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# *Myrmecodia platytyrea* tuber aqueous extract exacerbates neuroinflammation

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## Abstract

**Background:** *Myrmecodia platytyrea*, a member of Rubiaceae, has been traditionally used to treat inflammation-related diseases, including cancer and rheumatoid arthritis. However, its potential in managing neurodegenerative disorders remains unexplored. This study aimed to investigate the neuroprotective effect of *M. platytyrea* tuber aqueous extract (MPAE).
**Methods:** The cytotoxicity effect on astrocytes was assessed using the MTT assay. The effect of MPAE (0.025–0.5 mg/mL) on reactive oxygen species (ROS) and pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) were evaluated in $Fe_2SO_4$-, $H_2O_2$-, and LPS-stimulated astrocyte cell lines.
**Results:** MPAE was found to be non-cytotoxic to astrocytes, with no significant protective effect against $Fe_2SO_4$-, $H_2O_2$-, or LPS-induced stress. Instead, MPAE increased astrocyte cell death, as well as ROS and cytokine levels, in a dose-dependent manner.
**Conclusion:** Although MPAE is non-cytotoxic to astrocytes, its potential to exacerbate neuroinflammation *in vitro* raises concerns about its use, particularly among ageing individuals who consume this plant to manage inflammatory conditions. These findings highlight the need for caution and emphasise the importance of further research to evaluate its safety and efficacy before advocating for its medicinal use, especially for neurodegenerative disease therapy.
**Keywords:** Astrocytes, neuroinflammation, oxidative stress, pro-oxidant, *Myrmecodia platytyrea*

## Introduction

Historically, the substantial contributions of natural products and their structural analogues to pharmacotherapy cannot be overlooked. Their role in treating chronic ailments, including infectious diseases and cancer, is pivotal. However, in the 1990s, the pharmaceutical industry's pursuit of these natural products began to wane, attributed to challenges like isolation, screening, characterisation, and optimisation. In recent years, there has been renewed interest in these natural products, especially those from traditional medicinal plants, as potential leads for inflammation-related diseases [1, 2]. The accessibility, affordability, and historical efficacy of medicinal plants make them particularly attractive, especially in developing countries.
Neurodegenerative diseases, a spectrum of brain disorders encompassing Alzheimer's (AD), Parkinson's (PD), Huntington's (HD), and amyotrophic lateral sclerosis, are characterised by a steady decline in cognitive, physical, and social faculties. Such diseases, deeply rooted in neuroinflammation and chronic oxidative stress, significantly impair the quality of life [3, 4]. The brain's vulnerability to oxidative stress is heightened due to its high oxygen consumption and enrichment with polyunsaturated fatty acids [5-7]. This vulnerability underscores the significance of metabolic coupling between neurons and astrocytes in combating brain oxidative stress [8, 9]. Astrocytes, principal regulators of neuroinflammation, play a vital role both during neurogenesis and in the context of brain pathology

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[10], maintaining the health and function of the central nervous system (CNS) [11]. With their multifunctional capabilities, it's unsurprising that these cells are linked to the onset and progression of various neurodegenerative disorders [12, 13]. Recent research highlights astrocytes' protective roles against free-radical toxicity and their responsiveness to inflammatory signals [14, 15], emphasising their suitability as *in vitro* models for neurodegenerative diseases [16-18].

Interestingly, due to their diverse properties, natural compounds like flavonoids, alkaloids, resveratrol, and curcumin, are emerging as potential multitarget therapeutics for neurodegenerative disorders. *Myrmecodia platytyrea*, commonly referred to as ant-plant or *Sarang Semut*, is a myrmecophyte renowned in traditional medicine for its antioxidant, anti-inflammatory, and anticancer properties [19-21]. The tuber of this plant, rich in bioactive components such as flavonoids, tannins, polyphenols, and stigmasterol which contribute to its therapeutic potential [19, 22, 23]. Flavonoids, one of the main bioactive groups in *M. platytyrea*, are particularly noteworthy for their ability to cross the blood-brain barrier (BBB) and exert neuroprotective effects. These compounds demonstrate antioxidant and anti-inflammatory properties, critical in mitigating oxidative stress and neuroinflammation in the CNS [24-26]. Despite its traditional use in treating inflammation-linked ailments, research on the specific effects of *Myrmecodia sp*. decoction on neuroinflammation and its potential for treating neurodegenerative diseases remains sparse. This study aims to bridge this knowledge gap by elucidating the neuroprotective effects of *M. platytyrea* tuber aqueous extract (MPAE) on nerve cells, focusing on its potential to prevent or mitigate neuroinflammatory processes.

## Methods

### Plant extraction

*M. platytyrea* tubers were collected from Northern Sulawesi and were identified by Prof. Eko Baroto Walujo, from Herbarium Bogoriense, Research Centre for Biology, Indonesian Institute of Sciences, Bogor, Indonesia (voucher identification numbers BO1647929 and BO0009642). This study utilised a decoction method [27]. Briefly, the dried tuber of *M. platytyrea* was grounded into powder form and soaked in boiling distilled water (1:9) for 15 min and filtered through 4 different filter paper ranges from Whatman No. 1, 40, 42 and cellulose acetate membrane filter. The solvent in the filtrate was eliminated using a rotary evaporator (Heidolph, Germany) under reduced pressure at 100 mbar, 55°C. The concentrated filtrate was stored in a -80°C freezer for 2 days (Sanyo, Japan) and freeze-dried at a pressure of 0.007 mbar in a freeze-dryer (Labconco, UK) to get the dried powder of aqueous extract. The powdered extract was kept at -20°C (Sanyo, Japan) until further use.

### Astrocyte culture

The murine astrocyte cell lines, C8-D1A (ATCC® CRL-2541™), were purchased from ATCC. The C8-D1A cell line has the morphology of fibrous astrocytes and was isolated from the cerebellum of the mouse (*Mus musculus sp*.) brain. The complete growth medium for this cell line was Dulbecco's Modified Eagle's Medium (DMEM; Sigma-Aldrich) with foetal bovine serum (FBS; Thermofisher, UK) to a final concentration of 10 % in 75 $cm^3$ flask.

### Cytotoxicity of MPAE

Once confluent, MPAE was tested on the cells for cytotoxicity study using MTT assay [28]. Cells (100 µL) were seeded into each 96-multiwell plate (Corning, Sigma, USA). After 24 h, the MPAE extracts (0.0001, 0.001, 0.01, 0.1, 1, and 10 mg/mL) were added into the wells and incubated for 24, 48 or 72 h. At the end of each incubation period, 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich) solution was added to each well. After 4 h of incubation, the supernatant was removed, and 100 µL of DMSO was added into the wells to dissolve the formazon. MTT reduction was quantified at 550 nm using a microplate reader (Infinite M200, Tecan, Switzerland).

### Determination of the effect of MPAE on cell viability activity of $Fe_2SO_4$- and $H_2O_2$-induced oxidative stress and LPS-induced inflammation on astrocytes

Astrocytes ($2 \times 10^4$ cells/well) were seeded in a 96-well plate and incubated for 24 h. Then, the plated cells were treated with MPAE extract (*i.e*. 25, 50,125, 250, and 500 µg/mL) for 1 h. After that, the media containing $Fe_2SO_4$ (7.5 mM), $H_2O_2$ (0.2 mM) and LPS (1 µg/mL), respectively, were added into each well and then incubated at 37 °C, 5 % $CO_2$ for 24 h. Then, the MTT assay was carried out. Cell viability was determined after a 4-h incubation by measuring the absorbance at 550 nm using a microplate reader (Infinite M200 Tecan, Switzerland).

### Determination of the effect of MPAE on reactive oxygen species (ROS) level in $Fe_2SO_4$- and $H_2O_2$-induced oxidative stress

ROS accumulation was measured by the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA) [29]. Astrocytes ($2 \times 10^4$ cells/well) were seeded in a 96-well black plate and incubated for 24 h. Then, the plated cells were treated with various concentrations of MPAE extract (*i.e*. 25, 50,125, 250, and 500 µg/mL) for 1 h, after which the plate was incubated with $Fe_2SO_4$ (7.5 mM) or $H_2O_2$ (0.2 mM), respectively for 24 h. Then, the cells were washed with Kreb's buffer. Then, the measurement started by adding 100 µL of 5 µM of DCF-DA solution into the wells in the dark. After 30 min of incubation at 37ºC, ROS production was measured using a microplate reader at Ex = 485 and Em = 538 nm.

### Determination of effect of MP on pro-inflammatory cytokines of LPS-induced astrocytes

Astrocytes ($5 \times 10^5$ cells/well) were seeded in a 6-well plate for 24 h before being treated with MPAE (25, 50, 125, 250, and 500 µg/mL). After 24 h, LPS (1 µg/mL)

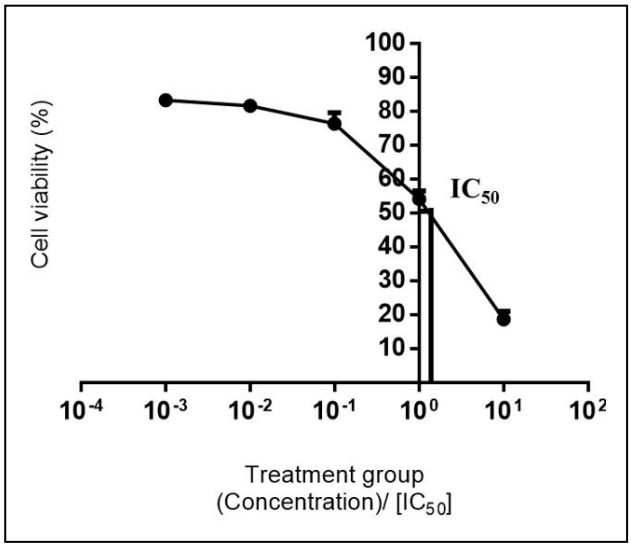


**Figure 1. Effect of *M. platytyrea* tuber aqueous extract on astrocyte viability**. Astrocytes ($2 \times 10^4$ cells/well) were seeded on 96-well plates and incubated with MPAE (0.001, 0.01, 0.1, 1, and 10 mg/mL) for 24 h. Cell viability was determined by MTT assay. Mean ± SD ($n$ = 3). $IC_{50}$ for MPAE was 1.54 ± 0.26 mg/mL.

was added to the cells, followed by a 24-h incubation [9]. Cells were then collected and centrifuged at 3000 rpm for 10 min at 4ºC. The activity of pro-inflammatory cytokines (*i.e.* TNF-α, IL-1β, and IL-6) was measured by an ELISA kit (eBioscience, Austria). The assays were carried out according to the manufacturer's protocol. The absorbances were measured using a microplate reader (Infinite M200, Tecan, Switzerland).

### Statistical analysis

Values are represented as mean ± SD of three parallel measurements. Statistical analysis was performed using One-way ANOVA in GraphPad Prism 7 software. Post-hoc comparisons between groups were made using the Bonferroni test. Value $P < 0.05$ was considered statistically significant.

## Results

*M. platytyrea* tubers (MPAE) were prepared as an aqueous extract using the decoction method to replicate traditional medicine practices. The percentage yield of MPAE was 16.05 ± 0.14 %.

The C8-D1A murine astrocyte cell line was the cell model employed to represent CNS functionalities, as these cells are key players in both physiological neuronal functions and the pathological process. Through the MTT assay, the $IC_{50}$ of MPAE in astrocytes was 1.54 ± 0.26 mg/mL (Figure 1) with maximal inhibition of 81.40 ± 2.33% at 10 mg/mL, indicating that it is not cytotoxic against normal astrocyte cell line. The criteria used for the classification of cytotoxicity is as follows: $IC_{50}$: < 20 µg/mL (high cytotoxicity), $IC_{50}$: 21-200 µg/mL (mo derate cytotoxicity), $IC_{50}$: 201-500 µg/mL (weak cytotoxicity), $IC_{50}$: >501 µg/mL (no cytotoxic activity) [30].

The effects of MPAE on astrocyte viability were evaluated under oxidative and inflammatory conditions induced by $Fe_2SO_4$, $H_2O_2$ or LPS for 24 h (Figure 2). Exposure to $Fe_2SO_4$ (7.5 mM), $H_2O_2$ (0.2 mM), and LPS (0.1 µg/mL) significantly ($P < 0.05$) reduced astrocyte viability to 51.39 ± 4.63% (Figure 2A), 52.25 ± 0.6% (Figure 2B), and 70.1 ± 3.4% (Figure 2C), respectively. Pretreatment with MPAE (25, 50, 125, 250, and 500 µg/mL) further exacerbated the reduction in cell viability in a concentration-dependent manner ($P < 0.05$) for all three inducers. No cytoprotection by MPAE was observed; instead, cell viability decreased further.

ROS accumulation in the astrocytes was measured by DCF-DA assay (Figure 3). $Fe_2SO_4$ (7.5 mM) alone induced a significant 4.70 ± 0.83-fold increase in ROS production compared to control cells ($P < 0.05$, Figure 3A). Pretreatment with MPAE (25–500 µg/mL) did not significantly alter ROS levels in $Fe_2SO_4$-treated cells, with fold changes ranging from 4.75 ± 0.33 to 5.7 ± 1.66, compared to cells treated with $Fe_2SO_4$ alone ($P > 0.05$).

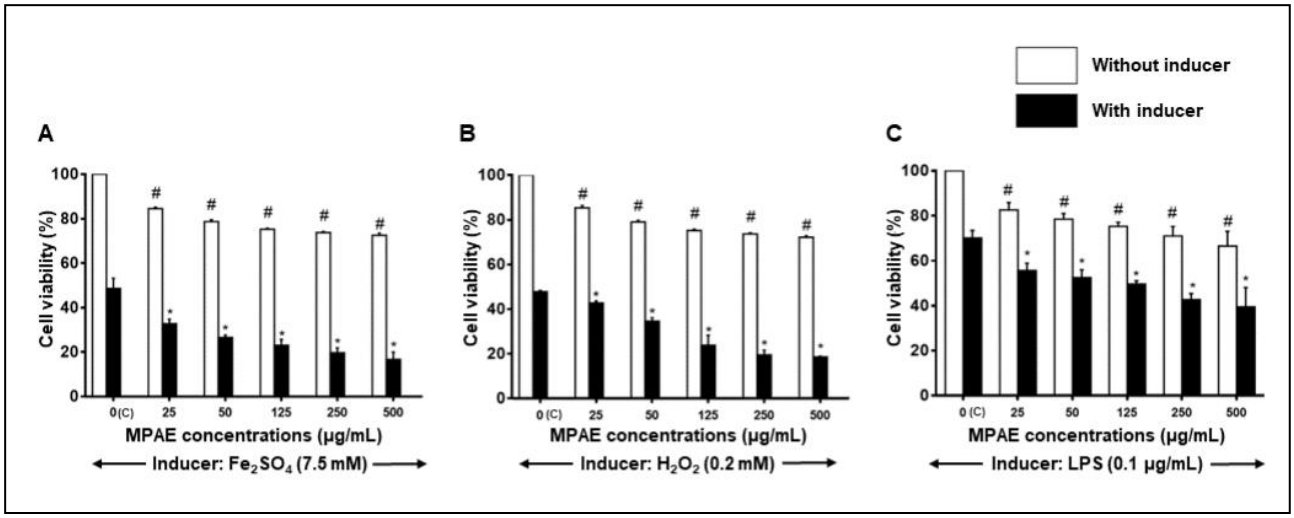


**Figure 2. Effects of MPAE on viability of astrocytes induced with (A) $Fe_2SO_4$, (B) $H_2O_2$ and (C) LPS**. Cell viability was determined by MTT assay. Mean ± SD ($n$ = 3). #Significantly different from control cells which were not treated with MPAE or inducer agent. *Significantly different from cells treated with inducer agent only ($P < 0.05$, One-way ANOVA + Bonferroni test).

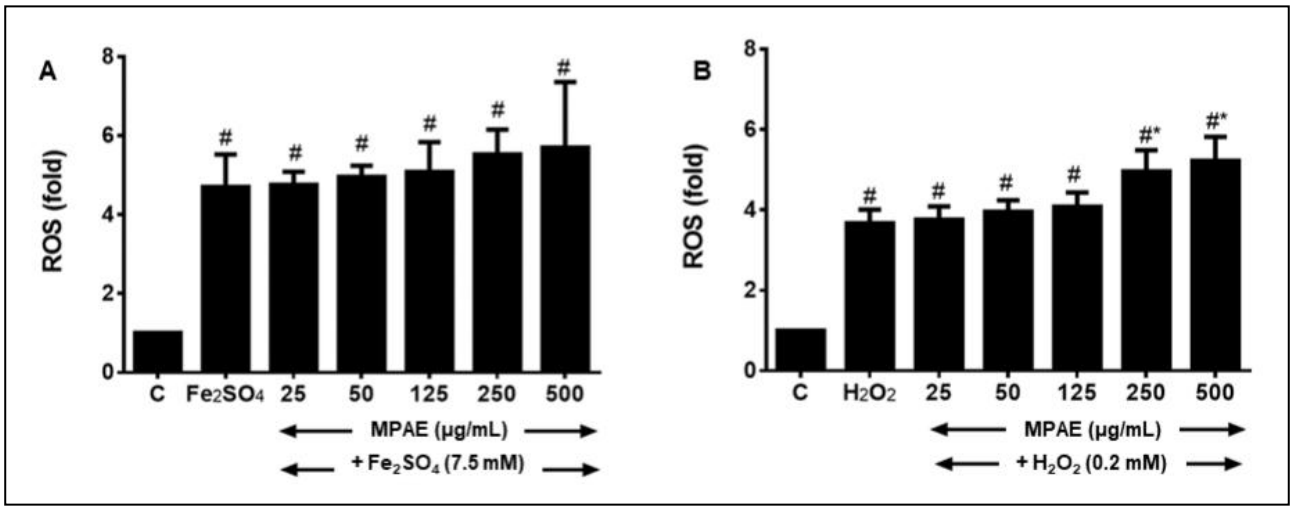


**Figure 3**. **Effect of MPAE on ROS production by astrocytes exposed to (A) $Fe_2SO_4$- or (B) $H_2O_2$-induced oxidative stress.** ROS was determined by DCF-DA assay. Mean ± SD ($n$ = 3). #Significantly different compared to control cells which were not treated with MPAE or inducer agent *Significantly different from cells treated with inducer agent only. Pretreatment of cells with MPAE did not affect ROS production by $Fe_2SO_4$ or $H_2O_2$ ($P$ > 0.05, One-way ANOVA + Bonferroni test).

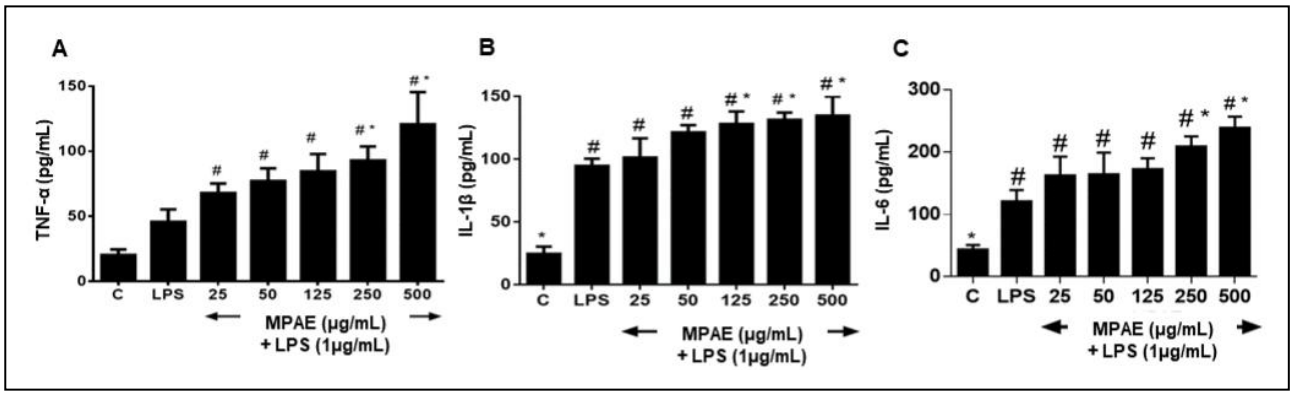


**Figure 4**. **Effect of MPAE on (A) TNF-α, (B) IL-1β and (C) IL-6 levels of astrocytes exposed to LPS-induced inflammation**. Cytokines were determined by ELISA assay. Mean ± SD ($n$ = 3). #Significantly different compared to control cells not treated with MPAE or LPS, *Significantly different compared to cells treated with LPS only ($P$ < 0.05, One-way ANOVA + Bonferroni test).

Similarly, $H_2O_2$ (0.2 mM) caused a significant 3.7 ± 0.3-fold increase in ROS production compared to control cells ($p$ < 0.05, Figure 3B). Pretreatment with MPAE (25–500 μg/mL) further increased ROS production, particularly at higher concentrations (250 and 500 μg/mL), eliciting 5.0 ± 0.5 and 5.2 ± 0.6-fold increases, respectively ($P$ < 0.05). This indicates that higher concentrations of MPAE exacerbated ROS accumulation in $H_2O_2$-treated cells.

LPS (1 μg/mL) significantly increased TNF-α (45.78 ± 9.61 pg/mL, Figure 4A), IL-1β (94.7 ± 5.8 pg/mL, Figure 4B), and IL-6 (121.0 ± 18.0 pg/mL, Figure 4C) levels compared to control cells ($P$ < 0.05). Pretreatment with MPAE further elevated these cytokine levels in a concentration-dependent manner. Significant increases in TNF-α and IL-6 beyond LPS alone were observed at 250 and 500 μg/mL of MPAE ($P$ < 0.05, Figure 4A and C). Additionally, higher concentrations of MPAE (125, 250, and 500 μg/mL) significantly increased IL-1β levels compared to LPS alone ($P$ < 0.05, Figure 4B).

## Discussion

Aqueous extraction was prioritised for its safety for consumption, while organic solvent (*i.e.*, methanol, acetone, chloroform, dichloromethane, *etc.*) extracts are highly likely to produce toxicity [31]. Additionally, aqueous extraction retains all the compounds of the plant material, while other extraction methods may retain only some compounds depending on the polarity of the solvent [32].

An oxidative stress model was created by inducing astrocytes with iron overload using $Fe_2SO_4$. Iron overload increases synaptic activity, leading to cytotoxic effects [31, 32] and may occur in AD, PD, and HD [33-35]. A preliminary study by a colleague who looked at the effect of MPAE on ferrous-ion chelating (FIC) assay showed that MPAE has good chelating activity with $IC_{50}$ of 147.62 ± 18.82 μg/mL [36]. More so, tuber extracts of *M. platytyrea* can impede the production of ROS and reduce the cytokine levels in HepG2 cells [36]. However, in this present study, MPAE caused an increase in cell death in a concentration-dependent manner, which showed no protective effect on the astrocytes against $Fe_2SO_4$ assault.

The second oxidative stress model was induced by $H_2O_2$. Excessive $H_2O_2$ mediates cell damage through the direct

oxidation of lipids, proteins, and DNA, or it acts as a signalling mechanism to trigger the cellular apoptotic pathway [37, 38]. Cell viability dropped dramatically when astrocytes were induced by 0.2 mM of $H_2O_2$. Pretreatment with MPAE increased the oxidative stress response in astrocytes and significantly accelerated cell death compared to untreated cells. MPAE, which has a high content of antioxidants, possibly acted as a pro-oxidant in the presence of $H_2O_2$, inducing oxidative stress either by the generation of ROS or by inhibiting antioxidants. Free radical scavengers, which are antioxidants, can be pro-oxidants unless linked to a radical sink. A radical sink means the antioxidant radical has to "sink" its unpaired electron in other reactions. Antioxidants such as ascorbic acid, vitamin E and polyphenols act as pro-oxidants in the presence of transition metals [39]. For example, resveratrol promotes oxidative DNA damage in the presence of $Cu^{2+}$ ions that may pose a problem as a neuroprotectant [40].

There is a likelihood that the antioxidative action of MPAE due to the extremely high antioxidant activity was changed to pro-oxidant action. Surprisingly, some common and eminent antioxidant flavonoids act as pro-oxidants [41]. Generally, the "double-edge sword" action was triggered in the presence of the metal ions and contributed by the chemical structure of the compounds in MPAE [42, 43]. Flavonoid antioxidant action may be connected to the hydroxyl (OH) functional group's electron-donating ability [44, 45]. Polyphenols without OH groups, like flavone and flavanone have no antioxidant or Fe/Cu-initiated pro-oxidant activity. The quantity of free OH groups in a flavonoid affects its Fe/Cu-initiated pro-oxidant activity [34, 46]. Pro-oxidant activity increases with OH groups. O-methylation and possibly additional O-substitutions of flavonoid OH groups inactivate antioxidant and pro-oxidant activity [39, 47]. Therefore, pro-oxidant activity in the astrocytes produces ROS, including $H_2O_2$. Excess $H_2O_2$ causes oxidative-nitrosative stress, oxidising lipids, proteins, and DNA, and damaging cells [48, 49]. $H_2O_2$ overload increases inducible nitric oxide synthase (iNOS) expression and cytokine release (TNF-α, IL-1β, IL-1, and IL-6) and lowers antioxidant defence (SOD, CAT, and GPx), causing mitochondrial membrane potential dysfunction, morphological changes, and apoptotic cell death [50, 51]. However, neuronal injury can be reduced by inhibiting these mediators [50].

A model of neuroinflammation was created by incubating astrocytes with 0.1 µg/mL LPS, which caused about 26% of cell death. A few studies have successfully used 0.1 µg/mL of LPS to induce inflammation in astrocytes but without apparent toxic effect (~25-30 % cell death) [9, 42, 43]. A previous study also reported that 0.1 µg/mL of LPS activates astrocytes by increasing cytokine production and the glial fibrillary acidic protein (GFAP) level that exerts TLR-4 expression [9].

Pretreatment of LPS-induced astrocytes with MPAE triggered cytokine release. Inflammation is initiated by activating pro-inflammatory signalling cascades, such as mitogen-activated protein kinase (MAPK) and NF-κβ [44]. Activated NFκβ stimulates the production of pro-inflammatory cytokines (*i.e.* TNF-α and interleukins), the release of which triggers inflammation and promotes cell death via necrosis [45]. This was shown by the increased loss of cell viability in this study in the presence of MPAE. Thus, treatment with MPAE triggers more oxidative damage and inflammation, leading to increased cell death in the presence of inducers.

The decoction of *M. platytyrea* tuber is claimed by the indigenous people of Papua New Guinea as an anticancer remedy [20]. A few studies have been conducted to determine the efficacies of this tuber, which indicated its potential in treating inflammation-related diseases such as cancer and diabetes, including pain inhibition [20, 21]. The benefits of the tuber were attributed to the presence of phenolic compounds (flavonoids, terpenoids, anthocyanins) and phytosterols (stigmasterol and β-sitosterol), as mentioned before [19, 22, 23]. These compounds possess strong antioxidant and anti-inflammatory properties [46-48].

The primary reasons for neurodegeneration appear to involve abnormal processing of proteins, genetic disorders, misfolding and aggregation of various proteins, activating cellular apoptosis, triggering mitochondrial dysfunction, neuroinflammation, production of free radicals, and oxidative stress [49]. Since the identified compounds in the tuber of *M. platytyrea* have the potential to block oxidative stress and inhibit neuroinflammation, there are great

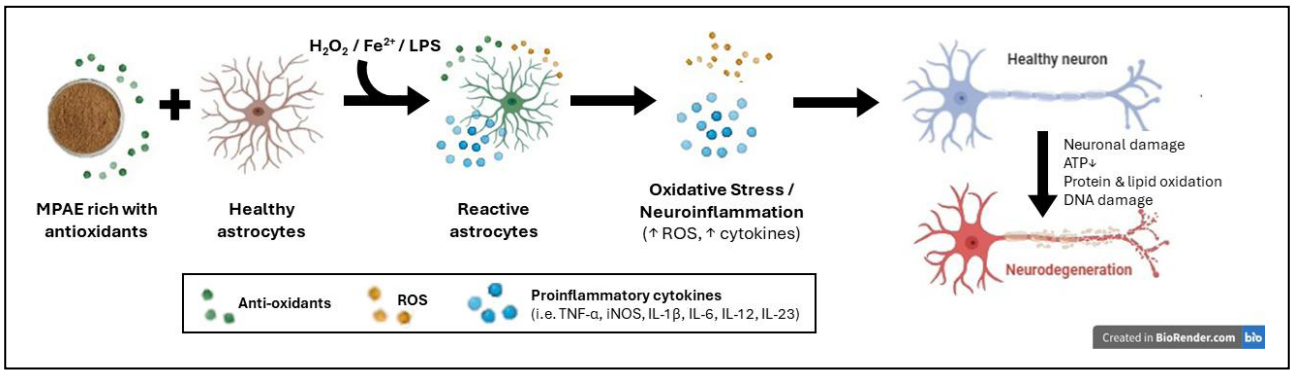


**Figure 5**. **Plausible mechanism of action of MPAE on astrocytes exposed to hydrogen peroxide/iron (II) sulphate/lipopolysaccharide that leads to neuronal damage**. Oxidative stress and neuroinflammation were aggravated due to the "double-edged sword" action of MPAE. The rich antioxidant MPAE may contain excessive phenolic antioxidant compounds, which, in the presence of the inducers, cause more damage because of the pro-oxidant activity.

possibilities for this plant to prevent the susceptibility of neurodegenerative disorders in the ageing society.

Myrmecophytes contain phenolic compounds (*i.e.* rosmarinic acid, procyanidin B1 and polymer of procyanidin B1) [50] and flavonoids (*i.e.* kaempferol, luteolin, apigenin, and quercetin). *M. platytyrea* tubers contain bioactive compounds such as stigmasterol and morindolide with a high antioxidant capacity [23]. Yet, in this study, MPAE worked differently in the astrocytes.

Several studies display controversial results on antioxidants. The type, dosage and matrix of antioxidants may be determining factors that affect the balance between their useful and harmful effects [37, 51]. Pro-oxidant activity on normal cells damages biomolecules such as DNA, proteins and lipids and induces lipid peroxidation and apoptosis as a consequence of cell death [52]. It can also initiate an intracellular signalling pathway that increases pro-inflammatory cytokine production, which leads to inflammation [53]. Due to the high content of polyphenolics in the MPAE, there is a high probability that it is a "double-edged sword" with activity, acting as antioxidants or pro-oxidants depending on the concentration/doses or presence of metal ions [54]. Hence, the proposed mechanism of action of MPAE in astrocytes (Figure 5). However, this study was limited because microglia were not used in its investigation. The comparison of findings from both cell types may provide a comprehensive understanding and treatment of neuroinflammatory disorders. Furthermore, it is recommended that the impact of MPAE should be extended to *in vivo* studies, which will help better understand the multifaceted origins of neurodegenerative diseases such as AD and PD.

## Conclusions

To our knowledge, this is the first study examining the impact of MPAE on neuroinflammation. Pretreatment with MPAE on astrocytes resulted in dose-dependent cytotoxicity with elevated ROS and cytokine levels, potentially attributable to its "double-edged sword" mechanism. While MPAE is non-cytotoxic to astrocytes, its capacity to aggravate neuroinflammation *in vitro* raises concerns regarding its application, especially in ageing individuals utilising this plant for inflammatory disorders. Before recommending its therapeutic usage, particularly for treating neurodegenerative diseases, these findings demonstrate the necessity of caution and stress the significance of more research to assess its safety and effectiveness. However, further studies must be conducted to confirm which compounds in MPAE exacerbate oxidative stress and inflammation in the astrocytes.

## Declarations

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**Authors' contributions:** Conceptualisation: SS, MHH. Investigation and Formal Analysis: AN, SS, MHH. Supervision: SS, AHJ, MHH. Visualisation: MFM, AN, MHH. Writing-original draft: AN, SS, MHH. Writing-review and editing: MFM, SS, AN, AHJ, HSJC, MHH. All authors have read and agreed to the published version of the manuscript.

**Availability of data and materials:** The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

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**Conflicts of interest:** All authors declare that there are no competing interests.

**Ethical approval and consent to participate:** Not applicable.

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