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Phyllanthus niruri leaves: Unveiling their reversible anti-inflammatory activity on MIF protein and macrophage cells

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ABSTRACT

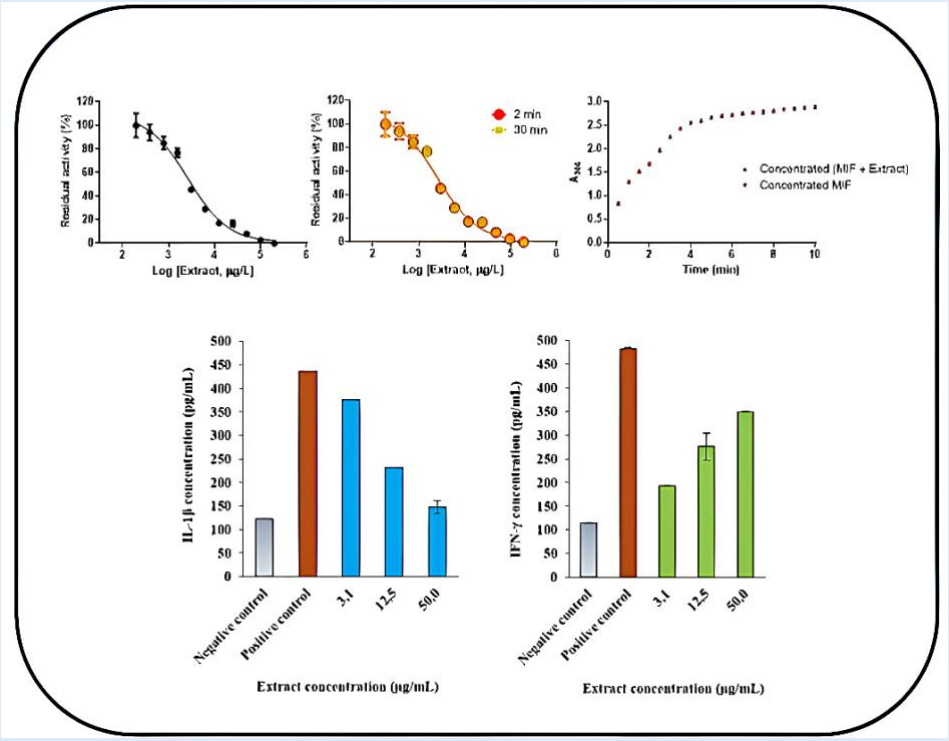
Background: *Phyllanthus niruri* leaves have been known for their various biological activities, including potential anti-inflammatory effects for prevention and treatment of chronic diseases. However, their anti-inflammatory effects have not yet been comprehensively elaborated. This study introduces the novel finding that *Phyllanthus niruri* leaf extract reversibly exhibits inhibition of macrophage migration inhibitory factor (MIF) activity, a key protein in chronic inflammation, with a low IC_{50}. Furthermore, it demonstrates the extract's ability to reduce the levels of pro-inflammatory cytokines IL-1β and IFN-γ in macrophage cells, suggesting a previously unelaborated mechanism for its anti-inflammatory potential.

Methods: The research innovatively combines the assessment of MIF tautomerase inhibition with the evaluation of pro-inflammatory cytokine reduction in macrophage cells to comprehensively characterize the anti-inflammatory activity of *Phyllanthus niruri*. The determination of the reversibility of MIF inhibition adds a crucial layer of understanding to its potential therapeutic action, suggesting a dynamic interaction with the target protein.

Results: *Phyllanthus niruri* leaf extract inhibited MIF activity reversibly with an IC_{50} in the low mg/L range and reduced the concentrations of IL-1β and IFN-γ pro-inflammatory cytokines in macrophage RAW 264.7 cells to different extents.

Conclusion: These findings suggest that *Phyllanthus niruri* leaf extract holds promise as a natural anti-inflammatory agent for managing inflammation-associated chronic diseases. The demonstrated *in vitro* efficacy warrants further investigation into its potential as a therapeutic or preventive agent, paving the way for future *in vivo* studies and clinical trials to validate its use in human health.

Keywords: inflammatory-associated chronic diseases, macrophage migration inhibitory factor (MIF), macrophage cells, *Phyllanthus niruri*, anti-inflammatory agent.



INTRODUCTION

Phyllanthus niruri Linns (*Phyllanthaceae*) is a plant known for its round, upright stem, which can grow taller than one meter in fertile soil. The leaves have an evenly pinnate structure, with each stem bearing small, oval-shaped compound leaves. The flowers grow at the leaf axils and face downward. It is typically not cultivated because it is considered a common weed. It thrives in moist environments, from lowlands to elevations of up to 1000 meters above sea level. The plant contains chemical compounds such as phyllanthin, potassium, resin, tannin, and flavonoid. *Phyllanthus niruri* are used empirically for treatment of dysentery, dyspepsia, constipation, coughs, fevers, asthma, cuts and bruises, corneal opacity, conjunctivitis, dropsy, itch, epilepsy, gonorrhea, excessive menstruation, vaginitis, and biolarvicide [1-2]. In addition, *Phyllanthus niruri* are also reported as having hepatoprotective activity [3-4], antitumor [5], immunomodulator [6], antibacterial [7], antidiabetic [8], and therapeutical effects on endometriosis [9].

The phytochemistry of *Phyllanthus niruri* has been identified in several studies: alkaloids, anthocyanins, coumarins, flavonoids, phenolic acids, saponins, tannins, lignans, terpenoids, and hypophyllanthin are reported among other bioactive components [10-12].

Although *Phyllanthus niruri* leaves are known for their various biological activities, including potential anti-inflammatory effects [1, 13-14], their full potential as an agent for anti-inflammatory has not yet been comprehensively evaluated. Therefore, our aim is to assess its impact on inhibiting the activity of macrophage migration inhibitory factor (MIF), a protein that exerts a key role in immune response and inflammation. Additionally, we will investigate its ability to reduce the levels of pro-inflammatory cytokines produced by macrophages, which are crucial in the inflammatory progression. Inhibiting the activity of MIF and reducing pro-inflammatory cytokine levels could enhance its anti-inflammatory effects, aiding in the development of treatments for chronic inflammatory diseases [15-16].

MIF exerts its effect on the immune response by inhibiting the migration of macrophages. MIF also exhibits tautomerase activity, which is frequently used to identify agents that can inhibit its activity, in reversible or irreversible manner [17]. In reversible manner, a substance is bound reversibly to the target protein and after some period it is dissociated, released, and excreted from the body. On the other hand, in an irreversible manner, a substance is bound irreversibly and difficult to dissociate from the target protein and can permanently modify the conformation of the target molecule, potentially leading to accumulation and adverse side effects.

When extracting bioactive compounds from *Phyllanthus niruri* leaves, it is important to consider their solubility in different solvents. These compounds generally have low solubility in water, high solubility in chloroform and ether, and blend well with ethanol and oils. In our study, ethanol 96 % was used to extract the bioactive compounds from *Phyllanthus niruri* leaves.

The study evaluates the potential of the *Phyllanthus niruri* leaf extract in inhibiting the activity of MIF, the reversibility of the inhibition, and its potential to decrease the concentrations of IL-1β and IFN-γ pro-inflammatory cytokines released by macrophage RAW 264.7 cells

MATERIALS AND METHODS

Materials: *Phyllanthus niruri* young leaves were sourced from the organic garden of HRL International Company in East Java Regency. Their identification was verified by a taxonomist at the Center for Information and Development of Traditional Medicine, Faculty of Pharmacy, University of Surabaya, located in Surabaya, East Java, Indonesia.

The samples were oven-dried at 40 °C for three days, ground with a herb grinder, and passed through an 80-mesh sieve to obtain a fine powder. This powdered sample was subsequently stored in plastic bags, sealed tightly in a closed container, and maintained at room temperature of 25 °C.

METHODS

Preparation for *Phyllanthus niruri* leaf extract: The process of obtaining the ethanolic extract from *Phyllanthus niruri* leaves followed the modified method described by Carmagnani et al. (2020). Specifically, 10.0 grams of *Phyllanthus niruri* leaf powder underwent maceration with 100 mL of ethanol 96 % (1:10 ratio) at a temperature of 30 °C for 3 days with agitation at 175 rpm in a shaker incubator. The resulting extract was filtered through filter paper, and the filtrate underwent evaporation using a rotary evaporator with a vacuum pump to eliminate ethanol residues. The extract was then weighed to determine the extraction yield, transferred into a vial bottle, and stored at 4 °C in the refrigerator until use [18].

Inhibition of MIF protein by *Phyllanthus niruri* leaf extract: The MIF protein was obtained following the procedure outlined by Kok et al. (2018a). To assess the potential inhibition by the *Phyllanthus niruri* leaf extract

on the tautomerase activity of MIF, we employed the methodology described by Nyotohadi and Kok (2023). This involved measuring the absorbance value of the borate complex formed with the enol form of 4-hydroxyphenylpyruvate (4-HPP) (Sigma Aldrich, Switzerland), which is the product of the tautomerase reaction. For the positive control measurement, the reaction mixture contained MIF protein in borate buffer pH 6.2, dimethyl sulfoxide (DMSO) solvent, and 1 mM 4-HPP substrate. In the meantime, for the extract inhibition assessment, the reaction components were identical to the positive control measurement, but the DMSO solvent was replaced with an extract solution in DMSO. For a definite inhibitor control, a Cu^{2+} solution of 50 µM was utilized instead of the extract solution. The negative control reaction mixture comprised all the components of the positive control measurement, except borate buffer pH 6.2 without MIF protein being used [1]. ISO-1 that has been reported as having definite inhibitory activity on MIF protein was used as a reference compound [19-22].

To evaluate the reversibility of the extract's inhibition on the tautomerase activity of MIF, we conducted pre-incubation and dilution assays. In the pre-incubation assay, the extract was combined with MIF for 2 minutes (standard period for such assay) and then incubated for 30 minutes before reacting with the substrate. For the dilution assay, we combined a 100-fold concentrated MIF without and with a 10-fold IC_{50} concentrated extract, and individual mixture was diluted 100-fold with boric acid buffer followed by 4-HPP in ammonium acetate buffer hence they were measured in the same standard condition of buffer [21].

Evaluation of macrophage RAW 264.7 cell viability during incubation with *Phyllanthus niruri* leaf extract: The viability of macrophage RAW 264.7 cells (Sigma Aldrich, Switzerland) was assessed following the protocol outlined by Kok (2023) using the MTS technique, which involves the conversion of yellow tetrazolium salt to a purple formazan product by the extract [23-24]. The treatment procedure with the *Phyllanthus niruri* leaf extract was as follows: Cells were seeded into a 96-well plate, with each well containing 5×10^5 cells in 180 µL of medium. They were then allowed to incubate at 37 °C for 24 hours with 5 % CO_2 until they reached confluency. Following this, the cells were exposed to lipopolysaccharide (LPS) at a concentration of 1 µg/mL for 18 hours. Subsequently, varying concentrations (ranging from 3.1 % to 12.5 % µg/mL) of *Phyllanthus niruri* leaf extract was introduced to the wells, while control wells received either only medium (negative control) or medium with LPS (positive control). Following another 24-hour incubation, 20 µL of the Cell Proliferation Assay Kit (Abcam ab197010, UK) was introduced to each well, and the absorbance was read at 490 nm after a 3-hour period using a microplate reader.

Reduction of pro-inflammatory cytokines concentrations by *Phyllanthus niruri* leaf extract: For the *Phyllanthus niruri* leaf extract treatment, cells were plated similarly and exposed to LPS for 18 hours. Subsequently, the extract was administered at concentrations optimized for cell viability in previous experiments. Following a 24-hour treatment period, cells were harvested, centrifuged, and the supernatant was utilized for ELISA evaluations to quantify the concentrations of IL-1β and IFN-γ cytokines (Elabscience, USA). The concentrations of cytokines were assessed both under inflammatory conditions induced by LPS (positive controls) and after treatment with the *Phyllanthus niruri* leaf extract.

Initially, each well of a 96-well plate was treated with 100 µL of *Phyllanthus niruri* leaf extract and then incubated for 90 minutes at 37 °C. Subsequently, any unbound components were removed, and the wells were washed with PBS buffer. Following this, 100 µL of

biotinylated detection antibody was introduced to individual well and incubated for 60 minutes at 37 °C. Following the removal of the unbound components and washing with PBS buffer, 100 µL of avidin-horseradish peroxidase conjugate was introduced to individual well and incubated for 30 minutes at 37 °C. Once again, following the removal of the unbound components and washing, 90 µL of tetramethylbenzidine substrate was introduced to individual well and incubated for 30 minutes at 37 °C. The reaction was halted by introducing 50 µL of H_2SO_4 solution to individual well, and the absorbance at 450 nm was read on a microplate reader, indicating the concentrations of IL-1β and IFN-cytokines. The calculations and graph constructions were done either using GraphPad Prism version 8.0 or using Microsoft Excel 365 software. Data were presented as mean ± SEM or mean ± SD.

RESULTS AND DISCUSSION

Inhibition of MIF protein by *Phyllanthus niruri* leaf extract: To assess the potential of *Phyllanthus niruri* leaf extract as an agent for anti-inflammatory, we measured the residual activity of MIF protein upon the addition of extract. The results indicated that the *Phyllanthus niruri* leaf extract exhibits inhibitory activity on MIF protein, with an IC_{50} of (2.6 ± 0.0) mg/L (Fig. 1A)

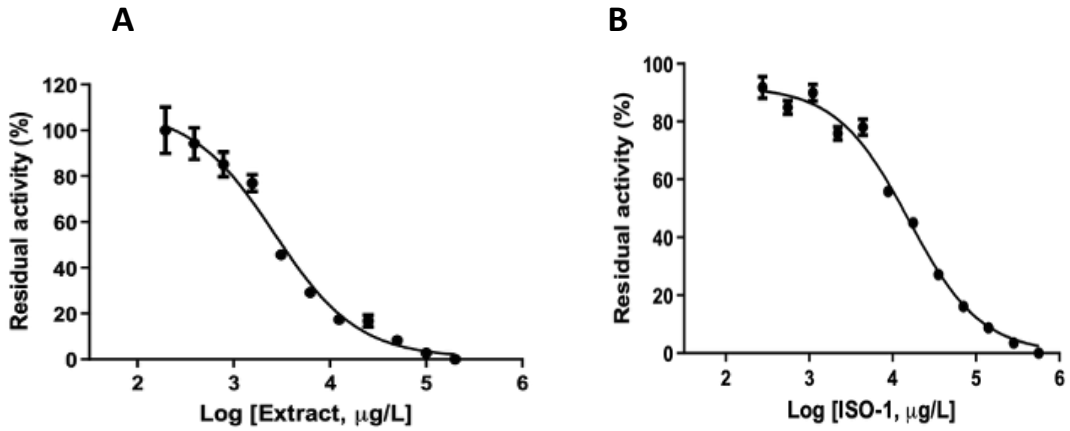


Figure 1. Inhibition of MIF tautomerase activity by (**A**) *Phyllanthus niruri* leaf extract, IC_{50} was (2649.0 ± 1.2) µg/L = (2.6 ± 0.0) mg/L and (**B**) ISO-1 reference compound, IC_{50} was (15526.0 ± 259.8) µg/L = (15.5 ± 0.3) mg/L. The variation of data was expressed as the standard error of mean (SEM) of 3 observations.

Previous reports on MIF tautomerase inhibition with ISO-1 as a reference compound gave IC_{50} value of 7 µM or 1647 µg/L = 1.6 mg/L [19], 18.2 µM or 4281 µg/L = 4.3 mg/L [20], 24 µM or 5646 µg/L = 5.6 mg/L [22]. The molecular weight of ISO-1 is 235.24. In-house study showed an IC_{50} of 15526 µg/L = 15.5 mg/L for the ISO-1 (Fig. 1B). The IC_{50} value of the *Phyllanthus niruri* leaf extract in the low mg/L range obtained in this work is among the IC_{50} values of the ISO-1 obtained by several studies. It suggests that the *Phyllanthus niruri* leaf extract has a potency to inhibit MIF protein thus preventing random migration of macrophage, thereby potentially reducing inflammation.

Reversibility of *Phyllanthus niruri* leaf extract's inhibition on MIF activity: To evaluate whether the inhibition by the extract on MIF tautomerase activity is

reversible, we conducted pre-incubation and dilution assays. In the pre-incubation assay, the results indicated no apparent difference between the sigmoidal curves of the 2-minute pre-incubation and the 30-minute pre-incubation (Fig. 2A) and in the dilution assay, the results showed that after dilution of solutions of (MIF + extract) and MIF, both absorbance curves were nearly identical (Fig. 2B)

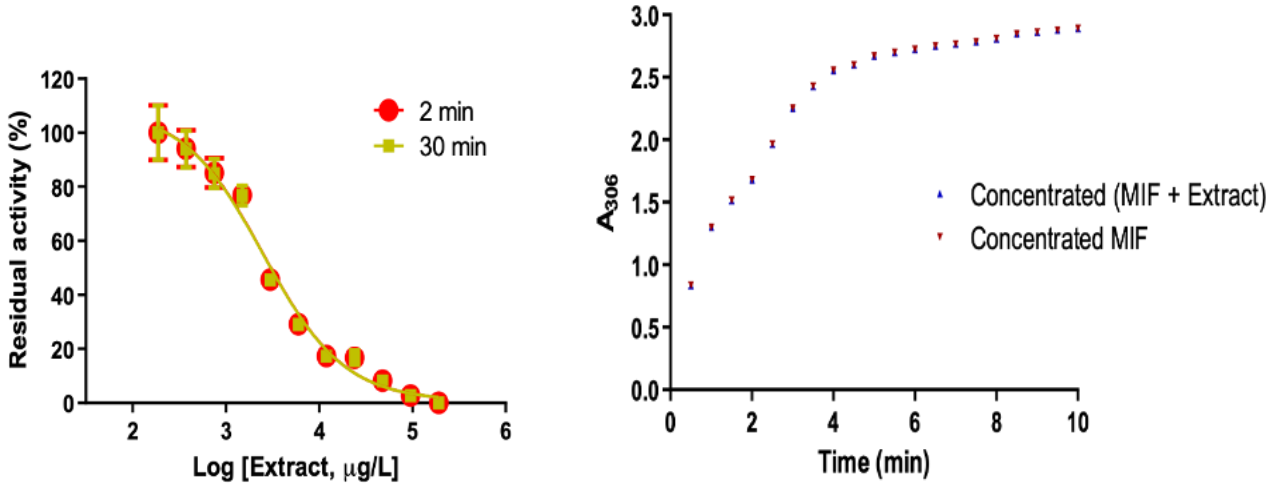


Figure 2. Inhibition of MIF tautomerase activity by (**A**) *Phyllanthus niruri* leaf extract, IC_{50} was (2649.0 ± 1.2) µg/L(2.6± 0.0) mg/L and (**B**) ISO-1 reference compound, IC_{50} was (15526.0 ± 259.8) µg/L = (15.5 ± 0.3) mg/L. The variation of data was expressed as the standard error of mean (SEM) of 3 observations

No apparent difference between the sigmoidal curves of the 2-minute pre-incubation and the 30-minute pre-incubation (Fig. 2A) suggests that the inhibition by the extract on MIF tautomerase activity is not dependent on the duration of pre-incubation and implies that the inhibition is reversible [21].

The nearly identical post-dilution absorbance curves of the solutions of (MIF + extract) and MIF in the dilution assay (Fig. 2B) indicates that the activity of MIF, when mixed with the concentrated extract, returns to its native state after a 100-fold dilution. This finding suggests further reversible inhibition. Most reversible inhibitors that bind to the active site of MIF enzyme produce hydrogen bonds with Ile-64, Lys-32, Pro-1, and/or Asn-97, and form aryl–aryl interactions with Tyr-95, Tyr-36, and/or Phe-113 [25]. In contrast, when combined with an irreversible inhibitor, MIF would exhibit no activity after being diluted, and the absorbance versus time curve of the mixture would be flat [17].

Macrophage RAW 264.7 cell viability during incubation with *Phyllanthus niruri* leaf extract: Given the extract's inhibition of the MIF target protein and the reversible nature of this inhibition, we proceeded to evaluate its effect on reducing pro-inflammatory cytokine concentrations in macrophage RAW264.7 cells. We began by examining the viability of macrophage RAW264.7 cells following the addition of the extract (Table 1).

Table 1. Macrophage RAW264.7 cells viability upon the addition of *Phyllanthus niruri* leaf extract. The variation of data was expressed as the standard of deviation (SD) of 3 observations.

Sample		***Viability (%)***	
		Average	***SD***
Negative control (i.e. medium only)		100.0	5.3
Positive control (LPS induction)		73.8	1.6
Extract concentration (µg/mL)	3.1	107.0	1.0
	6.3	101.2	4.2
	12.5	95.6	2.8
	25.0	93.6	3.3
	50.0	92.6	1.5

The MTS assay results, which measure cell viability by converting yellow tetrazolium salt to purple formazan, showed that the extract at concentrations up to 50.0 µg/mL showed cell viability above 80 %, outperforming the positive control (LPS-induced inflammation) (Table 1). This indicates that *Phyllanthus niruri* leaf extract at concentrations up to 92.6 µg/mL is safe for cells.

Reduction of pro-inflammatory cytokines concentrations by *Phyllanthus niruri* leaf extract: To assess the anti-inflammatory effects of *Phyllanthus niruri* leaf extract on macrophage RAW 264.7 cells, we used extract concentrations ranging from 3.1 to 50.0 µg/mL, which were previously shown as maintaining cell viability above that of the positive control (LPS-induced inflammation). The results indicated that at these concentrations, the *Phyllanthus niruri* extract could reduce the concentrations of the IL-1β and IFN-γ pro-inflammatory cytokines (Fig. 3).

An essential cytokine responsible for initiating and triggering the secretion of some other pro-inflammatory cytokines is IL-1β [26]. In the study, the expression level of IL-1β cytokine increased by approximately 250 % (from 125 to 435 pg/mL) following LPS-induced inflammation (Fig. 3A). The *Phyllanthus niruri* leaf extract reduced this level by approximately 14 % (from 435 to 375 pg/mL) at an extract concentration of 3.1 µg/mL, by about 47 % (from 435 to 230 pg/mL) at an extract concentration of 12.5 µg/mL, and by roughly 70 % (from 435 to 130 pg/mL) at an extract concentration of 50.0 µg/mL (Fig. 3A), exhibiting the anti-inflammatory effect of the extract.

One of the cytokines crucial in the progression of certain inflammatory disorders is IFN-γ [26]. IFN-γ can carry out this action by enhancing pro-inflammatory pathways and antagonizing anti-inflammatory signals. IFN-γ can activate macrophages to produce TNF-α and IL-6 pro-inflammatory cytokines [27]. In the meantime, IFN-γ can suppress the generation of Treg cells, hence inhibit the production of IL-10 anti-inflammatory cytokine [28]. In the study, the IFN-γ cytokine expression level rose approximately 300 % (from 115 to 485 pg/mL) during LPS-induced inflammation (Fig. 3B).

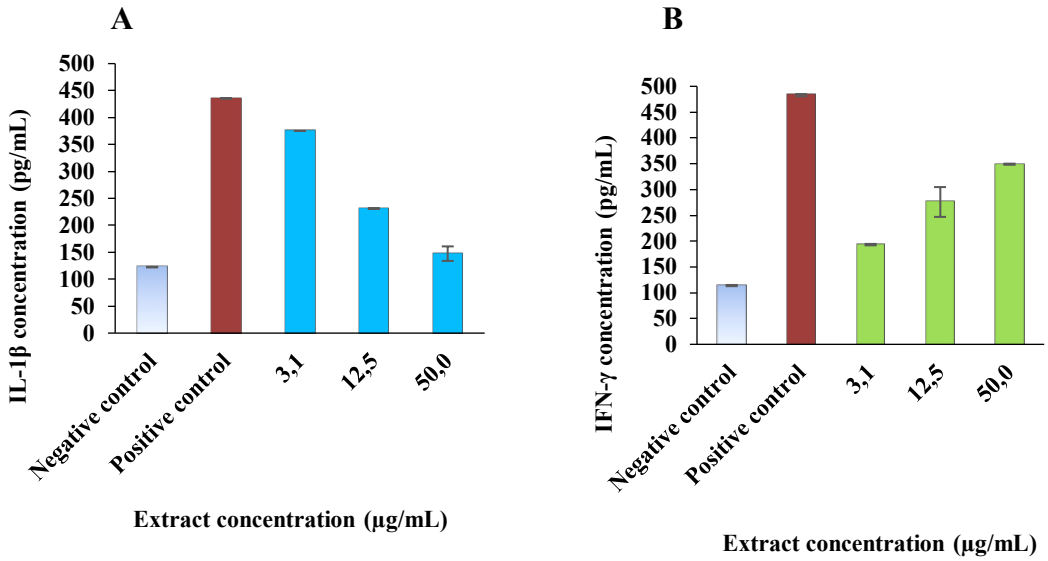


Figure 3. Inhibition of MIF tautomerase activity by (**A**) *Phyllanthus niruri* leaf extract, IC_{50} was (2649.0 ± 1.2) µg/L = (2.6 ± 0.0) mg/L and (**B**) ISO-1 reference compound, IC_{50} was (15526.0 ± 259.8) µg/L = (15.5 ± 0.3) mg/L. The variation of data was expressed as the standard error of mean (SEM) of 3 observations

Upon treatment with *Phyllanthus niruri* leaf extract, the IFN-γ cytokine expression level decreased by roughly 60 % (from 485 to 195 pg/mL) at an extract concentration of 3.1 µg/mL; the decrease was less pronounced at higher extract concentrations, i.e., 43 % (from 485 to 275 pg/mL) for an extract concentration of 12.5 µg/mL and 28 % (from 485 to 350 pg/mL) for an extract concentration of 50.0 µg/mL (Fig. 3B). This suggests that, concerning IFN-γ cytokine concentration, the anti-inflammatory effect of *Phyllanthus niruri* leaf extract is more pronounced at relatively lower concentrations compared to relatively higher ones.

In summary, LPS-induced inflammation prompts macrophage RAW 264.7 cells to release diverse pro-inflammatory cytokines, contributing to an inflammatory state. This study showed that *Phyllanthus niruri* leaf extract reduces the levels of IL-1β and IFN-γ pro-inflammatory cytokines released by macrophage RAW 264.7 cells. Thus, *Phyllanthus niruri* leaf extract is found to have potential anti-inflammatory properties.

CONCLUSION

The potential of *Phyllanthus niruri* leaf extract as an agent for anti-inflammatory was assessed. The findings revealed that the extract inhibits MIF tautomerase activity with an IC_{50} in the low mg/L range, in reversible manner, and decreased the concentrations of IL-1β and IFN-γ pro-inflammatory cytokines in macrophage RAW 264.7 cells to different extents. Therefore, *Phyllanthus niruri* leaves exhibit potential as an agent for anti-inflammatory in treating inflammation-associated chronic diseases.

Abbreviations: DMSO: dimethyl sulfoxide, ELISA: enzyme-linked immunosorbent assay, 4-HPP: 4-hydroxyphenylpyruvate, IC_{50}: inhibitory concentration-50, IFN-γ: interferon-γ, IL-1β:interleukin-1β, ISO-1: (S,R)-3-(4-hydroxyphenyl)-4,5-dihydro-5-isoxazole acetic acid methyl ester, LPS: lipopolysaccharide, MIF: macrophage migration inhibitory factor, MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, PBS: phosphate-buffered saline.

Competing Interests: The authors declare there is no competing interest in this manuscript.

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Products and Health Supplements for Degenerative Conditions University of Surabaya.

Authors' Contributions: TK conceived, designed and supervised the project, reviewed the manuscript; DGS acquired, analyzed and interpreted the data; FI supervised the project and prepared the manuscript; ZG critically reviewed and edited the manuscript.

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