming an increasing economic burden on society [12]. Therefore, there is an urgent to identify compounds that prevent neuronal cell death in order to facilitate the development of therapeutic strategies to combat this global health problem. Neuroinflammation and oxidative stress have been reported to be hallmarks of neurodegenerative diseases, which contribute towards disease progression [1,2]. Currently, researchers are investigating the therapeutic potential of natural antioxidants as treatments for neuroinflammation and oxidative stress and/or as preventatives of the neurodegenerative disorders that are present with these two conditions [13].

Table 1: Antioxidant activities of extracts from *A. marmelos* fruit and *M. oleifera* Lam leaves, showing the IC_{50} values for DPPH, ABTS and FRAP

Antioxidant	DPPH IC_{50} (mg/mL)	ABTS IC_{50} (mg/ml)	FRAP (mgTE/gExt)
A. marmelos fruit	0.0387±0.003	0.0007±0.000	406.110±0.227
M. oleifera leaf extract	0.0048±0.001	0.00028±0.000	621.071±0.930
Ascorbic acid	0.016±0.000	0.0250±0.001	-
Trolox®	0.044±0.001	0.0320±0.001	-

Units of the FRAP values are mgTE/gExt (DPPH: 2,2-diphenyl-1-picrylhydrazyl; ABTS: 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid); FRAP: ferric-reducing antioxidant power)

Table 2: Total phenolic **(**TPC**)** and flavonoid **(**TFC**)** content of ethanol extracts from *A. marmelos* fruit and *M. oleifera* Lam leaves

Extract	TPC (mgGE/gExt)	TFC (mgQE/gExt)
A. marmelos fruit	24.359±0.353	98.449.237±0.341
M. oleifera leaf	10.163±0.579	113.237±0.241

TPC was measured as gallic acid equivalents (mgGE/gExt), whereas TFC was measured as quercetin equivalents (mgQE/gExt)

Aegle marmelos and M. oleifera are widely cultivated in tropical and subtropical countries due to their important medicinal and dietary properties. Therefore, in the present study, three different *in vitro* (i.e., DPPH, ABTS, and FRAP assays) were performed to assess and compare the antioxidant potential of *A. marmelos fruit and M. oleifera leaf extracts.* The findings of the DPPH, ABPS and FRAP radical scavenging assays revealed that the *M. oleifera* leaf extract had more antioxidant capabilities than the *A. marmelos* fruit extract.

The results of ABTS IC_{50} values of *A. marmelos* and *M. oleifera* Lam leaf extracts were lower than those of the standards, i.e. ascorbic acid and Trolox. Furthermore, the DPPH IC_{50} values of *A. marmelos* and *M. oleifera* Lam leaf extracts were lower than Trolox, indicating that *A. marmelos* fruit and *M. oleifera* Lam leaf extracts possessed antioxidant activity. However, *M. oleifera* Lam leaf extract had higher antioxidant activity when compared with *A. marmelos* fruit extract. These results demonstrated that *A. marmelos* fruit and *M. oleifera* Lam leaf extracts had potent antioxidant activity than that which was reported by Rajan *et al* [14] and Xu *et al* [15]. The differences observed in activities, however, may be attributable to the different geographic regions. The *A. marmelos* fruit and *M. oleifera* Lam leaf extracts also had the ability to inhibit ROS production in LPS-activated BV2 microglial cells, although this effect was significantly decreased by treatment with the extract at high concentrations tested.

Furthermore, these extracts also suppressed neuroinflammation by decreasing the levels of NO, TNF-α and IL-6 in LPS-activated BV2 microglia. Nitric oxide production was significantly increased when BV2 microglial cells were exposed to LPS, whereas treatment of cells with the ethanol extracts of *A. marmelos* fruit and *M. oleifera* leaves at concentrations of 25 and 50 µg/mL, respectively led to a significant decrease in NO level in a concentration-dependent manner. However, treatment with *M. oleifera* reduced the NO level more than treatment with *A. marmelos.* These findings are consistent with those of a recent study which showed that the ethanol leaf extract of *M. oleifera* significantly inhibited the production of NO in LPS-stimulated murine macrophages [16]. In the central nervous system, NO is the inflammatory mediator derived from activated microglia. High concentrations of NO are involved in the onset of multiple sclerosis, Alzheimer's disease and Parkinson's disease, in addition to other neurodegenerative diseases [17]. The results of the present study have demonstrated that *A. marmelos* fruit and *M. oleifera* leaf extracts have the ability to mitigate the inflammation caused by excessive levels of NO.

In these experiments, LPS treatment led to significant increases in the release of TNF-α and IL-6 from BV2 microglia cells into the cell culture media after 24 h of treatment. However, the levels of TNF-α and IL-6 were significantly reduced in a dose-dependent manner when the BV2 cells were treated with *A. marmelos* fruit or *M. oleifera* leaf extracts. The leaf extracts of *M. oleifera* also yielded lower levels of TNF-α and IL-6 production compared with *A. marmelos* fruit. Finally, these results corroborated previous reports showing that treatment with the ethanol extract from *M. oleifera* leaves led to significant decreases in the levels of TNF-α and IL-6 in LPS-stimulated murine and human macrophage cells, as well as in an atherogenic diet-induced hyperlipidemic rat model [16,17]. Given that these pro-inflammatory cytokines have been reported to be neurotoxic factors driving progressive neuroinflammation and neuronal damage [17], this suggests that the *A. marmelos* fruit and *M. oleifera* leaf extracts may be potential sources of anti-neuroinflammatory compounds. The present study also demonstrated that total phenolic and total flavonoid contents of *M. oleifera leaf* and fruit extract was 10.163 ± 0.579 mgGE/gExt (mgGE/gram extract) and 113.237 ± 0.241 mgQE/gExt of dried extract, respectively. By contrast, the total phenolic and total flavonoid contents of *A. marmelos* fruit were 24.359 ± 0.353 mgGE/gExt and 98.449.237 ± 0.341 mgQE/gExt of dried extract, respectively. Therefore, a flavonoid-rich extract originating from either *A. marmelos* fruit and *M. oleifera* leaves could potentially act as a source of antioxidants and anti-neuroinflammatory compounds.

CONCLUSION

Lipopolysaccharide treatment leads to significant increases in the levels of ROS and secretion of inflammatory mediators from BV2 microglial cells, and these effects are significantly attenuated by treatment with extracts of either *A. marmelos* fruit or *M. oleifera* leaves. There is evidence of antioxidant and anti-neuroinflammatory properties of *A. marmelos* fruit and *M. oleifera* leaf extracts both in activated BV2 microglial cells and in an *in vitro* model. Therefore, *A. marmelos* fruit and *M. oleifera* leaf extracts have potential for use in the development of herbal medicines, or food supplement for combating oxidative stress and neuroinflammation in neurodegenerative diseases.

DECLARATIONS

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Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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