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Modulation of M1/M2 Cytokines and Inflammatory Enzymes by *Persicaria* Species Leaf Extracts in Lipopolysaccharide-stimulated RAW 264.7 Cell Macrophages

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ABSTRACT

Background: Investigating the impacts of plant-based substances on the regulation of pro-inflammatory M1 and anti-inflammatory M2 cytokines could have significant implications for immune-related health conditions. Seven *Persicaria* plant species from sub-Saharan Africa were specifically selected for analysis, based on their traditional use in treating inflammation.

Objective: To investigate the inhibitory effects of methanol leaf extracts from selected plants on enzymes involved in chronic inflammation.

Methods: The inhibition of nitric oxide production, acetylcholinesterase activity, and 15-lipoxygenase activity was assessed using the Griess reagent method, Ellman's colorimetric method, and the ferrous oxidation-xylenol orange assay. The quantity of M1/M2 cytokines released was quantified using a flow cytometer.

Results: At a concentration of 50 µg/mL, the methanol extracts of *P. limbata* exhibited the highest NO inhibition (97.67%), followed by *P. nepalensis* (93.06%) and *P. setosula* (92.78%). The NO inhibition caused by the plant extracts was correlated directly with the decrease in NO release by the LPS-stimulated macrophages. Furthermore, the pro-inflammatory enzyme assays indicated that the methanol extracts of *P. setosula* exhibited the highest enzyme inhibitory activity (LOX 89.59%, AChE 72.12 %). This was followed by *P. limbata* (with 92.76% for LOX and 56.93% for AChE) and *P. nepalensis* (with 88.16% for LOX and 47.17% for AChE). Cytokine assays revealed that the extracts of *P. limbata* had significant dose-dependent suppressive effects on IFN-γ and TNF-α expression while promoting the secretion of IL-2, IL-4, IL-6, and IL-10.

Conclusion: Extracts of *P. limbata* contain immunomodulatory compounds that could be further explored as potential remedies to target the molecular drivers of chronic inflammation.

Keywords: 15-lipoxygenase, Acetylcholinesterase, Cytokine, Macrophage, *Persicaria*

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INTRODUCTION

Inflammation is an essential physiological response coordinated by the immune system, acting as a protective mechanism against invading pathogens and tissue damage (1). However, dysregulated or sustained inflammation can trigger pathological conditions, resulting in the occurrence of different conditions such as autoimmune disorders, metabolic syndromes, and neurodegenerative disorders (2). By harnessing their bioactive constituents, plant extracts offer a rich source of pharmacophores capable of modulating the immune system, key inflammatory pathways, and molecular targets.

Plants belonging to the genus *Persicaria* exhibit various biological characteristics such as germ-killing, antifungal, antibiotic, diuretic, antinociceptive, and antirheumatic properties. They are used for healing ulcers, sores, skin infections, and for tightening the skin (3). Seven *Persicaria* species were selected for this study based on their traditional medical importance: *Persicaria limbata*, *P. decipiens*, *P. attenuata*, *P. acuminata*, *P. nepalensis*, *P. senegalensis*, and *P. setosula*. These plants have been used for centuries by people from various countries for medicinal purposes, particularly to alleviate inflammation and pain conditions like stomach pain and skin allergies (4, 5). The extracts of the *Persicaria* plant contain phytochemicals such as polyphenols, tannins, polyuronides, flavonoid triterpenes, glycosides, saponins, and essential oils (5). These secondary metabolites are believed to contribute to the biological effects of the natural compounds extracted from plants. For instance, portions of the upper parts of *P. acuminata* are commonly used to alleviate pathogens that cause wounds (3). Derita and Zacchino demonstrated the antimicrobial activity of the aerial parts of *P. acuminata* (6). Others reported the bioactivity of *P. limbata* methanol extracts (7). Additionally, previous studies have reported that portions of the upper parts of *P. decipiens* contain flavonoids (7) and flavonol glycosides (8) as the main constituents. These polyphenols have become a keyfocus in the search for new anti-inflammatory drugs because they Target inflammatory cytokines and enzymes which play a central role in the inflammatory response. Lipoxygenase (LOX) activity inhibition can suppress the production of leukotrienes and subsequent inflammation. Several research studies have shown thatplant extracts can reduce the activity of LOX, indicating their potential as anti-inflammatory agents (9). Moreover, acetylcholinesterase (AChE), another key enzyme that plant extracts can target, is involved in the degradation of acetylcholine, a neurotransmitter that regulates immune responses (10). Inhibition of AChE activity can modulate immune responses and attenuate inflammation (11). Furthermore, nitric oxide (NO), a free radical gas produced by macrophages, has been implicated in various inflammatory diseases. An excess of NO production can cause damage to tissues and contribute to the progression of inflammation. (12). Multiple research studies have shown that products derived from plants have the capacity to block the production of NO in LPS-stimulated macrophages. (13), suggesting their potential as anti-inflammatory agents. In addition to targeting key enzymes, inflammatory cytokines may also serve as potential targets for anti-inflammatory therapies because of their central role in mediating and propagating inflammatory responses in the body (14). M1 cytokines, such as IL-2, IFN-γ, and TNF-α, are produced in response to pathogens, cellular damage, and immune signals (15). They are involved in cell-mediated immunity and are essential for initiating and sustaining the inflammatory response (16, 17). M1 cytokines are primarily produced by M1 macrophages, also known as classically activated macrophages cells (18). On the other hand, M2 cytokines such as IL-4, IL-10, and IL-13, play a crucial role in resolving inflammation, promoting tissue repair, and maintaining tissue homeostasis (17,

19). They are released by M2 macrophages, also known as alternatively activated macrophages (18). Extracts from plants have also been demonstrated to regulate the productionof inflammatory cytokines. (20, 21). Some phytochemicals have been reported as modulators of M1 and M2 macrophages in inflammation (22). Although various products derived from *Persicaria* species have been known to exhibit an extensive array of biological effects, several species of the genus possess qualities that are exhibited both inside a laboratory environment and within a living organism. These properties includeanti-inflammatory, antidiabetic, neuroprotective, hepatoprotective and wound healing activities that have not been fully explored for their potential clinical uses. Further research ate needed to explore the potential applications of these *Persicaria* species. Therefore, the purpose of this study was to investigate how extracts from specific *Persicaria* species could reduce inflammation in LPS-stimulated RAW 264.7 macrophage cells. The study specifically aims to assess the modulatory effects of methanol extracts from seven *Persicaria* species on acetylcholinesterase (AChE), LOX inflammatory enzymes and mediators (NO, M1/M2 cytokines) that are implicated in the inflammatory response.

MATERIALS AND METHODS

We used reagents and chemicals of analytical grade that were obtained through standard methods and procedures, or in accordance with the manufacturer's instructions.

Chemicals

Lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 was acquired from Sigma-Aldrich in Darmstadt, Germany. Penicillin/streptomycin /fungizone (PSF), Dulbecco's Modified Eagle's Medium (DMEM), and fetal calf serum (FCS) were obtained from Highveld Biological Products in South Africa. Trypsin and phosphate-buffered saline (PBS) were provided by Whitehead Scientific in South Africa. MTT and quercetin were purchased from Sigma-Aldrich in St. Louis, MO, USA. Galantamine, acetylthiocholine iodide (ATCI), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), acetylcholinesterase (AChE) enzymes from Electrophorus electricus (electric eel) lyophilized (type VI-S lyophilized powder), bovine serum albumin (BSA), sodium dodecyl sulfate, sodium chloride (NaCl), MgCl2·6H2O, ferrous sulfate, indomethacin, 15-lipoxygenase from Glycine max, and sodium nitrite were sourced from Sigma in Germany. Lastly, Tris (hydroxymethyl) aminomethane was obtained from Sigma in Switzerland.

Plant Material and Extraction

The plant material used in this work was a gift kindly offered by Professor Jean Paul Dzoyem from the University of Dschang in Cameroon. Leaves of seven *Persicaria* species were collected from Balatchi (5° 37′ 40″ N, 10° 11′ 00″ E) and Santchou (5° 16′ 55″ N, 9° 58′ 27″ E) villages in the western region of Cameroon in March 2021. The plants being considered are not endangered species, and were harvested from an open community field, therefore not requiring prior authorization. The plants were identified by the curator at the National Herbarium of Cameroon in Yaoundé, where voucher specimens were stored and catalogued with specific reference numbers as follows: *P. limbata* 38852/HNC, *P. decipiens* 25044/SFRCam, *P. attenuata* 19717/SFRCam, *P. acuminata* 25031/SFRCam, *P. nepalensis* 16818/SRFCam, *P. senegalensis* 17014/SRFcam, and *P. setosula*: 32356/HNC. After drying, the leaves were pulverized into a fine powder. Methanol extraction was performed on 200 g of powdered leaves in 1000 mL of methanol. The extraction process included a 72-hour incubation period at temperature. The final product was filtered and then concentrated under low pressure using a rotary evaporator at 45°C to produce the methanol extracts. Additionally, the

botanical designations *P. limbata* (Meisn.) H. Hara, *P. decipiens* (R.Br.) K.L.Wilson, *P. attenuata* (R.Br.) Soják, *P. acuminata* (Kunth) M.Gómez, *P. nepalensis* (Meisn.) Miyabe, *P. senegalensis* (Meisn.) Soják, *P. setosula* (A.Rich.) K.L.Wilson (Polygonaceae) were further validated through cross-referencing with the plant index database, World Flora Online. The corresponding identities are: wfo-4000028848, wfo-0000488020, wfo-0001250565, wfo-0000488001, wfo-0000488438, wfo-0000488300, and wfo-0000483758.

Nitric Oxide Assay Using Lipopolysaccharide-stimulated RAW 264.7 Macrophage Cells Maintenance and Culture

The cell line RAW 264.7, consisting of mouse macrophages, was obtained from the American Type Culture Collection in Rockville, MD, USA. It was cultured following conventional cell culture protocols. The cells were kept alive in DMEM with 10% fetal calf serum (FCS) and 1% penicillin/streptomycin/fungizone (PSF). The standard maintenance temperature was set at 37°C, with a CO2 level of 5% in a humidified atmosphere.

Prevention of Nitric Oxide (NO) Generation in RAW 264.7 Cells Activated by LPS

RAW 264.7 macrophage cells were cultured in 96-well microtiter plates at concentration of 2×10^6 cells/mL. The cells were then activated by exposure to a medium with a concentration of 1 µg/mL LPS alone (control) or LPS along with different concentrations of extracts (50 µg/mL, 25 µg/mL, 12.5 µg/mL, and 6.25 µg/mL) dissolved in dimethyl sulfoxide. Quercetin was used as a control to produce an expected positive result for inhibiting NO production (23). The Griess reagent was used to quantify the nitric oxide released by the macrophages, as previously described (24).

Cell Viability

To verify that the decrease in nitric oxide was not caused by the extract's harmful effects, we performed the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay, on the macrophage cells. This assay is based on Mosmann's method (25) with minor adjustments. After removing the medium from each well, the cells were washed with PBS and fresh medium (200 µL) was added to each. Next, 30 µL of MTT (5 mg/mL in PBS) was added to the cells, and the plates were placed in an incubator at 37°C for 4 hours. After incubation, the media was removed from the wells, and DMSO was used to dissolve the formazan crystals that had formed. The absorbance at 570 nm was read using a SpectraMax 190 device from Molecular Devices. The percentage of cell inhibition was determined in comparison to the control group with unaffected cells considered as 100% viable.

Soybean 15-LOX Inhibition Assay

The 15-LOX test was conducted using a previously established method with slight modifications (26). The procedure included measuring the formation of the Fe^{3+}/xylenol orange complex at 560 nm with a spectrophotometer. After mixing 15-LOX from Glycine max with extracts or standard inhibitors, the mixture was incubated at 25°C for 5 minutes. The extracts were tested at a fixed concentration of 200 µg/mL, and those showing over 50% inhibition were further evaluated at varying concentrations ranging from 200 µg/mL to 1.56 µg/mL. The inhibition of 15-LOX activity was compared to quercetin as a reference (27). Linoleic acid was then added to a Tris-HCl buffer at a final concentration of 140 µM, and the mixture was left to incubate at 25°C for 20 minutes in the absence of light. The test was stopped by adding 100 µL of FOX reagent, which consisted of sulfuric acid, xylenol orange, and iron (II) sulfate dissolved in a methanol/water solution. Only the LOX solution and buffer were placed in the control wells. We used LOX enzyme without the substrate as a blank. To assess LOX inhibitory activity, the percentage

of hydroperoxide production inhibition was calculated based on changes in absorbance readings at 560 nm over a 30-minutes period at 25°C. The formula used for this calculation is as follows: % inhibition=$[(A_{control} - A_{blank}) - (A_{sample} - A_{blank})/(A_{control} - A_{blank})]$ x100, where $A_{control}$ represents the absorbance of the control well, A_{blank} represents the absorbance of the blank well, and A_{sample} represents the absorbance of the sample well.

Acetylcholinesterase Inhibition Activity

The inhibition of Acetylcholinesterase (AChE) was assessed using Ellman's colorimetric method, with minor modifications (28). In a 96-well plate, 25 µL of a 15 mmol/L solution of ATCI in water, 125 µL of a 3 mmol/L solution of DTNB in Buffer A (containing 50 mmol/L Tris-HCl at pH 8.0, along with 0.1 mol/L NaCl and 0.02 mol/L $MgCl_2.6H_2O$), 50 µL of Buffer B (with 50 mmol/L at pH 8 and 0.1% bovine serum albumin), and 25 µL of a sample (diluted in a series: 500-3.9 µg/mL for extracts and 10-0.078 µg/mL for Eserine as a standard AChE inhibitor) were added. AChE (0.2 U/mL) was then added to the wells, and the absorbance at 405 nm was measured using a BioTek Synergy microplate reader. Distilled water served as the negative control. The percentage of inhibition was calculated using the formula: $V=\Delta_{Abs}/\Delta_t$, % enzyme activity=$(V/V_{max})\times100$, % enzyme inhibition=100–% enzyme activity. In this formula, V represents the reaction rate with an inhibitor, while V_{max} is the reaction rate without an inhibitor. The IC_{50} value of the extract, which indicates 50% inhibition, was determined by comparing the inhibition percentage with the concentration.

Measurement of M1/M2 Cytokines

The RAW264.7 cells were first seeded in a 48-well microplate at a density of 2×105 cells/mL and were permitted to adhere for 24 hours. Subsequently, the cells were treated with lipopolysaccharide (LPS) at a concentration of 0.1 µg/mL, in addition to an extract with concentrations that corresponded equivalent to the IC_{50}, $IC_{50}/2$, or $IC_{50}/4$ values against RAW 264.7 cells. Control groups consisted of untreated cells and cells treated with indomethacin at a concentration of 5 µg/mL. After 48 hours of incubation, the supernatant was collected to measure the levels of pro-inflammatory cytokines (IFN-γ, TNF-α, and IL-2) and anti-inflammatory cytokines (IL-4, IL-6, and IL-10) released. The experiment was carried out following the manufacturer's instructions using the BD™ Cytometric Bead Array (CBA) Human Cytokine Kit from BD Biosciences. The data obtained were analyzed using a BD LSR Fortessa™ cell analyzer flow cytometer.

RESULTS

In our study, we selected seven *Persicaria* species based on a review of plants traditionally used for chronic inflammation-related conditions. We investigated their leaf extracts to determine their potential as modulators of LOX inflammatory enzymes, AChE, NO and M1/M2 cytokines.

Preventing the Production of Nitric Oxide (NO) in RAW 264.7 Macrophages Activated by LPS

Table 1 presents data on the inhibitory activity of extracts from various *Persicaria* plant species, as well as the positive control quercetin on nitric oxide (NO) production and cell viability.

Across different *Persicaria* species, a clear dose-response relationship is observed. In general, higher concentrations of the extracts lead to greater inhibition of NO production both in terms of the amount of NO produced and the percentage of NO inhibited. In the most concentrated level of extracts (50 µg/mL), *Persicaria limbata* extract demonstrates the most potent inhibition of NO production, with 97.67% inhibition. *Persicaria attenuata, Persicaria acuminata,* and other species exhibit varying degrees of NO production inhibition at different concentrations. They show moderate to high inhibitory activity,

Table 1: The inhibitory activity of extracts from several *Persicaria* plant species on Nitric oxide production in LPS-activated RAW 264.7 macrophages.

Plant species	Concentration (µg/mL)	NO (µM)	NO inhibition (%)	Cell viability (%)
Persicaria limbata	50	0.14±0.04	97.67±7.00	89.45±7.16
	25	0.21±0.07	95.28±3.32	91.00±6.35
	12.5	0.53±0.22	93.19±2.34	103.52±9.05
	6.25	0.76±0.26	88.98±4.01	113.62±1.33
Persicaria decipiens	50	0.93±0.34	87.99±4.11	94.76±5.90
	25	0.90±0.27	88.48±3.97	105.10±1.13
	12.5	1.64±0.44	78.95±5.45	114.83±8.53
	6.25	3.01±0.68	61.37±3.76	121.64±6.23
Persicaria attenuata	50	0.62±0.15	92.07±5.04	66.55±2.70
	25	0.64±0.27	91.83±4.32	78.19±7.43
	12.5	1.56±0.48	79.94±4.87	81.79±4.12
	6.25	4.17±0.69	46.38±5.54	85.95±4.10
Persicaria acuminata	50	0.27±0.07	90.53±3.21	61.24±7.73
	25	0.44±0.03	88.30±3.75	73.57±1.16
	12.5	1.15±0.08	85.26±3.09	83.76±6.74
	6.25	3.29±0.40	57.77±1.55	88.69±8.02
Persicaria nepalensis	50	0.46±0.05	93.06±4.99	60.05±6.52
	25	0.58±0.08	92.57±7.11	75.81±7.72
	12.5	1.45±0.25	81.30±4.03	82.79±8.89
	6.25	3.88±0.52	50.10±.45	92.76±8.06
Persicaria senegalensis	50	0.80±0.45	89.72±7.65	70.62±6.66
	25	1.12±0.33	85.64±5.32	77.90±5.83
	12.5	1.48±0.35	80.93±6.04	83.90±9.27
	6.25	1.73±0.57	77.71±6.58	101.45±9.20
Persicaria setosula	50	0.25±0.07	92.78±7.17	57.00±5.19
	25	0.26±0.02	91.66±8.49	60.62±5.16
	12.5	0.88±0.17	88.73±4.63	62.83±4.87
	6.25	2.50±0.44	67.93±3.02	67.02±3.64
Quercetin	25	0.35±0.10	90.54±8.56	49.33±3.44
	12.5	0.30±0.08	86.16±7.21	60.69±2.11
	6.25	0.69±0.05	71.08±5.01	73.76±5.21
	3.12	2.50±0.48	67.93±5.43	73.10±4.12

NO: Nitric oxide

particularly at higher concentrations, indicating their potential as anti-inflammatory agents. The lowest inhibition of NO production was observed with *Persicaria attenuata,* showing a percentage of 46.38%. Each species demonstrated a decrease in NO production that was directly proportional to the concentration of the extract used. This indicates that as the concentration of the extract increased, the inhibition of NO production became more pronounced.

Soybean 15-LOX and AChE Inhibition

Fig. 1 shows how extracts from different *Persicaria* species inhibit LOX and AChE activities, with quercetin and eserine as the respective reference compounds. Higher percentages indicate greater inhibition of the enzymes' activities, suggesting potential anti-inflammatory properties of the extracts.

Results of LOX inhibitory activity revealed that, five species *P. limbata*, *P. attenuata*, *P. acuminata*, *P. nepalensis*, and *P. setosul*a demonstrated relatively high percentages of lipoxygenase activity inhibition, ranging from 79.9% to 92.76%. The two other species *P. decipiens* and *P. senegalensis* showed moderate levels of LOX activity inhibition

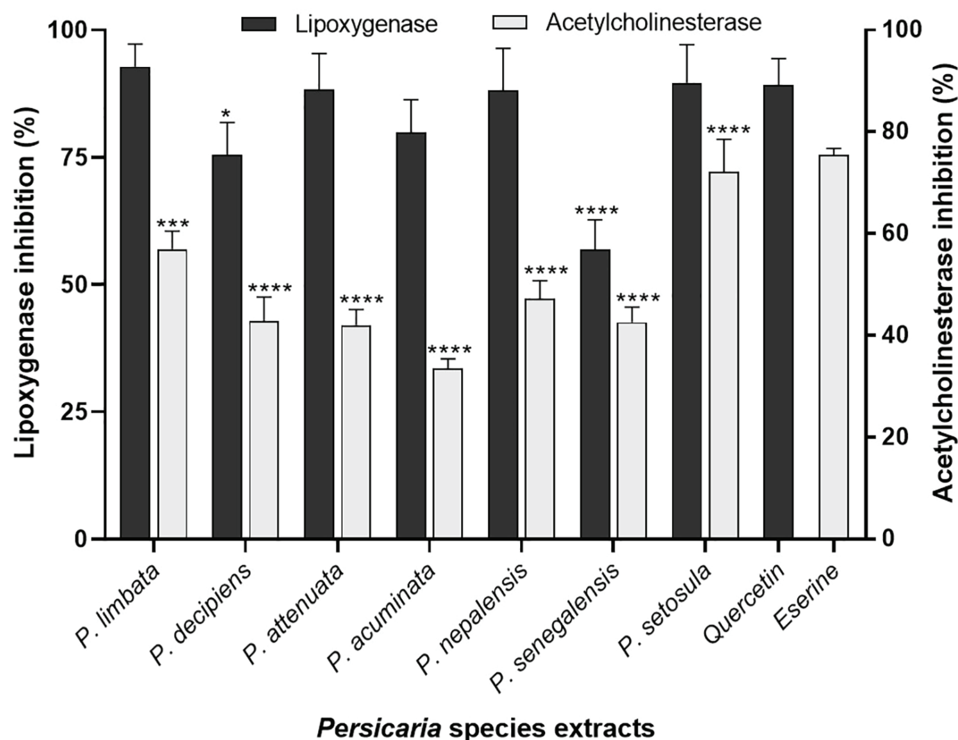

Fig. 1. Percentages of 15-LOX- and AChE-inhibitory activities of extracts from selected *Persicaria* species. For the 15-LOX assay, extracts were tested at a single concentration of 200 µg/mL while quercetin was tested at 25 µg/mL. For the AChE inhibitory activity, extracts were tested at a single concentration of 500 µg/mL and eserine at 10 µg/mL. The values represent the means of experiments conducted in triplicate (n=3)±standard deviation. Statistical analysis was carried out using two-way ANOVA with Dunnett's multiple comparison tests. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$

(75.51% and 56.84%, respectively). They still exhibit significant inhibition compared to the control group, with a noteworthy difference at $p<0.05$ for *P. decipiens*. However, their inhibition percentages are comparatively lower than those of other *Persicaria* species and quercetin. Quercetin, a well-known flavonoid with potent anti-inflammatory properties, demonstrates a high rate of LOX activity inhibition (89.24%). Its inclusion in the table serves as a reference point, emphasizing its effectiveness in inhibiting LOX activity and its potential as an anti-inflammatory agent.

In terms of AChE inhibitory activity, six out of seven plants (*P. decipiens*, *P. attenuata*, *P. acuminata*, *P. nepalensis*, *P. senegalensis*, and *P. setosula*) exhibited moderate levels of inhibition, with percentages ranging from 33.51% to 56.93%. Although they showed some degree of inhibition, it was lower compared to eserine and *P. limbata* demonstrated the highest percentage of AChE activity inhibition (72.12%), indicating stronger inhibition than the other *Persicaria* species listed. Eserine, also known as physostigmine, is a reference compound used to benchmark the potency of other compounds in inhibiting AChE activity. It exhibited a high percentage of AChE activity inhibition (75.5%) with a significant difference compared to all the extracts ($p<0.001$ with *P. limbata* extract and $p<0.0001$ with others).

All samples that exhibited more than 50% inhibition were selected for a dose-response assay to determine their IC_{50} values. The results can be found in Table 2.

The IC_{50} value represents the concentration of the extract required to inhibit 50% of the enzyme activity. Lower IC_{50} values indicate higher potency, meaning that lower concentrations of the extract are needed to achieve the desired level of inhibition. *P. decipiens*, *P. attenuata*, *P. acuminata*, *P.*

Table 2: IC_{50} values of LOX and AChE inhibitory activity of extracts from *Persicaria* species.

Plant species	LOX IC_{50} (µg/mL)	AChE IC_{50} (µg/mL)
Persicaria limbata	39.25±5.93	346.70±15.20
Persicaria decipiens	85.66±9.44	>500
Persicaria attenuata	73.36±7.15	>500
Persicaria acuminata	81.69±9.08	>500
Persicaria nepalensis	73.39±6.81	>500
Persicaria senegalensis	112.76±13.01	>500
Persicaria setosula	71.46±1.84	439.21±25.08
Quercetin	15.85±0.18	nd
Eserine	nd	4.94±0.05

nd: not determined; AChE: Acetylcholinesterase; LOX: Lipoxygenase, IC_{50}: Inhibitory concentration 50

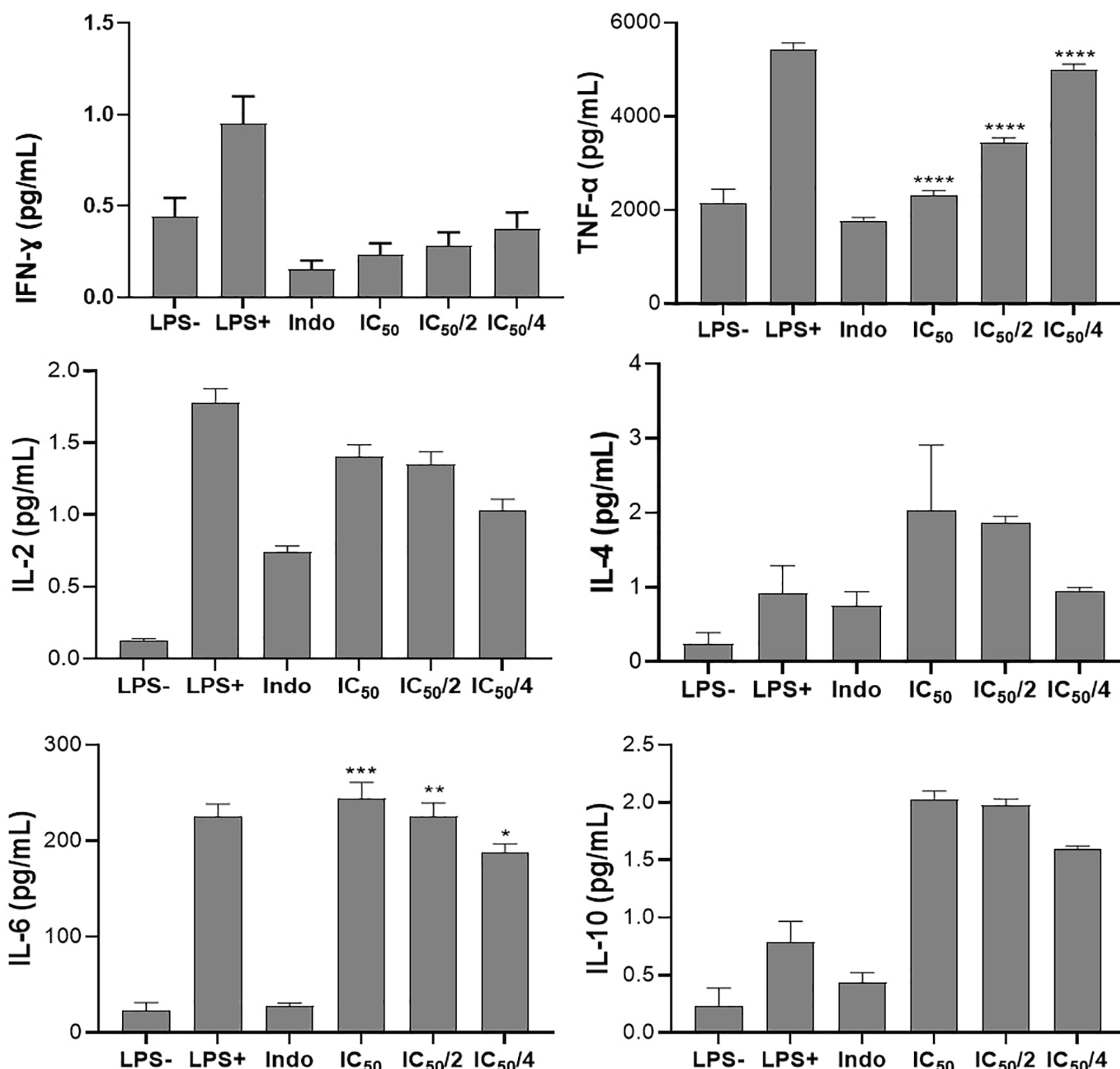

Fig. 2. Effect of methanol extracts from *P. limbata* on M1/M2 inflammatory cytokines in LPS-stimulated Raw 264.7 macrophages. Cells were treated for 24 hours at concentrations equal to the IC_{50}, $IC_{50}/2$, and $IC_{50}/4$ values against Raw 264.7 cells. Cytokine secretion levels were then measured. Indomethacin (Indo) was tested at 5 µg/mL. The values presented are the means of experiments performed in triplicate (n=3)±standard deviation. Statistical analysis was performed using two-way ANOVA with Dunnett's multiple comparison tests. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$

nepalensis, *P. senegalensis*, and *P. setosula* exhibited IC_{50} values greater than 500 µg/mL, while *P. limbata* showed an IC_{50} value of 346.70 µg/mL, indicating relatively higher potency compared to the other *Persicaria* species listed. In LOX activity, *P. limbata* had the lowest IC_{50} value (39.25 µg/mL) among the listed *Persicaria* species. Compared to quercetin (IC_{50} value of15.85 µg/mL), *P. decipiens*, *P. attenuata*, *P. acuminata*, *P. nepalensis* and *P. setosula* exhibited moderate IC_{50} values ranging from 71.46 µg/mL to 85.66 µg/mL while *P. senegalensis* showed the highest IC_{50} value (112.76 µg/mL).

Effect of Persicaria Species Extracts on the Modulation of M1/M2 Cytokines

Based on the results of NO, LOX, and AChE activities, the extract of *P. limbata* was found to be the most potent among the *Persicaria* species that were tested. As a result, *P. limbata* was chosen for further investigation of M1/M2 cytokines. The effects of methanol extracts on inflammatory cytokines in LPS-stimulated RAW 264.7 macrophages are presented in Fig. 2. The results of the effects of *P. limbata* extracts on pro-inflammatory cytokines showed that as the concentration of *P. limbata* extract increases the levels of INF-γ, TNF-α, and IL-2 decrease. Respective values of 0.37 pg/mL, 4987.762 pg/mL, and 1.03 pg/mL were obtained at the lowest concentration, while values of 0.234 pg/mL, 2316.16 pg/mL, and 1.406 pg/mL were obtained at the highest concentration. The inhibition of TNF-α by *P. limbata* extract at the three tested concentrations was statistically significant compared to indomethacin ($p<0.0001$).

In contrast to the effects on pro-inflammatory cytokines, the effects of *P. limbata* extracts on anti-inflammatory cytokines showed that higher concentrations of *P. limbata* extract lead to increased levels of IL-4, IL-6, and IL-10 cytokines, suggesting an enhanced anti-inflammatory response. Indomethacin, a well-known anti-inflammatory drug used as a positive control, reduced the levels of both pro- and anti-inflammatory cytokines compared to the untreated and stimulated control. In comparison to indomethacin, the inhibition of IL-6 by *P. limbata* extract at the three tested concentrations was statistically significant (∗∗∗$p<0.001$, ∗∗$p<0.01$ and ∗$p<0.05$ at IC_{50}, $IC_{50}/2$, and $IC_{50}/4$, respectively).

DISCUSSION

Nitric oxide (NO) serves as a potent signaling agent with significant implications in amplifying inflammatory processes and immune reactions (29). In cases of chronic inflammation, the inducible nitric oxide synthase (iNOS) enables ongoing and increased production of NO, resulting in an inflammatory reaction in various tissues and organs. (13). Plant extracts that have the ability to scavenge NO radicals or inhibit the activities of iNOS could potentially be used as templates for treating chronic inflammation (29). Previous studies have shown the effectiveness of plant extracts in regulating the synthesis and release of NO in macrophages that were stimulated with LPS in an in vitro setting (29). In our study, six plant leaf methanol extracts were tested and demonstrated dose-dependent inhibition of NO from LPS-induced macrophages in vitro (Table 1). This observation was crucial as it validated the scavenging activities of the tested leaf methanol extracts. Furthermore, the methanol extracts of *P. limbata* had the highest NO inhibition of 97.67%, followed by *P. nepalensis* (93.06%), and *P. setosula* (92.78%), respectively at a concentration of 50 µg/mL. The NO inhibition exhibited by the plant extracts was directly proportional to the decreased NO release by the LPS-stimulated macrophages. This work further supports previous claims that the plants' leaf extract contained secondary metabolites capable of inhibiting NO release in LPS-activated cells (29). The findings presented reinforce the idea that secondary compounds derived

from *Persicaria* species could effectively act as natural inhibitors of inducible nitric oxide synthase (iNOS) (30). Furthermore, these compounds can potentially be used as prototypes for developing new therapeutic approaches to reduce excessive nitric oxide (NO) production. In a study conducted by Tsai et al., a particular compound isolated from *P. multiflorum* showed a suppressive effect on NO release from cells activated by lipopolysaccharides (LPS), depending on the dosage administered (30). Similarly, the leaf extracts of *P. odoratum* using dichloromethane demonstrated an inhibitory effect on NO in LPS-activated cells, also dependent on the dose used (31). The observed bioactivity of the plant extracts appeared to be primarily concentrated in organic solvents such as methanol, n-hexane, and dichloromethane. This observation is not unexpected as these organic solvents are known for their efficacy as lipid-soluble agents, and numerous anti-inflammatory agents exert their effects primarily through lipid peroxidation (32).

In the pro-inflammatory enzyme assays, the methanol extracts of *P. setosula* showed the highest enzyme inhibitory activity (LOX=89.59%, AChE=72.12%), followed by *P. limbata* (92.76%, 56.93%) and *P. nepalensis* (88.16%, 47.17%) respectively (Fig. 1). These results support earlier studies on the potency of extracts derived from *Persicaria* species in their anti-inflammatory activities (33, 34). Yu et al's (33) research findings showed that the compounds they extracted had significant anti-inflammatory effects. These compounds were able to decrease the synthesis and release of substances such as nitric oxide, tumor necrosis factor-α, interleukin-6, and monocyte chemotactic protein-1. In our initial study, we tested the extracts at concentrations of 128 µg/mL for lipoxygenase inhibition and 500 µg/mL for acetylcholinesterase inhibition in order to determine the percentage of enzyme inhibition, as illustrated in Fig. 1. Plant extracts that have shown activity against LOX and AChE are potential candidates for treating the chronic inflammation observed in Alzheimer's disease. Enzyme inhibitors and nanoproducts are current therapeutic strategies to alleviate symptoms associated with chronic inflammation of brain cells in Alzheimer's disease (35). However, complications such as brain swelling, confusion, headaches, dizziness, and falls are some of the factors that have limited the achievement of therapeutic goals (36). Dual inhibitors of LOX and AChE with lower cytotoxicity could serve as targeted treatment options for individuals with chronic inflammation of the nerve cells occurring in Alzheimer's disease.

The extracts that demonstrated more than 50% enzyme inhibition were selected to determine the IC_{50} in a subsequent dose-response experiment. The findings showed that *P. limbata* extracts demonstrated the most effective anti-inflammatory properties overall, with an IC_{50} of 39.25 µg/mL for LOX and 439.21 for AChE. This was followed by *P. setosula* with an IC_{50} of 71.46 for LOX and 346.70 for AChE, and *P. attenuata* with an IC_{50} of 73.36 for LOX and >500 for AChE (Table 2). The results further validated the activities of *P. limbata* extracts shown in Fig. 1 in agreement with a study reported in the literature (37). Dzoyem and collaborators (37) found that the methanol extracts from P. *limbata* contained unique flavonoids that were only found in the species. This may explain the observed biological activity. To further investigate the bioactivity of the methanol extracts of *P. limbata* on inflammatory mediators and explore the possible target(s) of its action. The extracts were tested for their ability to inhibit/release signaling molecules (cytokines) involved in the activation, sustenance, and resolution of inflammation. The findings showed that the methanol extracts of *P. limbata* had a noticeable, dosage-dependent effect on suppressing the expression of IFN-ɣ and TNF-α, while also increasing the release of IL-2, IL-4, IL-6, and IL-10, as depicted in Fig. 2. The methanol extracts of *P. limbata*

showed significant dose-dependent inhibition of TNF-α and warrant further exploration. Similarly, IL-6 is a versatile cytokine that serves both pro-inflammatory and anti-inflammatory functions (38). It stimulate the production of acute phase proteins and facilitate the final stage of B cell differentiation, resulting in T cell activation andantibody production and (38). The anti-inflammatory role of IL-6 is important in B-cell maturation and differentiation in the humoral immune response (39). Methanol extracts from *P. limbata* increased the production of IL-6, IL-2, IL-4, and IL-10 in a dose-dependent manner compared to the anti-inflammatory agent indomethacin. It is well known that flavonoids are the primary chemical components found in the genus *Persicaria* (4, 40). *P. limbata* has been previously reported to contain flavonoid compounds with anti-inflammatory activity (7, 37). Therefore, the activity of *P. limbata* observed here might be related to its richness in flavonoid constituents. Moreover, the observed activity is desirablefor promoting the mitigation of inflammatory action and slowing down or eliminating the progression of chronic ailments.

CONCLUSION

Plant extracts have demonstrated the ability to reduce the secretion of cytokines by LPS-induced macrophages in vitro. Our research provides additional evidence supporting the traditional medicinal use of extracts derived from *Persicaria* species as remedies for inflammation and pain. Specifically, the potential of *P. limbata* extracts warrants further investigation as potential candidate for botanical medicine to address chronic inflammation. The significant inhibitory effect of the extracts on factors contributing to chronic inflammation suggests the presence of anti-inflammatory agents in the methanol extracts. It is essential to continue efforts to isolate, identify, and characterize the bioactive components responsible for these properties.

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AUTHORS' CONTRIBUTION

Conceptualization of this research idea, methodology development, experiments, data collection, data analysis, editing, interpretation, writing of the original draft and final revision were performed by Adamu Imam Isa.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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