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Phytochemistry, Cytoprotective and Anti-Inflammatory Effects of *Myrmecodia platytyrea* Ethyl Acetate Extract on Lipopolysaccharide-induced RAW 264.7 Cells

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ABSTRACT

Inflammation is a hallmark pathology for many diseases, including cancer, diabetes mellitus, atherosclerosis, and neurodegeneration. *Myrmecodia platytyrea* (Rubiaceae), an epiphytic plant native to Southeast Asia, Australasia, and Papua Island, has been investigated for its ethnomedicinal applications. However, the potential effects of ethyl acetate extract from *M. platytyrea* against inflammation are yet to be widely studied. This present study sought to evaluate the phytochemical components of *M. platytyrea* ethyl acetate extract and investigate its cytoprotective and anti-inflammatory effects on RAW 264.7 cells. Phytochemical screening was performed based on standard qualitative tests, while the IC_{50} value of the extract in RAW 264.7 cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) method. Subsequently, RAW 264.7 cells were pretreated with various extract concentrations (2, 4, and 8 µg/mL) and induced with 1 µg/mL lipopolysaccharide (LPS) for 24 h. The effect of the extract on cell viability and tumor necrosis factor-alpha (TNF-α) levels were measured using MTT and enzyme-linked immunosorbent assay (ELISA), respectively. Phytochemical screening identified the presence of alkaloids, phenolics, flavonoids, terpenoids, and phytosterols in the extract. The extract displayed moderate cytotoxicity against RAW 264.7 cells, with an IC_{50} value of 61.53 ± 1.26 µg/mL. It protected RAW 264.7 cells against LPS-induced cell growth inhibition and significantly reduced TNF-α levels dose-dependently. As a result, this study demonstrated the potential of *M. platytyrea* as a natural source for developing a therapeutic agent for inflammation-related diseases.

INTRODUCTION

Inflammation is the host's defence mechanism against harmful stimuli, such as viral and bacterial pathogens, irritants, and chemicals. Thus, the body's system requires an acute inflammatory response to

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remove these triggering factors while stimulating the repair mechanism [1]. As a result, the inflammatory response should be limited to the invaded area in the early stages rather than becoming chronic and systemic. Excessive and prolonged inflammatory processes often result in chronic systemic inflammatory diseases due to the disruption of tissue healing and repair [1]. Cancer, diabetes mellitus, coronary artery diseases, neurodegenerative diseases, and peripheral vascular diseases are among inflammation-related diseases that occur because of prolonged systemic inflammation [1], [2].

Furthermore, risk factors, both controllable (poor eating habits, smoking, stress, obesity) and uncontrollable (age, gender, family history, genetic predisposition), play an important role in the pathogenesis of these chronic diseases [2]. According to recent statistics on premature mortality among Malaysian adults, up to 3.5 million years of life were lost (YLL) in 2018 due to ischaemic heart disease, lower respiratory tract infections, diabetes mellitus, and cancers, among other causes [3], [4]. These findings highlighted the significant impact of inflammation on the disease burden among Malaysians, necessitating effective interventions, including using natural resources as a potential cure for inflammation-related diseases.

Tumour necrosis factor-alpha (TNF-α) is a pro-inflammatory cytokine released by macrophages, T-lymphocytes, and natural killer cells upon inflammation. It regulates various inflammatory mediators as a protective response during the acute stage of inflammation. However, the persistent release of TNF-α results in the pathogenesis of inflammatory diseases by activating fibroblasts, leading to fibrosis and stricture formation [5]. To date, infliximab, etanercept, adalimumab, certolizumab, and golimumab are the five clinically approved TNF-α inhibitors used to treat autoimmune diseases caused by fibroblast activation, such as rheumatoid arthritis, psoriasis, and Crohn's disease [5]. This class of drugs is classified as one of the Disease Modifying Antirheumatoid Drugs (DMARDs), a controlled drug that requires a thorough evaluation of the condition of patients before being prescribed due to potential side effects [6]. Studies have also associated the long-term use of TNF-α inhibitors with an increased risk of infections, such as tuberculosis and malignancies, leading to the discontinuation of therapy [7].

Non-steroidal anti-inflammatory drugs (NSAIDs) are the mainstay therapy for inflammation-related diseases commonly used as analgesics and antipyretics. These anti-inflammatory drugs on the market are mostly of synthetic origin, with potential complications after prolonged use. Non-selective cyclooxygenase (COX) inhibitors, including ibuprofen and indomethacin, are commonly associated with adverse effects like upper gastrointestinal bleeding manifested as haematemesis or melaena and cardiovascular-related complications [8]. The severity of these incidents cannot be neglected. It should be immediately tackled with strict prescription rules and continuous education on the safe use of medication among healthcare workers (physicians, nurses, and pharmacists) and patients. At the same time, persistent efforts to develop a naturally derived anti-inflammatory agent with an improved safety profile and enhanced efficacy through preclinical and clinical research should be encouraged. The World Health Organisation (WHO) reported that at least 11% of the 252 essential drugs are organically produced from plants [9]. Hence, plant-based medicinal extracts could be developed as therapies for numerous diseases in modern medicine.

M. platytyrea, also locally known as *sarang semut* or ant nest plant, belongs to the Rubiaceae family and is known to contain a variety of secondary metabolites, such as polyphenols, flavonoids, indole alkaloids, anthraquinones and terpenoids [10]. In preclinical research, *M. platytyrea* aqueous extract has been proven to be non-toxic to several cell lines and animals while demonstrating its medicinal benefits, including anticancer [11], antidiabetic [12] and cholesterol metabolism-regulating properties [13] in both in vitro and in vivo settings. Nonetheless, the therapeutic potential of *M. platytyrea* ethyl acetate extract, particularly against inflammation targeting TNF-α, has not been widely studied. In preclinical studies, the LPS-induced RAW 264.7 cell culture represents a well-established in vitro inflammation model commonly

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utilized for screening prospective compounds with anti-inflammatory properties. LPS, a component of Gram-negative bacterial cell walls like *Escherichia coli*, can induce an inflammatory response in the RAW 264.7 cell line, which is a cell line derived from mouse macrophage cells, resulting in an increased production of inflammatory mediators, including TNF-α [14]. Hence, this study aims to identify the presence of phytochemicals in *M. platytyrea* ethyl acetate extract and analyse its cytoprotective and anti-inflammatory effects against LPS-induced RAW 264.7 cells.

EXPERIMENTAL

Preparation of ethyl acetate extract of *M. platytyrea*

Clean dried tubers of *M. platytyrea* were subjected to extraction using a simple maceration method [15] by soaking in ethyl acetate in a 1:9 ratio (w/v) for 24 h. The soaking and filtration using Whatman filter paper were performed twice in successive days, and the filtrates were then pooled. The solvent of the crude extract was evaporated using a rotary evaporator (Heidolph, Germany). After that, the ethyl acetate extract of *M. platytyrea* was collected, weighed, and stored at -80 °C. The percentage yield of the dried extract was calculated using the following formula:

$$Extraction\ yield\ (\%) = \frac{W1}{W2} x\ 100 \quad (1)$$

Where $W1$ is the final weight of the extract after solvent extraction, while $W2$ is the initial weight of the dried tubers before extraction.

Assessment of solubility of the extract in different solvents

The solubility of the extract was tested in different solvents, namely ethanol, methanol, and dimethyl sulfoxide (DMSO), by mixing the extract with each solvent starting from the highest concentration, ranging from 100 mg/mL to 50 mg/mL. The maximum concentration of the dissolved extract was determined under standard laboratory conditions using pipetting and vertexing techniques at room temperature. The solvent that managed to dissolve the extract in a clear, homogenous solution without precipitation or sedimentation for up to 48 hours was selected as the appropriate solvent.

Qualitative phytochemical screening

The preliminary phytochemical tests to identify the presence of alkaloids, phenolics, flavonoids, terpenoids, and phytosterols were performed according to the standard methods modified from those described by Shaikh and Patil [16]. For this purpose, the extract was prepared in DMSO at a 5 mg/ml concentration to allow chemical reactions between the tested extract and the reagents. The methods for detecting phenolics, alkaloids, flavonoids, terpenoids, and phytosterols are described in Table 1.

Table 1. Chemical tests for qualitative phytochemical screening of plant extract.

Component	Test Name	Method	Observation of a Positive Test
Phenolics	Ferric chloride test	2 mL of 2% ferric chloride was added to 3 mL of extract	Change of colour to black-bluish, dark green, or reddish-purple
Alkaloids	Dragendorff's test	2 mL of Dragendorff's reagent was added to 3 mL of extract	The presence of orange or brownish precipitates in the solution

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Flavonoids	Alkaline reagent test	2 mL of 2% sodium hydroxide solution was added to 3 mL of extract. When the solution turned yellow, 1 mL of diluted hydrochloric acid was added to the mixture	When the yellow solution turned colourless
Terpenoids	Modified Salkowski's test	2 mL of chloroform was added to 1 mL of extract, followed by the addition of 2 mL of concentrated sulphuric acid	Appearance of the brownish ring
Phytosterols	Salkowski's test	The extract was mixed with 2 mL chloroform and 2 mL of concentrated sulphuric acid. The test tube was shaken and allowed to stand for 5 min.	The lower layer of the mixture solution turned reddish

RAW 264.7 cell culture

RAW 264.7 murine macrophage cell line (TIB-71) was purchased from the American Type Culture Collection (ATCC) (Virginia, United States). Cells were maintained in a Dulbecco's Modified Eagle's Medium (DMEM) of Dulbecco containing 4.5 g/dL glucose, L-glutamine, and sodium pyruvate (Nacalai Tesque, Japan) supplemented with 10% foetal bovine serum (FBS) (Tico Europe, The Netherlands) and 1% penicillin-streptomycin mixed solution (Nacalai Tesque, Japan) in a humidified incubator with 5% CO_2 at 37 °C. Upon reaching 70–80% confluence, the cells were passaged at a 1:5 ratio using 2.5 g/L trypsin solution (Nacalai Tesque, Japan).

Cytotoxicity study for determination of IC_{50}

In this study, a 24 h timepoint was chosen for the experiment since the RAW 264.7 cell line has a high proliferative rate, with cells growing exponentially in a logarithmic fashion at around 24 h after seeding and a doubling rate of approximately 12 h after that. As such, a 24-hour incubation for initial cell seeding and an additional 24 hours with treatment enables the cells to achieve an optimal density for an adequate cellular metabolic response before being analysed [17]. Briefly, RAW 264.7 cells (1.5×10^4 cells/well) were plated in a 96-well plate and treated with *M. platytyrea* ethyl acetate extract at a concentration range of 0.05 to 500 µg/mL for 24 h. Subsequently, an MTT assay was performed by adding 50 µL of MTT reagent to each well, and the plate was incubated for 2 h at 37 °C with 5% CO_2. Then, the supernatant was removed from each well and replaced with 100 µL of 100% DMSO to dissolve the formazan dye. Absorbance readings were immediately measured at 570 nm using the Infinite M200 Pro microplate reader (Tecan Life Sciences, Switzerland). A logarithmic dose-response curve was then constructed using GraphPad Prism 9 (GraphPad Software, USA) to determine the extract's half-maximal inhibitory concentration (IC_{50}) on RAW 264.7 cells.

Cell viability study

To evaluate the effect of the extract on the viability of LPS-induced RAW 264.7 cells, the cells were pretreated with *M. platytyrea* ethyl acetate extract at 2, 4, and 8 µg/mL for 1 h. Next, the cells were induced with LPS at 1 µg/mL for another 24 h. Ibuprofen (200 µM), which is a classical NSAID, was included as the reference drug. Then, an MTT assay was performed to measure the percentage cell viability of the control and treated cells, as previously described in the method for cytotoxicity study.

Measurement of TNF-α levels

TNF-α is a pro-inflammatory cytokine released upon induction of inflammation. To evaluate the TNF-α production effect of the *M. platytyrea* ethyl acetate extract in LPS-induced RAW 264.7 cells, an ELISA kit (Invitrogen, USA) was used following the manufacturer's protocol. In short, the RAW 264.7 cells were treated with *M. platytyrea* ethyl acetate extract (2, 4, and 8 µg/mL) or ibuprofen (200 µM) and stimulated with LPS (1 µg/mL) for 24 h. The supernatant from each sample group was then collected, while the levels of TNF-α were measured according to the principle of a sandwich ELISA. The absorbance readings were recorded at 450 nm using the Infinite M200 Pro microplate reader (Tecan Life Sciences, Switzerland).

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Statistical analysis

Microsoft Excel (Microsoft Corporation, Washington) and GraphPad Prism 9 (GraphPad Software, USA) were used for statistical analysis. The results were expressed as the mean ± standard error of the mean (SEM) calculated from at least three independent experiments. A non-linear regression curve fit was used to determine the IC_{50}. In contrast, one-way ANOVA and post-hoc Tukey's test were used to determine the statistical significance between the treated and untreated groups. The statistical significance was established at $p < 0.05$.

RESULTS AND DISCUSSION

Extraction yield and solubility

The percentage yield of the *M. platytyrea* ethyl acetate extract was 1.23% ± 0.11. However, the extract could not be directly dissolved into a cell culture medium due to the moderate polarity of ethyl acetate. Thus, its solubility was examined in several organic solvents to identify a suitable drug vehicle. The three commonly used solvents as drug vehicles are ethanol, methanol, and dimethyl sulfoxide (DMSO), which can dissolve a wide range of polar and non-polar compounds with considerably low cytotoxicity [18]. The solubility test revealed that the extract could be dissolved in 100% DMSO at a maximum 50 mg/mL concentration. In contrast, ethanol and methanol failed to dissolve the extract fully despite the lower concentrations. Thus, for cell culture studies, the extract in DMSO was further diluted in DMEM 100 times to 500 µg/mL to reduce the DMSO concentration to 1%. The use of DMSO as a drug vehicle in cell culture media at concentrations of up to 1% was shown to cause no apparent cytotoxicity [19].

Qualitative phytochemical screening

The result of the phytochemistry analysis of the extract is presented in Table 2. In this study, the phytochemical screening demonstrated the presence of phenolics, alkaloids, flavonoids, terpenoids, and phytosterols. The presence of these phytochemicals in the extract suggests that the extract may possess therapeutic potential that might be effective against a range of pathological disorders, including cancer and inflammation [20].

Table 2. Phytochemistry of *M. platytyrea* ethyl acetate extract.

Components	Phenolics	Alkaloids	Flavonoids	Phytosterols	Terpenoids
Result and Observation	 Dark green solution	 Precipitation	 Colourless solution	 Reddish lower layer	 A brown ring at the interface
Conclusion	Present	Present	Present	Present	Present

Half-maximal inhibitory concentration (IC_{50}) of the extract

The cytotoxicity of *M. platytyrea* ethyl acetate extract was evaluated on RAW 264.7 cells using an MTT assay performed at concentrations of 0.05, 0.5, 5, 50, and 500 µg/mL, with 500 µg/mL being the maximum concentration attained from the solubility test. The half-maximal inhibitory concentration (IC_{50}) of the extract on RAW 264.7 cells was determined to be 61.53 µg/mL ± 1.26, which inhibited the cells' growth by 50% after 24 h, as shown in Fig. 1. The IC_{50} values for cytotoxicity of plant extract are classified

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as follows: <20 µg/mL (strong), 20-100 µg/mL (moderate), 201-500 µg/mL (weak), and >500 µg/mL (no cytotoxic activity) [21]. Thus, *M. platytyrea* ethyl acetate extract possessed moderate cytotoxicity against RAW 264.7 cells. Based on the cytotoxicity experiment, three different concentrations of the extract at 2, 4, and 8 µg/mL were selected for further studies. The assay revealed that these dosages inhibited the proliferation of RAW 264.7 cells by 20% or less.

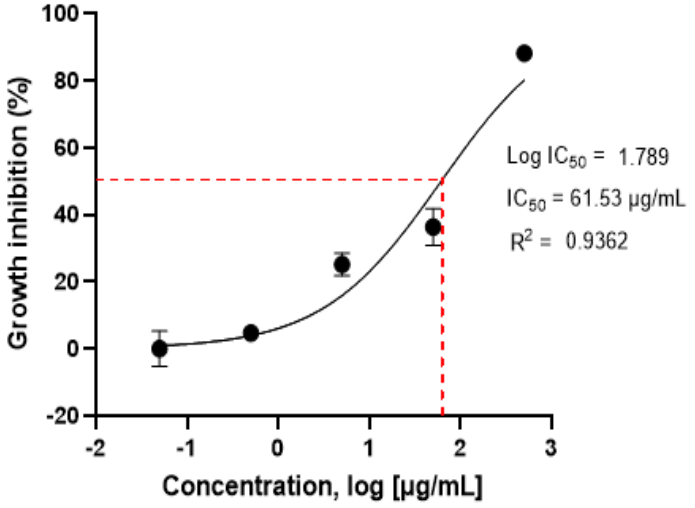

Fig. 1. A logarithmic dose-response curve of *M. platytyrea* ethyl acetate extract on RAW 264.7 cells showing the IC50 value. RAW 264.7 cells were treated with the extract at 0.05, 0.5, 5, 50, and 500 µg/mL for 24 h. An MTT assay was performed to determine the cytotoxicity of the extract, with results presented as the mean percentage of growth inhibition ± SEM (n = 3).

Cytoprotective effect

In evaluating the cytoprotective effect of *M. platytyrea* ethyl acetate extract on LPS-induced RAW 264.7 cells, an MTT assay was performed by pre-treating LPS-induced cells with the extract at 2, 4, and 8 µg/mL. The cell viability assay (Fig. 2) displayed a significant reduction in LPS-induced RAW 264.7 cells to 69.53 ± 5.35% compared to untreated cells (100%) ($p<0.0001$). Pretreatment of LPS-induced cells with the extract at 2, 4, and 8 µg/mL did not significantly alter cell viability compared to the LPS-treated cells alone. A similar pattern was observed in the treatment with ibuprofen, which suggests that the extract conferred cytoprotection of the RAW 264.7 macrophages against LPS-induced growth inhibition. In contrast, treatments with extract and ibuprofen did not aggravate the action of LPS in the cells. Several studies have also reported on the cytoprotective activity of various medicinal plant extracts on macrophages, such as *Adenanthera pavonina* [22], *Echinacea purpurea,* and *Humulus lupulus* [23], and *Cudrania tricuspidata* [24].

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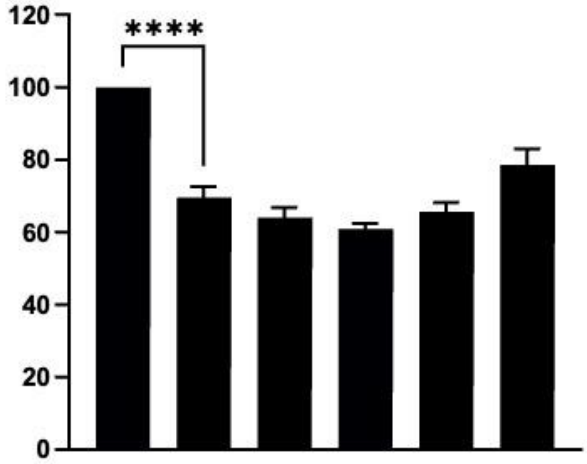

Fig. 2. Cytoprotective effect of *M. platytyrea* ethyl acetate extract on LPS-induced RAW 264.7 cells. RAW 264.7 cells were pretreated with 2, 4, and 8 µg/mL of extract or 200 µM ibuprofen and induced with LPS (1 µg/mL) for 24 h. Cell viability was measured using an MTT assay and presented as a percentage of control (mean ± SEM, n = 3). ****p<0.0001, compared to the LPS group.

Anti-Inflammatory Effect

TNF-α is an important pro-inflammatory cytokine released upon macrophage activation with LPS, aiding immune regulation [25]. As such, TNF-α levels were determined after pretreatment of the LPS-induced RAW 264.7 macrophages with *M. platytyrea* ethyl acetate extract to investigate its potential anti-inflammatory effect. The TNF-α levels were interpolated from a standard curve constructed using ELISA (Appendix 1). Fig. 3 demonstrated that LPS had significantly increased ($p < 0.0001$) the TNF-α level to 85.98 ± 8.26 ng/mL compared to the untreated control (0.13 ± 0.02 ng/mL). Meanwhile, treatment with *M. platytyrea* ethyl acetate extract at 2, 4, and 8 µg/mL significantly reduced the TNF-α level in a dose-dependent manner to 55.77 ± 0.93 ($p = 0.0065$), 53.69 ± 2.94 ($p = 0.0038$) and 48.73 ± 5.49 ng/mL ($p = 0.0001$), respectively. Similarly, ibuprofen-treated LPS-induced cells displayed significantly lower TNF-α levels at 26.87 ± 3.21 ng/mL ($p < 0.0001$) compared to the LPS control group. Meanwhile, there were significantly lower TNF-α levels in ibuprofen-treated groups compared to those treated with *M. platytyrea* ethyl acetate extract at 2, 4, and 8 µg/mL ($p = 0.0092$, $p = 0.0158$, and $p = 0.0389$, respectively).

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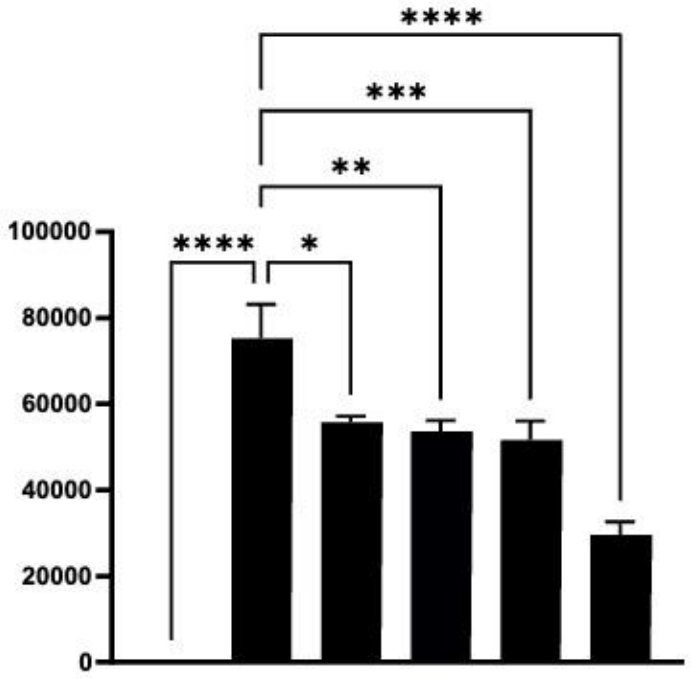

Fig. 3. Effect of *M. platytyrea* ethyl acetate extract on TNF-α production in LPS-induced RAW 264.7 cells. RAW 264.7 cells were pretreated with 2, 4, and 8 µg/mL of extract or 200 µM ibuprofen and induced with LPS (1 µg/mL) for 24 h. TNF-α levels were measured using an ELISA method and presented in pg/mL (mean ± SEM, n = 3). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, compared to the LPS group.

The results demonstrated the therapeutic potential of *M. platytyrea* ethyl acetate extract against acute inflammation targeting TNF-α, which is more or less comparable to that of ibuprofen despite being a plant extract comprising mixtures of secondary metabolites. Flavonoids (myricetin, luteolin, kaempferol and quercetin), terpenoids (santamarine, reynosin, and cynaropicrin), iridoids (aucubin and nardostachin), phytosterols (ergosterol peroxide and retinoic acid) and alkaloids (lycorine and berberine) are examples of natural products with known anti-TNF-α properties [26].

These natural products have been extensively explored for their anti-inflammatory activities, as they could be the sources of pure compounds with synergistic anti-inflammatory activity across multiple pathways. The findings of this study could be a stepping stone toward investigating the potential effects of bioactive compounds in *M. platytyrea* ethyl acetate extract, which may exert synergistic mechanisms against inflammation.

CONCLUSION

M. platytyrea ethyl acetate extract has demonstrated cytoprotective and anti-inflammatory effects against TNF-α in an in vitro inflammation model using LPS-induced RAW 264.7 cells by maintaining cell viability and reducing TNF-α levels in murine macrophages during an LPS-induced inflammatory response. The phytochemical composition of *M. platytyrea* ethyl acetate extract, namely polyphenols, alkaloids, flavonoids, terpenoids, and phytosterols, may contribute to its anti-inflammatory properties, which are still under investigation. Further research is required to comprehend the mechanisms underlying the anti-

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inflammatory effects of *M. platytyrea* ethyl acetate extract toward developing a naturally derived therapeutic agent for clinical applications in the future.

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CONFLICT OF INTEREST STATEMENT

The authors agree that this research was conducted without any self-benefits or commercial or financial conflicts and declare the absence of conflicting interests with the funders.

AUTHORS' CONTRIBUTIONS

Aisyah Jaafar conducted the research, analyzed the data, and wrote and revised the article. Mizaton Hazizul Hassan conceptualized the central research idea, provided resources, and acquired funding for the research. Aisyah Hasyila Jahidin, Fazleen Haslinda Mohd Hatta, and Nurul Alimah Abdul Nasir co-supervised and validated the research progress; Mizaton Hazizul Hassan approved the article submission.

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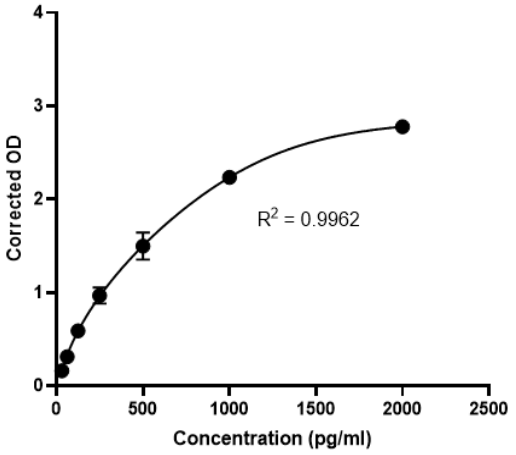
APPENDIX

A. ELISA standard curve for TNF-α



https://doi.org/10.24191/scl.v18i4.9671