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Cytotoxicity Study of *Plantago major* L. Extracts on RAW 264.7 Macrophages

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ABSTRACT

The use of medicinal plants in modern medicine is on the rise, particularly in addressing diabetes and its associated complications, such as persistent inflammatory wounds. *Plantago major* L. (*P. major*) is known for its rich distribution of bioactive compounds and its efficacy in treating diabetes, wound healing, inflammation, and oxidative stress. Before *in vitro* drug efficacy testing, it is essential to assess the toxicity of *P. major* extract. This study aims to evaluate the toxicity of *P. major* extracts on RAW 264.7 cells cultured in high-glucose DMEM media. This preliminary assessment is crucial for determining safe extract concentrations for subsequent anti-inflammatory activity testing using macrophage cells cultured in a hyperglycemic condition. Extracts were obtained from both leaf and non-leaf parts using maceration and UAE methods, resulting in four extract types: macerated leaf (DM), UAE leaf (DU), macerated non-leaf (NM), and UAE non-leaf (NU). Each extract was prepared in seven concentration series ranging from 7.81 to 500 µg/mL. Toxicity was assessed using the MTT method to determine cell viability after a 4-hour incubation. Significance against the control was analyzed using one-way ANOVA, and the effects of different plant parts, extraction methods, and their interaction were evaluated using two-way ANOVA. Results indicated that concentrations of 7.81-500 µg/mL for all four extracts did not significantly reduce cell viability ($p > 0.05$) compared to the control, with leaf extracts exhibiting higher viability percentages than non-leaf extracts, especially with the UAE extraction method at a concentration of 250 µg/mL (106.73 ± 4.20%). Variations in plant parts significantly affected ($p < 0.05$) cell viability percentages, whereas differences in extraction methods and their interaction did not have a significant effect. In conclusion, this study demonstrates that the four extracts at concentrations of 7.81-500 µg/mL are non-toxic to RAW 264.7 cells. Therefore, they are safe to use in anti-inflammatory activity testing.

Keywords: Cytotoxicity; MTT Assay; *Plantago major* L; Cell viability; RAW 264.7 Cell

INTRODUCTION

According to the World Health Organization (WHO), 75% of the world's population relies on medicinal plants for basic healthcare needs (Subramanian et al., 2018), and it is estimated that around 80% use herbal medicinal products for their therapeutic properties (Jităreanu et al., 2023). Medicinal plants have long been an option in medicine to treat various diseases and are now increasingly used as alternatives in modern medicine. One such medicinal plant, widely used in communities and recognized worldwide for its medicinal properties for hundreds of years, is *Plantago major* L. (*P. major*) (Adom et al., 2017).

P. major, commonly known as daun sendok in Indonesia, is a widely distributed herb with broad pharmacological activities (Zhakipbekov et al., 2023). *P. major* is recognized as a potential medicinal plant with diverse therapeutic properties, including anti-diarrheal, antibacterial, antiviral, antioxidant, wound healing, antidiabetic, and antiinflammatory effects. This species contains a wide array of bioactive compounds, including phenolic compounds (such as caffeic acid derivatives), flavonoids, iridoid glycosides, terpenoids, alkaloids, fatty acids, vitamins, and polysaccharides. These compounds are found in

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almost every part of the plant, including the flowers, leaves, roots, and seeds (Triastuti et al., 2022). The high content of active compounds in *P. major* presents a significant opportunity for further development. According to Zhakipbekov et al. (2023), one of the most renowned and effective studies on *P. major* focuses on its anti-inflammatory activity in various animal models. However, *in vitro* scientific data on this subject remains limited, particularly regarding its anti-inflammatory activity in diabetic wounds.

In the past decade, the development and discovery of new drugs from plants have often started with *in vitro* assays to test cytotoxicity, as this approach is simple, economical, and reliable. Cytotoxicity tests evaluate the toxic potential of chemicals and natural substances in cell culture models. Natural compounds with cytotoxic effects can significantly reduce cell viability, making this approach useful for preliminary studies to screen and identify toxic compounds (Gavanji et al., 2023). The MTT assay remains one of the most versatile and popular methods for evaluating cell viability (Kumar et al., 2018). This method has long been regarded as the gold standard in cytotoxicity assays due to its high sensitivity and suitability for High Throughput Screening (HTS) (van Tonder et al., 2015).

Recently, there has been a significant increase in the advancement of current extraction technologies, and one method that has gained prominence is ultrasonically aided extraction (UAE). The UAE technique facilitates the effective separation of specific bioactive substances by using its cavitational impact. This method provides benefits such as decreased extraction duration, greater quality of the extract, and increased yield. This novel methodology shows potential for isolating bioactive compounds from plants, enabling efficient extraction with enhanced product purity in a short time and with consistent results while also reducing the need for organic solvents. Researchers can enhance extraction efficiency and encourage sustainable practices in a more eco-friendly manner by using the UAE (Shen et al., 2023). Various modern extraction methods are currently under development, one of which is cavitation extraction (Chaves et al., 2020; Panda & Manickam, 2019). Therefore, a key novelty of this research lies in optimizing the Cavitation method to obtain flavonoid and phenolic-rich *P. major* extracts effectively. The phenolic and flavonoid compounds in *P. major* contribute significantly to its wound healing, antioxidant, and anti-inflammatory properties (Zhakipbekov et al., 2023).

Currently, there are no reports on the cytotoxicity testing of various parts of the *P. major* plant, including both leaves and non-leaves (a combination of roots, stems, and seeds), using a comparison of different extraction methods conventional (maceration) and modern (ultrasound-assisted extraction, UAE). These methods have undergone a series of optimization processes involving parameters such as temperature, time, Simplicia-solvent ratio, and solvent concentration, using the Box-Behnken Design (BBD) from Design Expert. The optimal conditions, based on the response values of total phenolic content (TPC) and total flavonoid content (TFC), are then applied to the maceration and UAE extraction processes for both the leaf and non-leaf parts of *P. major*.

Therefore, it is necessary to conduct a comparative study on the cytotoxicity of *P. major* leaf and non-leaf extracts obtained through maceration and UAE methods on RAW 264.7 cells in a hyperglycemic environment. This preliminary study aims to determine the non-toxic dose of *P. major*, which is crucial for further exploration of its pharmacological effects as an anti-inflammatory agent in diabetic conditions. Additionally, this research has the potential to significantly contribute to the development of independent procurement of herbal raw materials with potential as sources for anti-inflammatory drugs for treating diabetic wounds, inspiring hope for a new approach to diabetic wound treatment.

MATERIALS AND METHODS

Materials

P. major is a herb that is harvested when it reaches 3 months of age, during its generative period, and is sourced from Ballitro, West Java, Indonesia. Dulbecco's Modified Eagle's Medium (DMEM) high glucose (Sigma-Aldrich D5796 Lot No. RNBM0070), sterile Fetal Bovine Serum (FBS) (Sigma-Aldrich F0804 Lot No. 083M3395), sterile Phosphate Buffered Saline (DPBS Elabscience PB 180329), Antibiotic Antimycotic Solution (Sigma-Aldrich A5955), Sodium Bicarbonate ($NaHCO_3$), Sterile Water for Injection (SWI), 70% alcohol, MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], Sodium Dodecyl Sulfate (SDS) stopper solution 10%, triphenyl blue, and DMSO (Sigma-Aldrich D2650 Lot No. RNBL1502) were used in the study.

Methods

Optimization Extraction Methods of Leaf and Non-Leaf of *P. major*

The selected extracts of *P. major* plants were obtained through an optimization process using a

Response-Surface Methodology Box-Behnken (RSM) study. This was facilitated by Design Expert® software, which employs statistical models to elucidate the relationship between factors (independent variables) and responses within a 3-level factorial design. The primary goal of this study was to determine extraction process conditions with a combination of factors that yield optimal responses, thereby enhancing the efficiency and effectiveness of plant extraction processes. Optimization was conducted on both leaf and non-leaf (root, petiole, and seed) parts using ultrasound-assisted extraction (UAE) methods. The extraction process involved optimizing four factors: temperature (20-50°C), extraction time (20-60 minutes), sample-solvent ratio (1:5-1:25), and ethanol content in the solvent (10-90%). A total of 27 randomized experimental trials were conducted to obtain the actual TPC and TFC response values. The response values, in the form of actual TPC and TFC levels from each experiment (27 research designs), were analyzed through regression modeling. This analysis provided predicted TPC and TFC levels, which describe the optimum conditions for the *P. major* extraction optimization process. Optimum conditions are achieved at 50°C temperature, 20 minutes extraction time, 71.41% ethanol solvent content, and a 1:5 simplisia:solvent ratio. This specific condition was chosen based on preliminary research (unpublished).

Preparation of *P. major* Extract

Each plant part, including both leaves and non-leaf parts, underwent separate extraction processes using two methods: conventional extraction (maceration) and ultrasound-assisted extraction (UAE). The extraction is carried out under optimum condition For UAE, the extraction was conducted in an ultrasound bath at 50°C for 20 minutes, utilizing a solvent ratio of 1:5 with 71.41% ethanol). Conventional extraction followed the same conditions but without ultrasound assistance. Subsequently, the extract was filtered, and the resulting supernatant was evaporated using a rotary evaporator to obtain a concentrated extract. Based on this study, four optimum extracts were obtained, denoted as macerated leaf extract (DM), UAE leaf extract (DU), macerated non-leaf extract (NM), and UAE non-leaf extract (NU).

Determination of Total Phenolic Contents.

The Total Phenolic Content (TPC) was quantified using the reliable Folin–Ciocalteu method, as described by (Sari et al., 2023). In this procedure, 0.1 mL of the diluted extract and 0.1 mL of the Folin–Ciocalteu reagent were mixed in a 5 mL volumetric flask and allowed to react for 5 minutes. To initiate the colorimetric reaction, 1 mL of a 7% sodium carbonate solution was then added, and the flask was filled to volume with deionized water. The mixture was left to incubate in darkness at room temperature for 120 minutes. Subsequently, the absorbance of the samples was measured at 750 nm using a UV–Vis spectrophotometer. To account for background absorbance, the measurement was corrected by subtracting the absorbance of a control solution, which consisted of water in place of the extract or standard. The phenolic content was then calculated as gallic acid equivalents (GAE) per gram of extract, using a standard curve of gallic acid with concentrations ranging from 10 to 60 mg/L.

Determination of Total Flavonoid Contents.

The Total Flavonoid Content (TFC) was measured using the aluminum trichloride method, a straightforward procedure described by (Shraim et al., 2021; Sari et al., 2023). In this method, 500 µL of the diluted extract was combined with 2 mL of methanol and 200 µL of a 10% $AlCl_3$ solution. The mixture was then incubated at room temperature for 3 minutes. After incubation, 200 µL of a 1 M CH_3COONa solution was added, and the final volume was brought up to 5 mL with methanol. The solution was left to incubate in darkness at room temperature for 40 minutes, after which the absorbance was measured at 430 nm. A calibration curve using quercetin was generated under the same conditions. The flavonoid content was expressed as milligrams of quercetin equivalent per gram of dry extract (mg QE/g).

RAW 264.7 Cell Culture

Mouse RAW 264.7 monocyte/macrophage cells, purchased from the European Collection of Authenticated Cell Cultures (ECACC) with catalog number 91062702 and lot number 17K027, were cultured at the Laboratorium Riset Terpadu (LRT), FKKMK, Gadjah Mada University. The cells were grown in a complete medium consisting of DMEM high glucose (D-glucose: 25 mM, Sigma-Aldrich, USA), 10% fetal bovine serum (FBS) (Sigma-Aldrich, USA), and an antibiotic-antimycotic solution (Sigma-Aldrich, USA). The culture was incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air, providing reliable conditions for cell growth. Cells were grown to confluence, covering 80-90% of the flask volume. Cell counts were performed to ensure sufficient numbers for use. A cover glass was placed on the hemocytometer/colony counter, and 10 µL of cells

were introduced to each side of the hemocytometer. Cell counts were then performed under an inverted microscope.

RAW 264.7 Cell viability assay

The MTT assay was employed to assess cell viability and determine the non-toxic dosage for RAW 264.7 cells. Initially, A total of 1 x 10^4 RAW 264.7 cells per 100 µL were plated in each well of a 96-well plate and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. Afterwards, the complete medium, consisting of high glucose DMEM, FBS, and antibiotic-antimycotic solution, was discarded. The cells were then re-incubated under the same conditions with each test material extract (NM, NU, DM, and DU) at various concentrations (7.81, 15.63, 31.25, 62.5, 125, 250, and 500 µg/mL) in the medium for 24 hours. After the incubation period, the medium was aspirated, and the cells were washed with PBS.

Subsequently, 10 µL of a 5 mg/mL MTT solution was added to each well, and the cells were then further incubated for 4 hours. The resulting formazan crystals were dissolved in 100 µL of SDS Stopper solution. Absorbance was subsequently measured using a microplate reader at a wavelength of 570 nm. Cell viability was determined by calculating the percentage of absorption in the test material group relative to the cell control. Each concentration was tested in triplicate, and the presented data represent the average percentage of viability along with the standard deviations (SD).

DATA ANALYSIS

The cell viability test using the MTT method involved assessing the significance of differences between each treatment group and the cell control group through statistical analysis. One-way ANOVA was conducted with a confidence level of 95% using GraphPad Prism 10 to determine significance. In addition, the impact of different plant parts and extraction methods, as well as their interaction on the viability of RAW 264.7 cells, were statistically analyzed using two-way ANOVA with Minitab statistical software.

RESULTS

Based on the results of subculture, harvesting, and cell counting, a total of 2 x 10^6 cells were obtained. From these, 1 x 10^6 cells were utilized, comprising cell control, solvent control (DMSO), and treatment groups with four extracts across seven concentration series for cytotoxicity testing via the MTT method. The tests aimed to determine whether concentrations ranging from 7.81 to 500 µg/mL of optimal leaf and non-leaf extracts obtained through different extraction methods were nontoxic or safe for the cells.

The cytotoxic effect of *P. major* plant extracts on RAW 264.7 cell survival is expressed as percentage cell viability, as depicted in Figure 1 and Figure 2. Analysis of the four extracts revealed that percentage viability showed relatively fluctuating with increasing extract concentration. The percentage of cell viability for each extract group (DM, DU, NM, and NU) ranged from 95.45 ± 3.31% to 105.24 ± 6.01%; 88.23 ± 1.65% to 106.73 ± 4.20%; 90.46 ± 2.78% to 95.11 ± 5.82%; and 89.49 ± 7.35% to 98.53%, respectively.These results indicate that extracts obtained from non-leaf parts of *P. major* generally exhibit lower percentage cell viability compared to leaf extracts. Overall, the treatment with extracts at concentrations up to 500 µg/mL maintained RAW 264.7 cell viability above 85%.

Based on the statistical analysis of cell viability data for extract treatments DM, DU, NM, and NU, it was determined that the data exhibited normal distribution and homogeneous variance. A one-way ANOVA statistical test was conducted at a 95% confidence level to assess the significance of the effect of administering *P. major* leaf and non-leaf extracts through maceration and UAE methods on percentage cell viability compared to the cell control. The results indicated that the DM, DU, NM, and NU groups did not exert a significant effect ($p > 0.05$) on % cell viability. This suggests that administering the four extracts (DM, DU, NM, and NU) at concentrations ranging from 7.81 to 500 µg/mL does not induce toxicity in RAW 264.7 cells.

The influence of each variable individually and its interaction on percentage cell viability was examined through two-way ANOVA with Tukey's multiple comparison test at a 95% confidence level using Minitab Statistical Software. A single-effect analysis was performed to quantify the influence of each variable level. In contrast, interaction analysis was employed to assess the combined effect of one variable with others on percentage cell viability. Two variables were included in this analysis, each was divided into specific levels as outlined in Table I.

Based on the results of the two-way ANOVA analysis Table II, it is evident that plant parts exert a significant effect ($p = 0.006$, $p < 0.05$) on percentage cell viability. This finding aligns with the analysis depicted in (Figure 1 and Figure 2), indicating that the percentage viability of non-leaf parts tends to be lower than that of leaves. However, the extraction method had no significant effect on percentage cell viability ($p = 0.776$, $p > 0.05$). Similarly, there was no significant interaction between the two variables ($p = 0.67$,

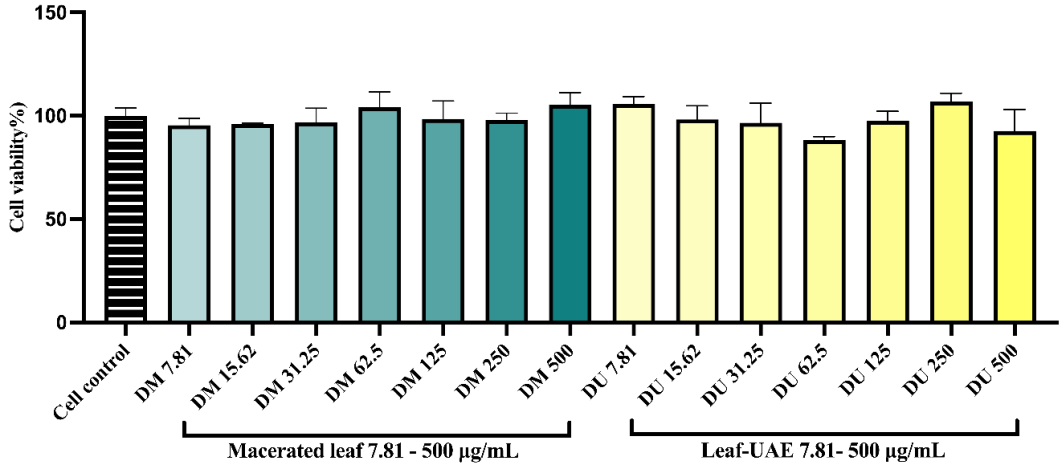

Figure 1. Percentage of viabiality cell RAW 264.7 of *P. major* leaf with macerated and UAE extraction methods.

DM refers to the ethanolic extract of *P. major* leaves obtained by the maceration extraction method. In contrast, DU refers to the ethanolic extract of *P. major* leaves obtained by the ultrasound-assisted extraction (UAE) method. The administration of DM and DU extracts in the concentration range of 7.81-500 µg/mL showed no significant difference compared to the cell control ($p > 0.05$, one-way ANOVA). Data are express as cell viability as mean ± SD (n = 3).

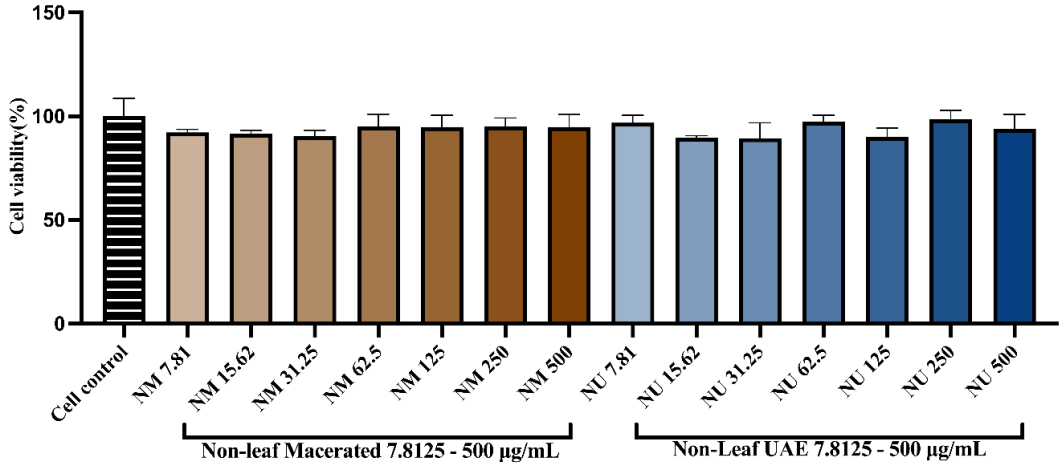

Figure 2. Percentage of viabiality cell RAW 264.7 of *P. major* non-leaf with macerated and UAE extraction methods.

NM refers to the ethanolic extract of non-leaf *P. major* obtained by the maceration extraction method, while NU refers to the ethanolic extract of non-leaf *P. major* obtained by the ultrasound-assisted extraction (UAE) method. The administration of DM and DU extracts in the concentration range of 7.81-500 µg/mL showed no significant difference compared to the cell control ($p>0.05$, one-way ANOVA). Data are express cell viability as the mean ± SD (n=3).

$p > 0.05$), indicating the absence of an interaction effect between different plant parts and extraction methods.

DISCUSSION

The selection of *P. major* leaf and non-leaf samples in this study is informed by recent innovations aimed at exploring the pharmacological potential of all plant parts. Previous research has shown that various parts of *P. major* are rich in bioactive compounds. Building on these findings, this study seeks to further investigate the pharmacological potential by grouping the plant into two categories—leaves and

Table I. Variables in the two-way *ANOVA* analysis.

Variable	Type	Levels	Values
Parts of the plant	Fixed	2	Leaf and Non-leaf
Extraction methods	Fixed	2	Maceration and UAE

Table II. The results of the two-way ANOVA analysis of variance on the effect of different plant parts and extraction methods on RAW 264.7 cell viability.

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Parts of the plant	1	173.992	173.992	8.92	0.006
Extraction methods	1	1.77	1.77	0.09	0.766
Parts of the plant*Extraction methods	1	3.623	3.623	0.19	0.67
Error	24	468.133	19.506		
Total	27	647.518			

non-leaves—for preliminary testing. Specifically, the study focuses on assessing anti-inflammatory activity related to diabetic wound healing *in vitro*, using RAW 264.7 macrophage cells cultured in high-glucose media containing 4,500 mg/L (25 mM) of D-glucose, a concentration that simulates the clinical conditions of uncontrolled diabetes as referenced in Cantuária et al., 2018.

This study adopts a comprehensive approach that not only aims to identify bioactive compounds from various parts of *P. major* but also seeks to optimize its use in broader therapeutic applications. The research is particularly focused on enhancing the independence in the procurement of medicinal raw materials, especially for anti-inflammatory drugs related to diabetic wounds. This work also provides valuable preliminary data that could support further exploration of *P. major*'s pharmacological activities, building on previous studies. Notably, Kartini et al. (2017) reported the anti-inflammatory activity of methanol extracts from the seeds, roots, stems, and leaves of *P. major*. Additionally, Kartini et al. (2018) demonstrated that ethanolic extracts of *P. major* leaves and seeds effectively exhibit anti-inflammatory activity, with leaves showing superior results in diabetic rats. Further research by Kartini et al. (2021) highlighted the wound-healing properties of *P. major* ethanol extracts in diabetic rats. Farid et al. (2022) found that ethanolic extracts of *P. major* leaves and seeds effectively provide anti-inflammatory activity, with leaves containing higher levels of total phenolic content (TPC) and total flavonoid content (TFC) compared to seeds. Ghanadian et al. (2022) also reported the wound-healing activity of *P. major* ethanol extracts in treating diabetic ulcers and decubitus ulcers. Adom et al. (2017) reported that the compounds responsible for *P. major's* anti-inflammatory activity include iridoid glycosides, such as aucubin, and flavonoid compounds, such as baicalein and hispidulin, which also possess anti-diabetic properties. Furthermore, Zhakipbekov et al. (2023) noted that the topical application of *P. major* extract accelerates the healing of infected wounds due to its content of phenols and flavonoids.

The UAE method was selected for this study over traditional maceration extraction due to its ability to extract targeted bioactive components efficiently through cavitation effects. This innovative technique not only significantly reduces extraction time but also enhances the quality and yield of extracts, specifically *P. major* extracts rich in phenolic and flavonoid compounds. As reported by Shen et al. (2023), the UAE method maximizes the total yield of phenolics and flavonoids and is more efficient than conventional extraction methods. Ultrasonication enables complete extraction with higher purity of the final product in just a few minutes, with high reproducibility, and minimizes the use of organic solvents.

The concentration range of *P. major* extract (7.81 to 500 µg/mL) was selected based on prior research by Kartini et al., (2021). They performed RAW 264.7 cell viability tests under the medium with high glucose that represent hyperglycemic conditions using an ethanol extract of *P. major* leaves. However, their findings indicated that extract concentrations up to 500 µg/mL did not significantly reduce cell viability compared to controls. Our study corroborates these results, showing no significant decrease in RAW 264.7 cell viability across all tested concentrations of extracts from both leaf and non-leaf parts of *P. major*. However, our study introduces several key differences: we utilized a more efficient cell density of 1 x 10^4 RAW 264.7 cells per well, a significant improvement over the 1 x 10^5 cells per well in the previous study, which enhances the validity and reliability of our findings. Additionally, our

research explored extracts from both leaf and non-leaf parts obtained through optimized extraction methods, including both maceration and UAE. At the same time, the previous study relied solely on leaf extracts prepared by maceration. Moreover, there were differences in the ethanol solvent concentrations used. Overall, our findings suggest that *P. major* extracts from both leaf and non-leaf parts, obtained through optimized maceration and UAE methods, are safe to use within the concentration range of 7.81 to 500 µg/mL.

In this study, RAW 264.7 cells were employed because they serve as a representative model for macrophage cells due to their ease of differentiation. These cells are particularly suitable for macrophage modeling as they exhibit phagocytic and pinocytic activities (Hartley et al., 2008). RAW 264.7 cells are commonly utilized in inflammation research because of their superior phagocytic capacity compared to THP-1 and U937 cells (Tarique, 2016).

The assessment of a safe dose devoid of toxic effects is a crucial preliminary step before pre-clinical testing. Cytotoxicity testing using the reduction compound 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is widely regarded as the "gold standard" and is therefore commonly employed (Pintor et al., 2020). Therefore, the MTT assay was selected in this study to assess the viability of RAW 264.7 cells due to its accuracy and sensitivity in measuring cellular metabolic activity. This method is not just suitable, but perfectly aligned with our research objectives, which aim to evaluate the potential cytotoxicity and anti-inflammatory effects of plant extracts. The MTT assay enables a quantitative assessment of cell viability, making it highly relevant for investigating cellular responses in the context of inflammation.

Figure 3 provides a visual representation designed to enhance the comprehension of our study. The MTT assay was employed to assess the viability of RAW 264.7 macrophage cells following treatment with *P. major* leaf and non-leaf extracts under conditions simulating uncontrolled hyperglycemia. These conditions were meticulously replicated by culturing the cells in high-glucose DMEM media, consistent with previous studies (García-Hernández et al., 2012; Cantuária et al., 2018; Kartini et al., 2021; Sousa et al., 2023. This setup mimics the cellular environment of a diabetic individual with uncontrolled blood sugar levels, ensuring the accuracy and reliability of our results.

After the MTT reagent was administered, it penetrated the cells and was reduced to purple formazan crystals by the NADPH-dependent cellular oxidoreductase enzyme, a key player in our research, indicating metabolic activity. The formazan crystals were subsequently dissolved in SDS-HCl or DMSO, and the optical density (OD) was measured at a wavelength of approximately 570 nm. Our findings, which are novel and significant, indicated that the concentration of *P. major* leaf and non-leaf extracts influenced the OD readings. As explained by Ghasemi et al. (2021), higher OD values correlate with a greater number of viable cells.

The results obtained from determining the concentration of *P. major* extracts, including both leaf and non-leaf parts, using different extraction methods collectively indicate that treating the extracts at the highest concentration range limit of 500 µg/mL ensures RAW 264.7 cell viability of more than 85%. Thus, it is deemed safe and recommended that *P. major* extracts be widely used in pharmacological activity tests, particularly in this study, as preliminary data to determine the appropriate concentrations for evaluating anti-inflammatory activity under diabetic conditions. This finding aligns with previous research by Joo et al., (2014), which suggests that sample concentrations yielding RAW 264.7 cell viability of over 85% are suitable for NO inhibition testing, an assessment of antioxidant and anti-inflammatory properties. Similarly, Auliafendri et al., (2019) also supported this criterion, demonstrating that RAW 264.7 cell viability exceeding 85% is conducive for further analysis in cytokine gene expression inhibition assays. Consequently, both leaf and non-leaf extracts of *P. major* may potentially shield against cytotoxic effects on RAW 264.7 cells, and the concentration range of 7.81-500 µg/mL applied in this study has been shown to influence fluctuations in cell viability (Figure 1).

Similar investigations have been undertaken by Kartini et al., (2021), who explored the wound-healing potential of *P. major* leaf extract under hyperglycemic conditions both *in vivo* using mice and *in vitro* using RAW 264.7 cells. As a preliminary examination, the viability of RAW 264.7 cells exposed to *P. major* leaf extract, using ethanol as the solvent (PMLE) within a concentration range of 7.81-500 µL/mL exhibited variable results in cell viability with increasing extract concentration. Notably, there was no significant decline in cell viability observed until the PMLE concentration reached 500 µL/mL In a separate study, Phumsuay et al., (2020) evaluated the cytotoxic effects of curcumin ester forms within the concentration range of 1-50 µM against RAW 264.7 cells. Their findings indicated a linear relationship, where an increase in compound

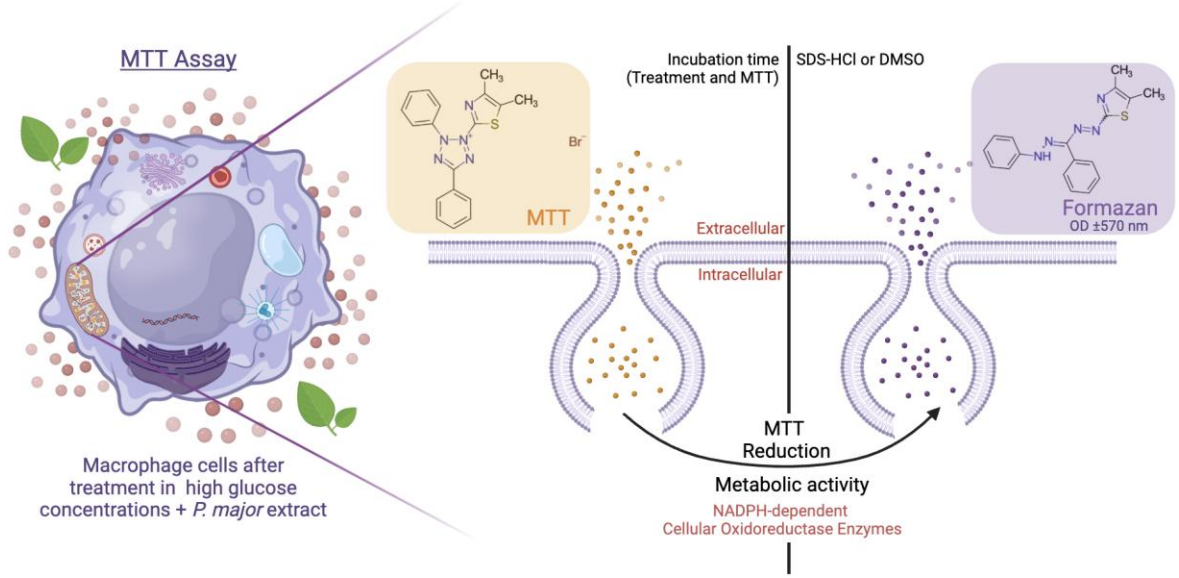

Figure 3. MTT assay performed on RAW 264.7 macrophage cells exposed to high glucose concentrations, following treatment with extracts from both the leaves and non-leaf parts of P. major. Created in BioRender. Hertiani, T. (2025) https://BioRender.com/ftcod8j

concentration corresponded to decreased cell viability, with a notable decline observed at a concentration of 10 µM. The pronounced decrease in cell viability relative to the control suggests that this concentration is unsuitable for *in vitro* testing, as it demonstrates a toxic effect on the experimental model.

In this study, extracts were obtained through the optimization of the extraction process using the UAE method. The optimal conditions identified for the four factors were then applied to the maceration method for a comprehensive comparison. The extraction optimization process was primarily focused on the UAE method due to its significant enhancement in the yield and quality of bioactive compounds extracted (Shen et al., 2023). Moreover, the UAE has been found to augment the likelihood of preserving desired biological activities and functions, thereby expanding its potential applications across various domains (Dzah et al., 2020). For instance, research conducted by (Sunarwidhi et al., 2022) illustrated that brown algae extract obtained through the UAE method exhibited greater cytotoxicity against B16-F10 melanoma cells compared to cold maceration. This study aligns with the rationale that extracts obtained via UAE may contain a higher abundance of bioactive compounds than those obtained via maceration. Consequently, extracts obtained using the UAE method may manifest increased toxicity or reduced cell viability at equivalent concentrations relative to maceration.

It is noteworthy that no significant difference in cell viability was observed between the two extraction methods. This may be attributed to the complex nature of plant extracts, which contain a multitude of compounds rather than single molecules, thus emphasizing the potential importance of compound interactions (Caesar & Cech, 2019). *P. major* extracts comprise compounds that can exhibit both toxic effects and cell-conditioning properties. While the UAE method yields a higher abundance of bioactive compounds than maceration, it is conceivable that the ratio of compounds, synergists, and antagonists affecting cytotoxicity remains similar between the two methods. Thus, this similarity in cell viability outcomes may stem from comparable compositions extracted by both methods. Additionally, it has been elucidated by Cantuária et al., (2018) that high glucose levels in the culture medium (4500 mg/L equivalent to 25 µM) do not alter the viability of RAW 264.7 cells. As far as the author's exploration, research on the cytotoxicity of *P. major* extract obtained from the UAE method against macrophage cells has never been reported.

CONCLUSION

P. major is a herb with considerable pharmacological potential and is characterized by a rich array of bioactive compounds distributed throughout its various plant parts. The cytotoxicity assessment of four optimized extracts derived from *P. major* leaves and non-leaves, employing different extraction methods at concentrations ranging from 7.81 to 500 µg/mL, revealed no toxicity to RAW 264.7 cells. Notably, leaf extracts exhibited higher cell viability than non-leaf

extracts, particularly when extracted using the UAE method. Two-way ANOVA analysis indicated a significant influence of *P. major* plant parts on the percentage viability of RAW 264.7 cells. However, the choice of extraction methods yielded opposite outcomes, with no observed interaction between their combinations.

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CONFLICT OF INTEREST

The authors declare that there are no financial interests or interrelated personal and the institution of Universitas Gadjah Mada relationships that could influence the work reported in this study.

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