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NYPA FRUTICANS WURMB LEAF FRACTIONS ENHANCE INNATE AND ADAPTIVE IMMUNE RESPONSES: IMMUNOMODULATORY EVALUATION AND LC-MS CHARACTERIZATION OF BIOACTIVE

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ABSTRACT

This study investigated the immunomodulatory activity of *Nypa fruticans* Wurmb leaf fractions and characterised their major bioactive constituents using LC–MS. The ethanolic leaf extract was successively fractionated with *n*-hexane, ethyl acetate, and ethanol, and each fraction was orally administered to male mice for 14 days. Immunomodulatory activity was evaluated through macrophage phagocytic activity, serum IL-12 and IFN-γ levels, and the expression of $CD4^+$, $CD8^+$, and $CD14^+$ markers. All fractions significantly enhanced immune responses compared with the negative control. The ethanol and ethyl acetate fractions showed the most consistent effects on cytokine production and lymphocyte modulation, whereas the *n*-hexane fraction produced the highest macrophage phagocytic activity. LC–MS profiling revealed that the active fractions were enriched with phenolic and flavonoid-related compounds, including emodin-8-*O*-glucoside, quercitrin, and 2,4-dihydroxybenzaldehyde, which may contribute to the observed immunostimulatory effects. These findings provide fraction-level scientific evidence supporting *N. fruticans* as a promising source of plant-derived immunomodulatory agents and help bridge traditional medicinal use with modern pharmacological and phytochemical validation. The study also offers a foundation for future investigations on compound isolation, mechanism of action, standardisation, and safety evaluation. Practically, phenolic-rich fractions of *N. fruticans* may be further developed as candidates for standardised herbal immunomodulators or supportive immune-enhancing therapies.

Keywords: *Nypa fruticans* Wurmb, Immunomodulatory Activity, Macrophage Phagocytosis, IL-12, IFN-γ, LC–MS, Phenolic Compounds, Flavonoids.

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INTRODUCTION

The immune system plays a pivotal role in protecting the host against pathogenic microorganisms and malignant cells through coordinated innate and adaptive responses. Disturbances in immune regulation may lead to chronic infection, autoimmune disease, or cancer, so agents capable of modulating immune function, known as immunomodulators, are increasingly explored to restore homeostasis and to support conventional therapy. Natural immunomodulators can act either as immunostimulants, enhancing host defence, or as immunosuppressants in conditions where excessive immune activation is detrimenta.[1–3] Medicinal plants represent one of the most important sources of immunomodulatory agents. They are rich in structurally diverse secondary metabolites such as flavonoids, terpenoids, saponins, alkaloids, tannins, and other polyphenols that may target multiple signalling pathways in immune cells.[1–3] Compared with synthetic immunomodulatory drugs, which can cause adverse effects, plant-derived products are widely explored as alternative sources of safer and more accessible therapeutic candidates, particularly in developing countries.[2,3] At the same time, there has been renewed interest in natural products as leads for drug discovery because they occupy a unique and biologically relevant chemical space and have contributed substantially to modern therapeutics.[4] *Nypa fruticans* Wurmb, commonly known as nipa palm,

is a member of the family Arecaceae and is a characteristic mangrove palm distributed in estuarine and brackish water habitats in Southeast Asia, India, and parts of Oceania. Traditionally, different parts of this plant have been used by local communities to treat fever, headache, respiratory problems, hypertension, diabetes, and other ailments, while its sap is fermented to produce beverages and vinegar.[5,6] Phytochemical investigations have shown that *N. fruticans* contains a wide array of bioactive compounds, including phenolic compounds, flavonoids, alkaloids, tannins, saponins, steroids, triterpenoids, and other polyphenols, which may underlie its diverse pharmacological activities.[5-8] A growing body of evidence indicates that *N. fruticans* exhibits strong antioxidant properties in different plant parts, including fruits, leaves, young shoots, and sap, with high total phenolic and flavonoid contents and potent free-radical scavenging capacity.[7–10] Antioxidant activity can support immunomodulatory effects by protecting immune cells from oxidative damage and by regulating redox-sensitive signalling pathways.[1–3] In addition to antioxidant activity, *N. fruticans* extracts have demonstrated antimicrobial, cytoprotective, anti-inflammatory, and other pharmacological effects, suggesting a broad spectrum of bioactivity relevant for host defence.[5,8,9,11] More recently, several studies have provided direct evidence of the immunomodulatory potential of *N. fruticans*. *N. fruticans* palm vinegar has been reported to influence macrophage phagocytic activity, while the hot-water extract of *N. fruticans* improved immune parameters in a forced-swimming-induced immune-compromised mouse model.[12,13] In vitro data also showed anti-inflammatory effects of the ethyl acetate fraction of *N. fruticans* in macrophages through modulation of nuclear factor-κB (NF-κB), Akt/IκB, and mitogen-activated protein kinase (MAPK) signalling pathways[11]. These findings support the ethnomedicinal use of *N. fruticans* and highlight it as a promising source of plant-based immunomodulators.[5,11–13] However, most available studies have focused on crude extracts, vinegar, shoots, fruits, or selected fractions of *N. fruticans*, and have not systematically explored how different solvent fractions of the leaves, enriched in compounds of varying polarity, contribute to its immunomodulatory effects.[9,11–13] From a drug discovery perspective, moving from crude extracts to more refined fractions is a critical step for enriching bioactive constituents, improving biological selectivity, and facilitating subsequent isolation of active compounds. Fractionation allows the separation of complex mixtures into groups of compounds with similar physicochemical properties, which can then be evaluated individually for biological activity.[14,15] Despite the recognised pharmacological potential of *N. fruticans*, the immunomodulatory activity of its leaf fractions has not been comprehensively evaluated, and there is limited information on which fractions are most responsible for stimulating innate and adaptive immune responses. Therefore, a clear research gap remains in understanding the fraction-specific immunomodulatory effects of N. fruticans leaves and linking these effects with their chemical profiles. Addressing this gap is important because fraction-level analysis can help identify the most active phytochemical groups, support the discovery of bioactive constituents, and provide a stronger scientific basis for the development of standardised natural immunomodulators. Detailed phytochemical characterisation is also essential to establish a chemical basis for the observed biological effects. Classical phytochemical screening and chromatographic techniques, such as thin-layer chromatography, provide preliminary information about metabolite classes present in plant extracts and fractions.[14] In recent years, liquid chromatography coupled with mass spectrometry (LC-MS or LC-MS/MS) has become a powerful tool for metabolite profiling of complex natural extracts and herbal matrices.[16,17] LC-MS-based approaches enable rapid detection, dereplication, and structural annotation of numerous constituents in a single run, thereby accelerating the identification of marker compounds that correlate with biological activity.[16,17] Applying LC-MS to bioactive fractions of *N. fruticans* is therefore expected to reveal candidate immunomodulatory molecules and to support rational standardisation of the plant material. On this basis, the present study was designed to evaluate the immunomodulatory activity of *N. fruticans* Wurmb leaf fractions through phagocytic, cytokine, and lymphocyte subset parameters, and to characterise the bioactive compounds present in the most active fractions using LC-MS analysis. By integrating fraction-level biological evaluation with LC-MS-based chemical profiling, this work aims to fill the existing research gap between crude-extract studies and compound-oriented investigations, and to strengthen the scientific evidence for *N. fruticans* as a source of immunomodulatory agents. This study is also aligned with Sustainable Development Goal 3, Good Health and Well-being, because it explores a locally available mangrove medicinal plant as a potential source of safe and accessible

immunomodulatory agents that may support future health-promoting interventions. By investigating both biological activity and chemical composition, this work contributes to the broader effort to develop evidence-based natural products for improving immune health and supporting the sustainable use of medicinal plant resources.

EXPERIMENTAL

Chemicals and Reagents

Ethanol 96%, *n*-hexane, and ethyl acetate of analytical grade were obtained from Merck, Darmstadt, Germany. Carboxymethyl cellulose sodium (Na-CMC) was used as a suspending agent. Stimuno® (Dexa Medica, Indonesia) served as the positive control immunomodulator. Giemsa stain, methanol, and phosphate-buffered saline (PBS) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Enzyme-linked immunosorbent assay (ELISA) kits for IL-12 and IFN-γ were obtained from eBioscience, San Diego, CA, USA. Monoclonal antibodies or ELISA kits for CD4, CD8, and CD14 were obtained from BD Biosciences, San Jose, CA, USA. LC-MS-grade methanol, acetonitrile, and formic acid were used for chromatographic analysis.

Plant Material and Fraction Preparation

Fresh leaves of *Nypa fruticans* Wurmb were collected from mangrove areas in Southeast Sulawesi, Indonesia, and taxonomically authenticated by a botanist. A voucher specimen was deposited in the university herbarium. The leaves were washed, air-dried at room temperature, and pulverised into a fine powder. Approximately 500 g of powdered leaves were macerated with 96% ethanol for three consecutive 24-h cycles. The combined filtrates were concentrated under reduced pressure at 50 °C using a rotary evaporator to obtain a crude ethanol extract. Fractionation of the crude extract was performed by liquid–liquid partitioning using *n*-hexane, ethyl acetate, and ethanol to obtain non-polar, semi-polar, and polar fractions, respectively, following standard phytochemical extraction and fractionation procedures[18]. The choice of solvent system and fractionation scheme was supported by previous phytochemical and pharmacological studies reporting that *N. fruticans* contains phenolic compounds, flavonoids, alkaloids, tannins, saponins, steroids, triterpenoids, and other bioactive secondary metabolites.[19,20] All fractions were stored at –20 °C in airtight vials until further analysis.

Experimental Animals

Male Swiss albino mice (*Mus musculus*), 8–10 weeks old and weighing 20–25 g, were obtained from the Animal Research Center. Animals were acclimatised for at least one week and maintained under standard laboratory conditions at 25 ± 2 °C with a 12-h light/dark cycle and free access to standard pellet diet and water *ad libitum*. All experimental procedures were carried out in accordance with institutional and international guidelines for the care and use of laboratory animals.[21,22] The study protocol was approved by the Institutional Animal Ethics Committee with ethical approval No. 009/KEP/UMW/III/2024.

Treatment Protocol

Mice were randomly allocated into six groups, with six animals in each group. The negative control group received 0.5% Na-CMC suspension, while the positive control group received Stimuno® at a dose of 0.13 mg/kg body weight. The remaining groups were treated orally with crude ethanol extract of *N. fruticans* leaves, *n*-hexane fraction, ethyl acetate fraction, or ethanol fraction once daily for 14 consecutive days. All test samples were freshly prepared in 0.5% Na-CMC before administration. The treatment duration and biological parameters were selected by considering previous studies on plant-derived immunomodulators and *N. fruticans*, including reports showing that *N. fruticans* extracts or fractions may affect macrophage activation, cytokine production, antioxidant activity, and inflammatory signalling pathways.[23–25]

Fractions selected for further chemical characterisation were determined based on their overall immunomodulatory responses, including macrophage phagocytic activity, IL-12 and IFN-γ levels, and $CD4^+$, $CD8^+$, and $CD14^+$ expression. Fractions showing the highest biological responses were considered the most active and subjected to LC-MS analysis, consistent with the principle of activity-guided fractionation in natural product discovery.[26]

Phagocytic Activity Assay

On day 15, each mouse received 0.1 mL Bacillus Calmette–Guérin (BCG) antigen intraperitoneally. Three hours after BCG administration, peritoneal macrophages were harvested by lavage with cold RPMI-1640 medium. The cell suspension was centrifuged, and the pellet was resuspended and smeared onto clean glass slides. Smears were air-dried, fixed with methanol, and stained with 10% Giemsa solution. The phagocytic index was determined by counting 100 macrophages per slide under a light microscope at 1000× magnification using immersion oil. The result was expressed as the percentage of macrophages containing phagocytosed particles. This assay was used as an indicator of innate immune response and macrophage activation in the evaluation of immunomodulatory activity.[23–25]

Cytokine and Lymphocyte Surface Marker Analysis

At the end of the treatment period, blood samples were collected from the retro-orbital plexus or by cardiac puncture under light anaesthesia. Blood was allowed to clot at room temperature, and serum was separated by centrifugation and stored at –20 °C until analysis. Serum concentrations of IL-12 and IFN-γ were quantified using commercial ELISA kits according to the manufacturer's instructions. IL-12 and IFN-γ were selected because these cytokines are important indicators of macrophage activation, Th1-type immune responses, and cellular immunity in immunomodulatory evaluation.[23–25] The expression levels of CD4, CD8, and CD14 surface markers were determined using ELISA-based assays or specific monoclonal antibody-based detection according to the kit protocol. These markers were used to evaluate helper T-cell-associated responses, cytotoxic T-cell-associated responses, and monocyte/macrophage-associated immune activation, respectively. Results were expressed as ng/mL based on calibration curves prepared from appropriate standards.

LC-MS Analysis of Bioactive Fractions

The most active fraction or fractions, as indicated by immunological parameters, were selected for LC-MS profiling. Approximately 10 mg of each selected fraction was dissolved in 1.0 mL of methanol, sonicated for 10 min, and filtered through a 0.22 μm PTFE membrane filter into LC vials before injection. LC-MS analysis was carried out using a high-performance liquid chromatography system coupled to an electrospray ionisation mass spectrometer (LC-ESI-MS). Separation was achieved on a reversed-phase C18 column, for example, 150 × 2.1 mm, 3 μm, maintained at 30 °C. The mobile phase consisted of solvent A, water containing 0.1% formic acid, and solvent B, acetonitrile containing 0.1% formic acid. The flow rate was set at 0.3 mL/min using the following gradient program: 0–5 min, 5% B; 5–25 min, 5–60% B; 25–30 min, 60–95% B; 30–35 min, 95% B; followed by re-equilibration to initial conditions. The injection volume was 5–10 μL. These chromatographic conditions were adapted from common LC-MS workflows used for plant metabolite profiling and natural product dereplication.[27] The mass spectrometer was operated in both positive and negative ionisation modes over an *m/z* range of 100–1000. Typical operating parameters included capillary voltage of approximately 3.5 kV, nebulising gas flow of 8–10 L/min, drying gas temperature of 300–350 °C, and fragmentor voltage adjusted to obtain informative fragmentation spectra. Data were acquired in full-scan mode, and, when available, data-dependent MS/MS was used to obtain product ion spectra of major peaks. Tentative identification of compounds was performed by comparing observed retention times, molecular ions such as $[M+H]^+$ and $[M–H]^-$, and fragment ion patterns with literature data, focusing on phenolic acids, flavonoids, and other polyphenolic constituents previously reported in *N. fruticans* and related plant materials.[19,20,27] The relative abundance of each component was estimated from peak areas in extracted ion chromatograms.

RESULTS AND DISCUSSION

LC–MS Profiling of *Nypa fruticans* Leaf Fractions

Ethanol Fraction

LC-MS analysis of the ethanol fraction of *Nypa fruticans* leaves showed a heterogeneous mixture of relatively polar compounds, dominated by phenolic- and flavonoid-type constituents such as quercitrin, emodin-8-*O*-glucoside, and 2,4-dihydroxybenzaldehyde, together with several nitrogen-containing molecules. These classes of metabolites are widely associated with antioxidant, anti-inflammatory, and

immunomodulatory properties; therefore, their presence provides a plausible chemical basis for the immunostimulatory effects observed in the *in vivo* assays of this study.[28–31]

Table-1: Compounds Tentatively Identified in the Ethanol Fraction of ***Nypa fruticans*** Leaf Extract by LC–MS

Peak (Cpd)	Tentative compound	Molecular formula	RT (min)	Measured mass (m/z)*	CAS No.	Relative abundance (%)
4	(Heptafluoropropyl)trimethylsilane	$C_6H_9F_7Si$	0.513	242.0342	3834-42-2	3.23
6	Sucrose	$C_{12}H_{22}O_{11}$	0.562	342.1163	57-50-1	4.96
16	N-Ethylaniline	$C_8H_{11}N$	1.840	121.0891	103-69-5	3.19
28	Niceritrol	$C_{29}H_{24}N_4O_8$	3.000	556.1608	1449-40-7	10.63
31	Altenusin	$C_{15}H_{14}O_6$	3.244	290.0793	31186-12-6	10.04
32	2,4-Dihydroxybenzaldehyde	$C_7H_6O_3$	3.255	138.0319	95-01-2	12.29
42	Quercitrin	$C_{21}H_{20}O_{11}$	3.854	448.1007	522-12-3	8.81
48	Emodin 8-glucoside	$C_{21}H_{20}O_{10}$	4.225	432.1059	23313-21-5	4.67
50	4-tert-Butylcyclohexyl acrylate	$C_{13}H_{22}O_2$	4.622	210.1622	84100-23-2	4.44
54	Pacrinidol	$C_{23}H_{28}N_2O_4$	5.777	396.2047	65655-59-6	3.45
56	Pemerid	$C_{15}H_{32}N_2O$	6.031	256.2520	50432-78-5	3.25
64	DEET (N,N-diethyl-m-toluamide)	$C_{12}H_{17}NO$	6.857	191.1311	134-62-3	2.32
65	N-Lauryldiethanolamine	$C_{16}H_{35}NO_2$	7.053	273.2670	1541-67-9	4.44
110	HT-2 toxin	$C_{22}H_{32}O_8$	11.369	424.2098	26934-87-2	8.44
124	Irinotecan	$C_{33}H_{38}N_4O_6$	13.024	586.2818	97682-44-5	15.84

Emodin-8-*O*-glucoside is an anthraquinone glycoside that has been reported to stimulate macrophage immune responses more strongly than its aglycone form, emodin. In RAW264.7 murine macrophages and THP-1 human monocytic cells, emodin-8-*O*-glucoside enhanced phagocytic activity and increased the production of inflammatory mediators through activation of TLR-2/MAPK/NF-κB signalling pathways.[28] The presence of emodin-8-*O*-glucoside in the ethanol fraction therefore supports the enhanced phagocytic response and cytokine production observed in treated mice. Activation of macrophage-related signalling pathways is consistent with increased IL-12 production, which may subsequently promote Th1-type immune responses and IFN-γ production. Another important constituent identified in the ethanol fraction was 2,4-dihydroxybenzaldehyde, a phenolic aldehyde reported to have anti-inflammatory, anti-angiogenic, and anti-nociceptive activities. Jung et al. showed that 2,4-dihydroxybenzaldehyde inhibited angiogenesis in the chick chorioallantoic membrane assay and reduced inflammatory responses in mouse models of vascular permeability and carrageenan-induced air-pouch inflammation.[29] These properties suggest that 2,4-dihydroxybenzaldehyde and related phenolic compounds may contribute to the regulation of inflammatory processes during immune activation. In the context of the present study, phenolic aldehydes in the ethanol fraction may help protect immune cells from oxidative stress while supporting balanced macrophage activation. Quercitrin, a rhamnoside derivative of quercetin, was also tentatively identified in the ethanol fraction. Quercitrin has been reported to ameliorate lupus nephritis-like disease in a murine systemic lupus erythematosus model by suppressing $CD4^+$ T-cell activation and reducing macrophage-mediated inflammatory responses.[30] In addition, quercetin and its glycosides are widely recognised as bioflavonoids with antioxidant, anti-inflammatory, and immunomodulatory activities, including regulation of cytokine production and modulation of inflammatory signalling pathways.[31] These findings are relevant to the present study, in which the ethanol fraction increased IL-12 and IFN-γ levels as well as $CD4^+$ and $CD8^+$ T-cell expression. Thus, quercitrin and related quercetin glycosides may contribute to the modulation of both innate and adaptive immune responses. Collectively, the detection of emodin-8-*O*-glucoside, 2,4-dihydroxybenzaldehyde, quercitrin, and other phenolic constituents supports a synergistic mechanism underlying the immunomodulatory activity of the ethanol fraction. Anthraquinone glycosides such as emodin-8-*O*-glucoside may directly activate macrophages and promote phagocytosis and cytokine production.[28] Phenolic aldehydes such as 2,4-dihydroxybenzaldehyde may provide anti-inflammatory and antioxidant support.[29] Meanwhile, flavonoid glycosides such as quercitrin may modulate T-cell activation

and macrophage-mediated immune responses.[30,31] This phytochemical profile is consistent with the observed enhancement of macrophage phagocytic activity, increased IL-12 and IFN-γ levels, and increased expression of $CD4^+$ and $CD8^+$T-cell subsets in the *in vivo* experiments. It is noteworthy that the LC-MS library search also suggested the presence of compounds such as HT-2 toxin and irinotecan. These compounds are unlikely to be genuine phytochemical constituents of *N. fruticans* leaves. HT-2 toxin is a type-A trichothecene mycotoxin associated with toxic effects in humans and animals, including immunotoxicity, while irinotecan is known as a synthetic anticancer drug rather than a plant secondary metabolite.[32] Therefore, these matches should be interpreted cautiously because they may reflect limitations of automated database matching, similarity of mass fragments, or possible contamination during sample preparation or analysis. Consequently, biological interpretation in this study should focus primarily on phytochemically plausible constituents, especially phenolic- and flavonoid-type compounds supported by previous pharmacological evidence. Overall, the LC-MS data indicate that the ethanol fraction of *N. fruticans* leaves contains bioactive phenolics, flavonoids, and anthraquinone glycosides compatible with the immunostimulatory pattern observed *in vivo*. These findings suggest that the ethanol fraction may serve as a promising source of phytochemical-rich immunomodulatory agents. However, further targeted LC-MS/MS analysis, purification, and compound isolation are required to confirm the structures and individual contributions of key compounds such as emodin-8-*O*-glucoside, quercitrin, and 2,4-dihydroxybenzaldehyde.

Ethyl Acetate Fraction

LC-MS analysis of the ethyl acetate fraction of *Nypa fruticans* leaves revealed a complex mixture of polar and semi-polar constituents. The most abundant peaks corresponded to *N*-lauryldiethanolamine, irinotecan, hematoporphyrin, altenusin, quercitrin, myo-inositol, 2,4-dihydroxybenzaldehyde, isomaltulose, and emodin-8-*O*-glucoside, together with smaller amounts of ionone, niceritrol, and *N*-ethylaniline. This profile indicates that the ethyl acetate fraction contains not only phenolic and flavonoid-type metabolites, but also carbohydrate-based molecules and several nitrogen-containing compounds. Several of the identified components belong to phytochemical classes that are widely associated with antioxidant, anti-inflammatory, and immunomodulatory properties.

Table-2: Compounds Identified in the Ethyl Acetate Fraction of ***Nypa fruticans*** Leaf Extract by LC–MS

Peak (Cpd)	Tentative compound	Molecular formula	RT (min)	Measured mass (m/z)*	CAS No.	Relative abundance (%)
4	(Heptafluoropropyl)trimethylsilane	$C_6H_9F_7Si$	0.503	242.0340	3834-42-2	3.19
5	Myo-inositol	$C_6H_{12}O_6$	0.526	180.0633	87-89-8	6.12
6	Isomaltulose	$C_{12}H_{22}O_{11}$	0.573	342.1160	13718-94-0	2.80
14	N-Ethylaniline	$C_8H_{11}N$	1.835	121.0891	103-69-5	3.31
23	Niceritrol	$C_{29}H_{24}N_4O_8$	3.011	556.1607	1449-40-7	5.59
25	Altenusin	$C_{15}H_{14}O_6$	3.250	290.0793	31186-12-6	7.57
26	2,4-Dihydroxybenzaldehyde	$C_7H_6O_3$	3.258	138.0319	95-01-2	5.91
36	Quercitrin	$C_{21}H_{20}O_{11}$	3.896	448.1006	522-12-3	7.18
42	Emodin 8-glucoside	$C_{21}H_{20}O_{10}$	4.225	432.1058	23313-21-5	4.23
46	Ionone (β-ionone-like)	$C_{13}H_{20}O$	4.625	192.1518	8013-90-9	3.44
47	4-tert-Butylcyclohexyl acrylate	$C_{13}H_{22}O_2$	4.640	210.1621	84100-23-2	3.42
63	N-Lauryldiethanolamine	$C_{16}H_{35}NO_2$	7.086	273.2670	1541-67-9	14.88
103	DIBP / Diisobutyl phthalate	$C_{16}H_{22}O_4$	10.874	278.1518	84-69-5	5.41
117	Irinotecan	$C_{33}H_{38}N_4O_6$	13.029	586.2814	97682-44-5	16.40
122	Hematoporphyrin	$C_{34}H_{38}N_4O_6$	13.401	598.2820	14459-29-1	10.55

Emodin-8-*O*-glucoside has been shown to prime macrophages more strongly than its aglycone, emodin, by enhancing phagocytic activity and increasing the production of TNF-α, IL-6, and nitric oxide through activation of TLR-2/MAPK/NF-κB signalling pathways.[28] Likewise, 2,4-dihydroxybenzaldehyde exhibits

significant anti-inflammatory and anti-angiogenic activities *in vivo*, including reduction of vascular permeability and leukocyte infiltration in experimental inflammatory models.[29] The presence of these compounds suggests that the ethyl acetate fraction may support macrophage activation while also contributing to the regulation of inflammatory processes. Quercitrin, a glycoside derivative of quercetin, was also detected in the ethyl acetate fraction. Quercitrin has been reported to attenuate lupus nephritis-like disease in a murine systemic lupus erythematosus model by inhibiting $CD4^+$ T-cell activation and macrophage-mediated inflammatory responses.[30] In addition, quercetin and its derivatives are known to possess broad antioxidant, anti-inflammatory, and immunomodulatory activities, including suppression of NF-κB activation and modulation of cytokine production.[31] These effects are relevant to the present study, in which the ethyl acetate fraction enhanced macrophage phagocytic activity, increased IL-12 and IFN-γ production, and modulated $CD4^+$ and $CD8^+$ T-cell expression. Altenusin was another notable compound detected in the ethyl acetate fraction. Altenusin is a microbial biphenyl metabolite that has been identified as a non-steroidal farnesoid X receptor agonist and reported to attenuate non-alcoholic fatty liver disease through regulation of lipid metabolism and inflammatory pathways.[33] Although altenusin is more commonly described as a microbial or fungal metabolite rather than a typical higher-plant constituent, its detection may be relevant because mangrove-associated plants can harbour diverse endophytic microorganisms. Therefore, the presence of altenusin or altenusin-like compounds may indicate a possible contribution from plant-associated microbial metabolites. This finding should be interpreted cautiously and confirmed by targeted LC-MS/MS analysis or isolation studies. The fraction also contained myo-inositol and isomaltulose. Myo-inositol has been reported to reduce oxidative stress and inflammatory mediator release in human endothelial cells exposed to hyperglycemic conditions, indicating its potential role in maintaining redox balance and vascular homeostasis.[34] Although carbohydrate-derived molecules such as myo-inositol and isomaltulose may not directly stimulate immune cells in the same manner as flavonoids or anthraquinone glycosides, they may support immune function indirectly by reducing oxidative stress, maintaining cellular energy balance, and protecting tissues during inflammatory activation. This supportive effect may contribute to a more balanced immune response in treated animals. Ionone-type apocarotenoids were also detected in the ethyl acetate fraction. β-Ionone and its derivatives have been associated with antioxidant and anti-inflammatory activities. In particular, 3-hydroxy-β-ionone has been reported to reduce inflammatory responses in endothelial cell models by inhibiting the IκB-α/NF-κB signalling pathway and decreasing monocyte transendothelial migration.[35] The detection of an ionone-related peak in the ethyl acetate fraction therefore provides an additional possible explanation for the modulation of cytokine production and macrophage function observed in the *in vivo* experiments. By contrast, some high-abundance LC-MS library hits, such as *N*-lauryldiethanolamine, di-isobutyl phthalate, irinotecan, and hematoporphyrin, should be interpreted with caution. These compounds are more plausibly associated with surfactants, plasticisers, laboratory contaminants, or pharmacological agents than with genuine plant metabolites. Hematoporphyrin and related porphyrin derivatives are widely known as photosensitizers used in photodynamic therapy, rather than typical constituents of mangrove palms.[36] Similarly, irinotecan is a semi-synthetic anticancer drug, and di-isobutyl phthalate is commonly associated with plastic materials. Therefore, the appearance of these compounds in LC-MS results may reflect automated library misassignment, similarity of mass fragments, background contamination, or sample-handling artefacts. For this reason, the biological interpretation of the ethyl acetate fraction should focus primarily on phytochemically plausible compounds, especially phenolics, flavonoid glycosides, anthraquinone glycosides, apocarotenoids, and carbohydrate-derived metabolites. Overall, the LC-MS profile indicates that the ethyl acetate fraction of *N. fruticans* leaves is rich in bioactive phenolic aldehydes, flavonoid glycosides, anthraquinone glycosides, apocarotenoids, and carbohydrate-related compounds. These classes of metabolites provide a coherent mechanistic explanation for the enhanced macrophage phagocytic activity, increased IL-12 and IFN-γ production, and modulation of T-cell subsets observed in the *in vivo* part of the study.[28–31,33–35] Further targeted LC-MS/MS analysis, purification, and isolation of key constituents such as emodin-8-*O*-glucoside, quercitrin, 2,4-dihydroxybenzaldehyde, myo-inositol, and ionone-related compounds are needed to confirm their structures and clarify their individual contributions to the overall immunomodulatory activity of the ethyl acetate fraction.

n-Hexane Fraction

LC-MS analysis of the *n*-hexane fraction of *Nypa fruticans* leaves showed a profile dominated by lipophilic compounds, including surfactant-type alkanolamines such as triisopropanolamine and *N*-lauryldiethanolamine, aromatic ester compounds such as methyl cinnamate, quinine ethylcarbonate, steroid-like molecules including norepiandrosterone and 17β-hydroxyandrost-3-one, and several LC-MS library hits corresponding to HT-2 toxin, irinotecan, hematoporphyrin, AB-CHMICA, and decamethonium. This chemical profile suggests that the *n*-hexane fraction contains a mixture of possible plant-derived non-polar constituents, environmental or fungal contaminants, and xenobiotic compounds that may have originated from analytical background, sample handling, or automated library misassignment.

Table-3: Compounds Identified in the n-Hexane Fraction of ***Nypa fruticans*** Leaf Extract by LC–MS.

Cpd	Compound name	Molecular formula	RT (min)	[M+H](^+) (m/z)	CAS number	Relative abundance (%)
6	Triisopropanolamine	$C_9H_{21}NO_3$	0.748	191.1523	122-20-3	1.58
19	AB-CHMICA	$C_{21}H_{29}N_3O_2$	3.469	355.2259	2219330-90-0	2.07
39	Quinine ethylcarbonate	$C_{23}H_{28}N_2O_4$	6.330	396.2051	83-75-0	1.88
43	N,N-Dibutylamphetamine	$C_{17}H_{29}N$	6.681	247.2302	59313-94-9	1.30
45	BHA (3-tert-Butyl-4-hydroxyanisole)	$C_{11}H_{16}O_2$	6.970	180.1151	121-00-6	2.48
47	N-Lauryldiethanolamine	$C_{16}H_{35}NO_2$	7.121	273.2669	1541-67-9	22.57
75	Decamethonium	$C_{16}H_{38}N_2$	9.093	258.3060	156-74-1	1.32
76	Norepiandrosterone	$C_{18}H_{28}O_2$	9.370	276.2090	10002-93-4	6.58
83	2,5-Dimethyl-2,5-di-(tert-butylperoxy)hexane	$C_{16}H_{34}O_4$	9.972	290.2459	78-63-7	5.33
86	HT-2 toxin	$C_{22}H_{32}O_8$	10.194	424.2098	26934-87-2	4.71
88	Methyl cinnamate	$C_{10}H_{10}O_2$	10.480	162.0680	103-26-4	5.08
93	Diisobutyl phthalate (DIBP)	$C_{16}H_{22}O_4$	10.885	278.1519	84-69-5	4.14
95	17β-Hydroxyandrost-3-one	$C_{19}H_{30}O_2$	11.150	290.2248	29873-50-5	3.57
110	Irinotecan	$C_{33}H_{38}N_4O_6$	13.011	586.2816	97682-44-5	26.23
115	Hematoporphyrin	$C_{34}H_{38}N_4O_6$	13.400	598.2818	14459-29-1	11.14

Among the identified compounds, methyl cinnamate is one of the most phytochemically plausible constituents. Methyl cinnamate is a cinnamic acid ester that occurs in several aromatic plants and has been associated with anti-inflammatory properties. Murakami et al. reported that methyl cinnamate showed anti-inflammatory activity in RAW264.7 macrophages with relatively lower cytotoxicity and lower pro-inflammatory effects than some related acrylate compounds.[37] Therefore, methyl cinnamate may be one of the more credible contributors to the biological effects of the *n*-hexane fraction, particularly through modulation of inflammatory mediator production in macrophage-related immune responses. The detection of quinine ethylcarbonate may suggest the presence of quinine-like alkaloid structures. Quinine is classically known as an antimalarial drug, but recent experimental evidence has also shown potential anti-inflammatory activity under hemolytic inflammatory conditions. Dou et al. reported that quinine or quinine-hemin complexes decreased inflammatory mediators such as IL-1β, IFN-β, IL-6, and TNF-α and reduced tissue damage in experimental sickle-cell disease models.[38] If quinine-like structures are truly present in the *n*-hexane fraction, they may contribute to the regulation of excessive inflammatory responses. However, because quinine ethylcarbonate is not a common reported metabolite of *N. fruticans*, this identification should be confirmed by targeted LC-MS/MS or comparison with authentic standards before being interpreted as a genuine plant-derived constituent. The surfactant-type alkanolamines, including *N*-lauryldiethanolamine and triisopropanolamine, should be interpreted cautiously. These compounds are more commonly associated with industrial, cosmetic, or formulation-related materials than with natural plant metabolites. Their amphiphilic structure may allow interaction with biological membranes, which could theoretically influence membrane fluidity, endocytosis, or phagocytic uptake by macrophages. However, such detergent-like behaviour may also produce

cytotoxicity at higher concentrations. Therefore, their contribution to immunomodulatory activity cannot be considered beneficial without further cytotoxicity testing and confirmation of their origin. The detection of HT-2 toxin is particularly important from a safety perspective. HT-2 toxin is a type-A trichothecene mycotoxin and is known to exert toxic and immunotoxic effects in animals and humans. Recent toxicological reviews describe T-2 and HT-2 toxins as compounds capable of disrupting protein synthesis, damaging rapidly dividing tissues, and affecting immune, hematopoietic, gastrointestinal, and nervous systems.[32] Therefore, HT-2 toxin is unlikely to be a desirable bioactive constituent of the extract. Its presence may indicate fungal contamination of plant material, carry-over during analysis, or an incorrect database match. Future work should include repeated LC-MS/MS confirmation and mycotoxin screening to ensure the safety of *N. fruticans* fractions intended for immunomodulatory development. Two high-intensity library hits in the *n*-hexane fraction corresponded to irinotecan and hematoporphyrin, both of which are more consistent with pharmaceutical xenobiotics than plant metabolites. Irinotecan is a semi-synthetic camptothecin derivative whose active metabolite SN-38 inhibits DNA topoisomerase I, leading to DNA damage and apoptosis in proliferating cells.[39] Hematoporphyrin and related porphyrin derivatives are known as photosensitizers used in photodynamic therapy and are not typical constituents of mangrove palms.[36] Because these compounds co-occurred with other xenobiotic-like hits such as AB-CHMICA and decamethonium, their detection most likely reflects contamination, carry-over from previous LC-MS runs, or limitations of automated spectral library matching. Overall, the *n*-hexane fraction appears chemically heterogeneous and is dominated by lipophilic molecules, surfactant-like compounds, and several questionable xenobiotic hits. Among the identified constituents, methyl cinnamate and possibly quinine-like alkaloid structures are the most plausible contributors to any modest anti-inflammatory or antimicrobial effects of this fraction.[37,38] In contrast, HT-2 toxin, irinotecan, hematoporphyrin, AB-CHMICA, and decamethonium should be treated as safety liabilities or analytical artefacts rather than as meaningful plant metabolites.[32,36,39] These findings reinforce the need for repeated LC-MS analysis using freshly prepared and carefully handled plant material, confirmation of key peaks using authentic standards, and bioactivity-guided fractionation to isolate genuine non-polar constituents responsible for the observed immunomodulatory effects. Taken together, the LC–MS findings provide important chemical evidence that supports the biological activity of *N. fruticans* leaf fractions. The detection of phenolic aldehydes, flavonoid glycosides, anthraquinone glycosides, and other bioactive constituents strengthens the scientific basis for the traditional use of this mangrove plant and contributes to the existing body of knowledge on plant-derived immunomodulators. Importantly, the fraction-level profiling presented in this study helps distinguish phytochemically plausible immunomodulatory constituents from possible xenobiotic or contaminant-related library hits, thereby improving the reliability of future natural-product investigations.

Phagocytic Activity of Macrophages

The phagocytic activity of peritoneal macrophages was evaluated after 14 days of oral administration of *Nypa fruticans* Wurmb leaf fractions. Significant differences were observed among treatment groups ($p < 0.05$). The n-hexane fraction demonstrated the highest phagocytic activity (75%), followed by the ethyl acetate fraction (74%) and the crude ethanol extract (72%). These values were comparable to the positive control Stimuno® (71%), whereas the negative control group (Na-CMC) displayed the lowest activity (35%). The detailed quantitative data are presented in Table 4, and the graphical distribution is shown in Fig.-1.

Table-4: Percentage of Macrophage Phagocytic Activity in Mice after Treatment with *Nypa fruticans* Leaf Fractions

Group	Mean ± SD (%)
Na-CMC (negative control)	35.00 ± 5.2
Stimuno® (positive control)	71.00 ± 6.3
Ethanol extract (250 mg/kg BW)	72.00 ± 5.8
n-Hexane fraction	75.00 ± 6.1
Ethyl acetate fraction	74.00 ± 5.6
Ethanol fraction	61.00 ± 4.9

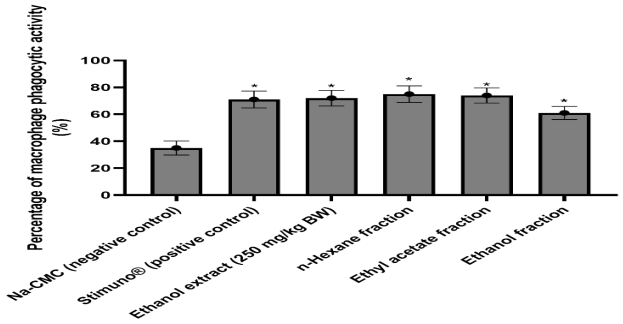

Fig.-1: Phagocytic Activity of Macrophages after Treatment with *Nypa fruticans* Wurmb Leaf Fractions. Data are expressed as mean ± SD (n = 5). Asterisks (*) Indicate Significant Differences Compared with the negative Control Group ($p < 0.05$, One-Way ANOVA followed by LSD post-hoc test)

These results clearly indicate that all fractions and the crude ethanol extract exhibited marked immunostimulatory activity, with phagocytic indices approximately two-fold higher than the negative control and nearly equivalent to the commercial immunomodulator Stimuno®. This enhancement of macrophage activity suggests that the fractions contain bioactive constituents capable of stimulating innate immune responses. Macrophages are central effector cells of the innate immune system and play essential roles in pathogen recognition, phagocytosis, cytokine secretion, and antigen presentation to adaptive immune cells, thereby acting as an important bridge between innate and adaptive immunity.[23,40] The increased phagocytic activity is likely related to the presence of flavonoids, saponins, phenolic compounds, and other secondary metabolites previously reported in *N. fruticans* leaves and related plant parts.[19,20] These compounds are known to modulate macrophage activation through immune-related signalling pathways, including NF-κB, MAPK, and TLR-associated pathways.[23,28,31] Similar observations have been described for immunomodulatory polysaccharides from other medicinal plants. For example, polysaccharides from *Abrus cantoniensis* were reported to enhance macrophage immunomodulatory activity, while polysaccharide fractions from fermented *Morinda citrifolia* increased nitric oxide production, cytokine release, and immune-related protein expression in RAW264.7 macrophages and BALB/c mice.[41,42] When considered alongside the LC-MS data, the phagocytic results suggest that not only polar and semi-polar constituents, represented by the ethanol and ethyl acetate fractions, but also non-polar constituents in the *n*-hexane fraction may contribute to macrophage activation. In the ethanol and ethyl acetate fractions, phenolic aldehydes, flavonoid glycosides, and anthraquinone glycosides such as 2,4-dihydroxybenzaldehyde, quercitrin, and emodin-8-*O*-glucoside are plausible contributors to macrophage stimulation. Emodin-8-*O*-glucoside has been shown to enhance macrophage phagocytosis and activate TLR-2/MAPK/NF-κB signalling, while quercitrin and quercetin derivatives are associated with the modulation of cytokine production and macrophage-mediated inflammatory response.s[28,30,31] Likewise, 2,4-dihydroxybenzaldehyde may contribute to the regulation of inflammatory responses through its anti-inflammatory and anti-angiogenic activities.[29] In contrast, the strong phagocytic response observed in the *n*-hexane fraction is more difficult to interpret because its LC-MS profile was dominated by surfactant-like molecules and several xenobiotic-like library hits. Among the more plausible constituents, methyl cinnamate and quinine-like compounds may contribute to the modulation of inflammatory responses, as cinnamate derivatives have been reported to influence macrophage inflammatory mediators, and quinine-related compounds have shown anti-inflammatory effects in experimental models.[37,38] However, the presence of compounds such as HT-2 toxin, irinotecan, hematoporphyrin, AB-CHMICA, and decamethonium suggests that further purification and confirmatory analysis are required to distinguish genuine plant-derived immunomodulators from contaminants or automated LC-MS misassignments[32,36,39].

Overall, the phagocytic assay supports the immunostimulatory potential of *N. fruticans* leaf extract and its solvent fractions. The ethanol and ethyl acetate fractions appear to have a more coherent phytochemical basis for macrophage activation because they contain phenolic, flavonoid, and anthraquinone glycoside constituents with documented immunomodulatory effects.[28-31] Meanwhile, the biological activity of the *n*-

hexane fraction should be interpreted more cautiously until the identities and origins of its non-polar constituents are confirmed. These findings suggest that *N. fruticans* may enhance innate immune defence primarily by promoting macrophage phagocytic activity, which may subsequently support antigen presentation, cytokine production, and activation of adaptive immune responses. The high phagocytic activity observed in all treated groups is biologically significant because macrophage phagocytosis represents an early and essential defence mechanism against invading pathogens. Compared with previous studies that mainly reported antioxidant or anti-inflammatory properties of *N. fruticans*, the present findings provide direct in vivo evidence that its leaf fractions can enhance innate immune function. This result expands the pharmacological relevance of *N. fruticans* from general bioactivity toward measurable immune-enhancing effects, particularly macrophage-mediated host defence.

Cytokine Profiles (IL-12 and IFN-γ)

The cytokine analysis showed that administration of *N. fruticans* fractions significantly enhanced the production of IL-12 and IFN-γ compared with the negative control group ($p < 0.05$). As presented in Table 5 the highest IL-12 concentration was observed in the positive control Stimuno® group (12.55 ± 0.001 ng/mL), followed by the ethanol fraction (10.60 ± 0.002 ng/mL). The n-hexane (9.15 ± 0.001 ng/mL) and ethyl acetate fractions (8.77 ± 0.002 ng/mL) produced moderate increases, while the crude ethanol extract induced a somewhat lower IL-12 level (8.82 ± 0.005 ng/mL). In contrast, the negative control exhibited the lowest IL-12 response (4.46 ± 0.001 ng/mL). For IFN-γ, the strongest stimulation was recorded in the ethyl acetate fraction (9.49 ± 0.001 ng/mL), which was almost identical to the positive control Stimuno® (9.48 ± 0.001 ng/mL), followed by the ethanol fraction (9.32 ± 0.002 ng/mL). The n-hexane fraction (8.35 ± 0.002 ng/mL) also showed a notable effect, whereas the ethanol extract group exhibited the lowest IFN-γ increase among the treated groups (7.22 ± 0.001 ng/mL), though still significantly higher than the negative control (6.19 ± 0.001 ng/mL). These findings are further supported by Fig.-2, which visually demonstrates the marked elevation of both cytokines in all treatment groups relative to Na-CMC.

Table-5: Serum Levels of IL-12 and IFN-γ in Mice Treated with *N. fruticans* Wurmb Fractions

Group	IL-12 (ng/mL)	IFN-γ (ng/mL)
Na-CMC (negative control)	4.46 ± 0.001	6.19 ± 0.001
Stimuno® (positive control)	12.55 ± 0.001	9.48 ± 0.001
Ethanol extract (250 mg/kg BW)	8.82 ± 0.005	7.22 ± 0.001
Ethanol fraction	10.60 ± 0.002	9.32 ± 0.002
Ethyl acetate fraction	8.77 ± 0.002	9.49 ± 0.001
n-Hexane fraction	9.15 ± 0.001	8.35 ± 0.002

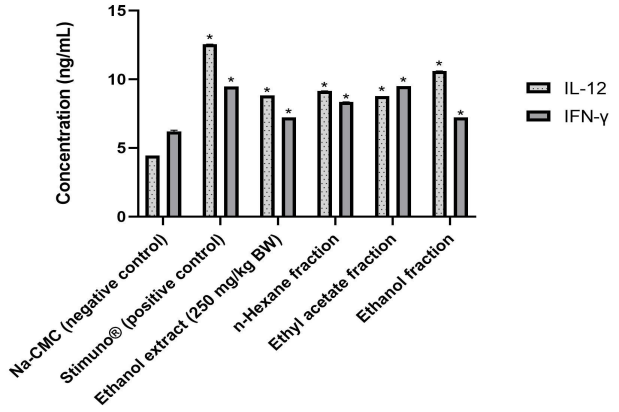

Fig.-2: Serum Cytokine Levels of (A) IL-12 and (B) IFN-γ after Treatment with *N. fruticans* Wurmb Fractions Compared with Controls. Data are expressed as Mean Values (n = 5). All Treatment Groups Showed Significant Increases Compared with the Negative Control ($p < 0.05$)

The elevation of IL-12 suggests that N. fruticans fractions can activate Th1-mediated immune responses and enhance IFN-γ secretion, thereby functionally linking innate and adaptive immunity.[43] IL-12 plays a crucial role in the differentiation of naïve T cells into Th1 cells and in the activation of natural killer cells, promoting the production of IFN-γ and supporting cell-mediated immune responses.[43,44] The ethanol fraction, which produced the highest IL-12 level among the treatments, is likely enriched in semi-polar phytochemicals such as flavonoids and phenolic acids, compound classes that have been repeatedly associated with the up-regulation of Th1-type cytokines.[28–31] Meanwhile, the pronounced increase of IFN-γ in the ethyl acetate fraction indicates strong stimulation of cell-mediated immunity and macrophage effector function. IFN-γ is a central cytokine that enhances macrophage microbicidal activity, up-regulates MHC expression and promotes antigen presentation.[44] Similar effects on IFN-γ production have been reported for other immunomodulatory plants, such as Astragalus membranaceus, which enhances IFN-γ release through NF-κB activation.[45] and Echinacea purpurea, which has been shown to stimulate Th1 cytokines in vivo.[46] Viewed together with the LC-MS findings, these cytokine data suggest that the ethanol and ethyl acetate fractions contain complementary sets of bioactive phenolic and flavonoid constituents that differentially shape Th1-type responses: the ethanol fraction appears more effective in driving IL-12 production, while the ethyl acetate fraction exerts a particularly strong effect on IFN-γ. This complementary pattern supports the hypothesis that N. fruticans has potential as a plant-derived therapeutic for fine-tuning both innate and adaptive immune responses.[28–31,43–46] The cytokine results further demonstrate that the immunostimulatory effect of *N. fruticans* is not limited to macrophage phagocytosis but extends to cytokine-mediated immune communication. The elevation of IL-12 and IFN-γ indicates activation of the macrophage–Th1 immune axis, which is essential for cellular immunity and host protection. These findings contribute to the current understanding of medicinal plants as modulators of coordinated innate and adaptive immune responses, and suggest that the ethanol and ethyl acetate fractions may contain complementary bioactive compounds capable of shaping Th1-type immunity.

Lymphocyte Subset Expression

Analysis of lymphocyte subsets revealed that administration of *N. fruticans* fractions significantly increased $CD4^+$, $CD8^+$ and $CD14^+$ cell expression compared with the negative control ($p < 0.05$). The results are summarised in Table 6 and illustrated in Fig.-3, showing that all treatment groups demonstrated higher lymphocyte subset expression relative to Na-CMC.

Table-6: Expression Levels of $CD4^+$, $CD8^+$ and $CD14^+$ Lymphocytes after Treatment with *N. fruticans* Wurmb Fractions

Group	CD4 (ng/mL) Mean ± SD	CD8 (ng/mL) Mean ± SD	CD14 (ng/mL) Mean ± SD
Na-CMC (negative control)	0.09 ± 0.076	15.57 ± 0.001	7.82 ± 0.001
Stimuno® (positive control)	1.73 ± 0.006	39.19 ± 0.002	10.81 ± 0.001
Ethanol extract	1.25 ± 0.001	31.97 ± 0.004	6.91 ± 0.002
n-Hexane fraction	1.30 ± 0.001	27.30 ± 0.001	8.86 ± 0.001
Ethyl acetate fraction	1.24 ± 0.764	31.82 ± 0.004	10.81 ± 0.001
Ethanol fraction	1.50 ± 0.004	36.96 ± 0.001	10.53 ± 0.001

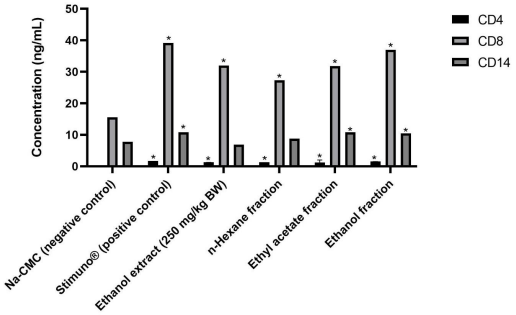

Fig.-3: Lymphocyte Subset Expression of $CD4^+$ (A), $CD8^+$ (B) and $CD14^+$ (C) Cells in Mice Treated with *N. fruticans* Wurmb Fractions Compared with Controls. Data are expressed as Mean Values (n = 5). All Treatment Groups Showed Significant Increases Compared with the Negative Control ($p < 0.05$)

For CD4, the ethanol fraction (1.50 ± 0.004 ng/mL) and the ethanol extract (1.25 ± 0.001 ng/mL) produced higher levels than the negative control (0.09 ± 0.076 ng/mL), although the strongest effect was observed in the positive control Stimuno® (1.73 ± 0.006 ng/mL). CD8 expression was most pronounced in the Stimuno® group (39.19 ± 0.002 ng/mL), followed closely by the ethanol fraction (36.96 ± 0.001 ng/mL) and ethanol extract (31.97 ± 0.004 ng/mL). The n-hexane fraction produced the lowest CD8 stimulation among the treatment groups (27.30 ± 0.001 ng/mL), yet still significantly higher than the negative control (15.57 ± 0.001 ng/mL). For CD14, a monocyte/macrophage surface marker, the ethyl acetate fraction (10.81 ± 0.001 ng/mL) and ethanol fraction (10.53 ± 0.001 ng/mL) showed higher expression compared with the negative control (7.82 ± 0.001 ng/mL) and the crude ethanol extract (6.91 ± 0.002 ng/mL). These data indicate that N. fruticans fractions are capable of enhancing innate immune cell activation, particularly monocytes/macrophages, which are critical for pathogen recognition and phagocytosis.[40] Overall, the pattern of lymphocyte subset modulation suggests that different fractions of *N. fruticans* exert complementary immunomodulatory effects. The ethanol fraction strongly stimulates $CD4^+$ helper and $CD8^+$ cytotoxic T-cell markers, indicating a robust impact on adaptive, cell-mediated immunity, while the ethyl acetate fraction is particularly effective at enhancing $CD14^+$ expression, pointing to strengthened monocyte/macrophage responses. These findings are consistent with the cytokine data, in which IL-12 and IFN-γ were elevated in a fraction-dependent manner, and together they support the hypothesis that *N. fruticans* can coordinately modulate both innate and adaptive immune responses.[40,43,44] The combined increase in $CD4^+$, $CD8^+$, and $CD14^+$ expression provides additional evidence that *N. fruticans* fractions affect both adaptive and innate immune components. This integrated immune response is important because effective immunomodulation requires not only macrophage activation but also communication with T-cell-mediated pathways. Therefore, the present results add new fraction-level evidence to the existing literature by showing that different solvent fractions of *N. fruticans* may exert complementary effects on macrophages, cytokines, and lymphocyte subsets.

CONCLUSION

Fractions of *Nypa fruticans* Wurmb leaves demonstrated clear immunomodulatory activity, as shown by increased macrophage phagocytic activity, elevated serum IL-12 and IFN-γ levels, and enhanced $CD4^+$, $CD8^+$, and $CD14^+$ expression compared with the negative control. The ethanol and ethyl acetate fractions showed the most consistent effects on cytokine production and lymphocyte subset modulation, indicating their potential role in enhancing both innate and adaptive immune responses. LC-MS analysis suggested that these active fractions contained phenolic and flavonoid-related compounds, including emodin-8-*O*-glucoside, quercitrin, and 2,4-dihydroxybenzaldehyde, which may contribute to the observed immunostimulatory effects. Overall, this study provides fraction-level evidence that *N. fruticans* leaf fractions, particularly the ethanol and ethyl acetate fractions, may serve as promising sources of plant-derived immunomodulatory agents. In line with Sustainable Development Goal 3, Good Health and Well-being, these findings support the exploration of locally available medicinal plant resources for future development of accessible and evidence-based immune-supporting preparations. Further studies are recommended to confirm the identified compounds using targeted LC-MS/MS, isolate the major active constituents, clarify their mechanisms of action, and evaluate their safety profiles before practical application.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest regarding the publication of this article.

AUTHOR CONTRIBUTIONS

The present work is related to *SDG-3: Good Health and Well-being*. All the authors contributed significantly to this manuscript, participated in reviewing/editing and approved the final draft for publication. The research profile of the authors can be verified from their ORCID ids, given below:

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