In vitro studies on the immunomodulatory effects of fulvic acid extract from palm oil empty fruit bunch (OPEFB)

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Abstract. This study evaluates the potential of Fulvic Acid (FA), derived from oil palm empty fruit bunches (OPEFB), as an immunomodulatory agent through in vitro analysis. Fulvic Acid, a component of humic substances, is known for its wide-ranging applications in agriculture, biotechnology, and medicine. With an increasing need for sustainable natural therapies, FA has shown promise in treating degenerative diseases by modulating immune responses. The research focused on assessing the immunomodulatory effects of FA by analysing its impact on cytokine production. In vitro studies used the MTT assay to determine cytotoxicity and ELISA tests to measure cytokines associated with natural immunity (TNF-alpha and Interferon-gamma) and adaptive immunity (IL-2 and TGF-beta). Comparative evaluations were performed using FA samples from Shilajit and a fertiliser product. Results demonstrated that FA derived from OPEFB significantly enhanced cytokine production, suggesting its potential as an immunomodulatory agent. These findings indicate that FA from OPEFB could be a promising candidate for developing treatments for degenerative diseases. Further studies are recommended to explore its therapeutic potential and commercial application in the health sector.

1 Introduction

Fulvic Acid (FA) is a component of humic substances (HS) formed during the decomposition of organic matter. While FA is less developed commercially than other components like Humic Acid (HA), it has been reported to offer superior benefits in agronomy, poultry, and medical treatments [1-6]. Although HA shows positive results under controlled conditions, its efficacy in field applications remains questionable [7]. Market-available FA sources include shilajit, containing approximately 60-80% HS, of which FA is a major component [8]. A Chinese company claims their lignite-based fulvic acid fertiliser contains 50-60% FA, but FA composition varies with different raw materials, influencing its chemical content [3, 9].

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Shilajit is a natural substance renowned for its significant fulvic Acid (FA) content. It is primarily found in specific mountainous regions, including the Himalayas, and is also known by other names such as mumie or mineral pitch. This resinous material originates through a prolonged humification process involving plant matter, with bryophytes being a predominant source [10]. Historically, shilajit has been used to treat various ailments, including genitourinary disorders, diabetes, digestive issues, neurological conditions, and respiratory diseases [11]. Today, shilajit products are widely available online. According to Vucskits et al. [12], FA has been shown to contribute to improved performance, enhanced immune response, and better thyroid function in mice.

In addition to shilajit and lignite, lignin is another promising source of FA [13]. Lignin is commonly obtained as byproducts like lignosulfonate and sulfonated lignin from the pulp and paper industries [14]. Humic substances, including fulvic acid (FA), are hypothesised to form in nature through the fungal-mediated breakdown of lignin. This natural transformation process highlights the role of lignin as a precursor in developing these complex organic substances. There is potential to develop controlled processes using fungi to produce FA [13]. Jeong et al. [15] demonstrated that technical lignin from kraft processing could be transformed into humic-like fertilisers simulating natural fungal degradation.

The palm oil industry produces significant quantities of solid wastes, among which Oil Palm Empty Fruit Bunches (OPEFB) are the major component. In 2023, Indonesia produced 46.9 million tons of palm oil, with OPEFB making up a significant portion of the waste [16,17]. OPEFB contains 22.2% lignin, 43.07% cellulose, and 33% hemicellulose [18]. Managing this waste poses environmental challenges, but its high lignin content makes OPEFB a potential raw material for FA extraction. Utilising OPEFB for FA production could help mitigate raw material shortages and environmental issues [13].

Ensuring natural FA's beneficial properties and quality is essential, as the raw material source significantly affects its efficacy. With 1.2 million Indonesians suffering from degenerative diseases, FA's immunomodulatory potential could significantly impact healthcare. Vucskits et al. [12] demonstrated that FA and HA can stimulate immune responses in mice, suggesting that FA also has potential as a mild hypothyroid treatment. Therefore, as a compound with potential as an immunomodulator, studies on the effect of FA on the immune system are necessary. This study evaluates FA derived from OPEFB for its immunomodulatory properties through in vitro analysis.

2 Materials and methods

Fulvic Acid (FA) extraction from OPEFB refers to [19]. The cytotoxicity of the extracted FA was evaluated using the MTT assay, which measures cellular metabolic activity. Furthermore, the production of cytokines, which included Tumor Necrosis Factor-alpha (TNF-α), Interferon delta (IFN-δ), Transforming Growth Factor beta (TGF-β), and Interleukin-2 (IL-2), was quantified via ELISA Assay. Other FA sources, including shilajit and fertiliser, were also evaluated using the same method.

2.1 MTT assay

MTT assay was conducted to measure cell viability. The method starts with the isolation of lymphocytes from spleen tissue. The procedure involved the aseptic separation of mononuclear cells, obtained by processing spleens from euthanised mice in a PBS buffer with antibiotics. The tissue was passed through a 40 µm strainer, and the resulting suspension was centrifuged at 1500 rpm for 10 minutes to collect cell pellets. After discarding the supernatant, the cells were washed with PBS, centrifuged again under the same conditions,

and treated with a lysis buffer to remove red blood cells, followed by a 1-minute room temperature incubation.

The remaining cells were redissolved in RPMI 1640 medium supplemented with 10% FBS and 1% antibiotics. Cell counts were conducted by mixing 10 μL of the suspension with 90 μL of 3% acetic acid to calculate the required concentration for downstream assays. After the next centrifugation step, the cells were set to a final density of 10×10^6 cells/ml.

For the assay, splenocytes were plated in well plates at a density of 1,000,000 cells per well and incubated at 37°C in 5% CO_2 for 18–20 hours. Samples were added at varying concentrations (800, 400, 200, 100, 50, 25, and 12.5 μg/mL), with untreated cells serving as controls. After 48 hours at 37°C in 5% CO_2, 50 μL of MTT reagent was dispensed into each well. Subsequent to incubation for 4 hours, the formazan crystals formed were solubilised in 100 μL of ethanol per well, and the optical density was quantitated at 595 nm. The percentage of cell viability was calculated using the formula:

$$Cell\ viability\ (\%) = \frac{sample\ absorbance - blank\ absorbance}{control\ absorbance - blank\ absorbance}\ x\ 100\% \quad (1)$$

2.2 ELISA (TNF-α, IFN-δ, TGF-β, and IL-2)

The cytokine analysis using ELISA began with cell preparation. Isolated spleenocytes were cultured in 24-well plates, with each well containing 1,000,000 cells. Cultures were treated with the FA samples at different concentrations of 800, 400, 200, 100, 50, 25, and 12.5 μg/mL. Untreated cells were negative controls, while Lipopolysaccharide (LPS) at 25 μg/mL was used as a positive control.

Cells were then incubated at 37°C in 5% CO_2 for 48 hours. Following incubation, the supernatant was collected for cytokine quantification. ELISA was performed to measure TNFα, IFNδ, TGFβ, and IL-2 levels using a commercially available kit. The procedure involved adding the supernatant samples to the ELISA plates, introducing secondary antibodies specific to the target cytokines, and adding chromogenic substrates to enable visualisation. The resulting optical density (OD) values were recorded and converted into cytokine concentrations using standard curves prepared during the assay.

2.3 Data analysis

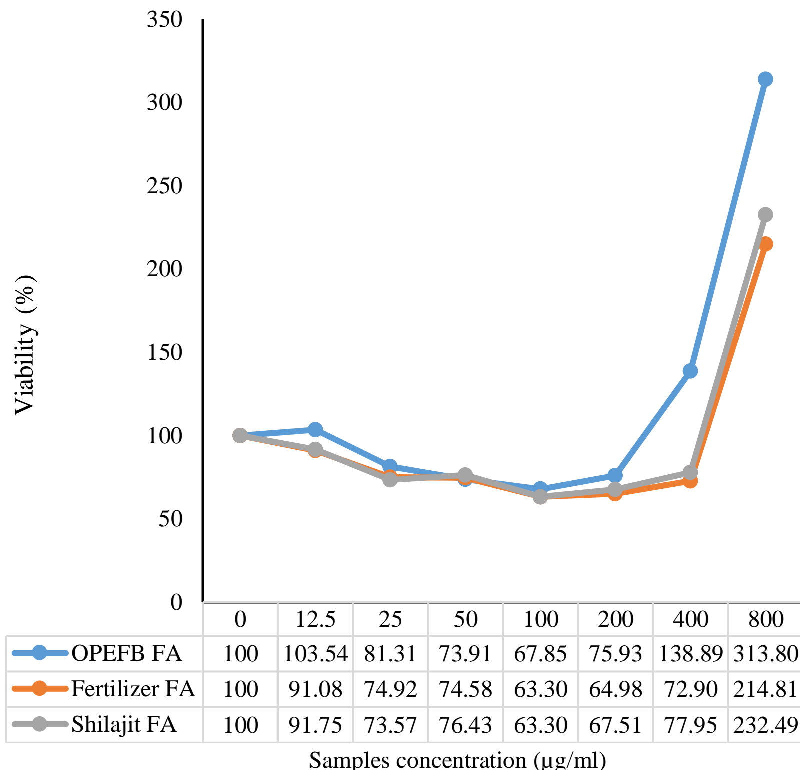
Descriptive statistics analysis was performed in this study to analyse the effect of FA samples on cytokines production. The statistical method used to compare the means value of samples was Analysis of Variance (ANOVA) with a 95% confidence interval (α=5%). Data processing was carried out using Microsoft Excel 2021.

3 Result and discussion

3.1 MTT assay

The population of spleenocytes treated with FA samples from OPEFB, Fertilizer and Shilajit showed cell colonies with lymphoblast-like morphology. The addition of FA from OPEFB, Fertilizer and Shilajit did not show toxic effects on the spleenocyte population of mice. The three samples showed the same trend of increasing viability percentage and exceeded the untreated control (Figure 1).

https://doi.org/10.1051/bioconf/202515402002



	0	12.5	25	50	100	200	400	800
OPEFB FA	100	103.54	81.31	73.91	67.85	75.93	138.89	313.80
Fertilizer FA	100	91.08	74.92	74.58	63.30	64.98	72.90	214.81
Shilajit FA	100	91.75	73.57	76.43	63.30	67.51	77.95	232.49

Fig. 1. The effect of FA addition on the splenocyte cell viability percentage and viability of treated cells exceeded the untreated cell (control), implying the potential of all FA samples to stimulate cell growth and indicate the potential of FA as an immunomodulatory agent.

The percentage of all treated cell viability that exceeds the untreated cell control indicates cell growth that exceeds cells without the addition of samples; this implies the potential of the three samples to stimulate cell growth. The finding also implicates FA's potential as an immunomodulatory agent. OPEFB FA treated cells showed a higher percentage of viability than Fertilizer FA and Shilajit FA, as can be seen in Figure 1.

3.2 ELISA (TNF-α, IFN-δ, TGF-β, dan IL-2)

Cytokines are chemical messengers that facilitate communication among cells within the immune system. Key cytokine groups are well-recognised for their critical roles. These compounds contribute to processes such as repairing tissue damage caused by chemical agents, tumor progression, cell proliferation and programmed cell death regulation, and the modulation of immune responses [20–22].

Given their potential as immunomodulatory agents, investigating the effects of Fulvic Acid (FA) on cytokine production is essential. This study focused on analysing the production of Tumor Necrosis Factor-alpha (TNF-α), Interferon-gamma (IFN-δ), Transforming Growth Factor beta (TGF-β), and Interleukin-2 (IL-2), each of which serves distinct functions within the immune system.

3.2.1 Tumor Necrosis Factor-alpha (TNF-α) analysis

Tumour Necrosis Factor-alpha (TNF-α) holds an important function as a cytokine in modulating acute inflammatory responses caused by Gram-negative bacteria and other microorganisms [20]. The primary sources of TNF-α are macrophages and antigen-activated T cells, NK cells, and mast cells [21]. The addition of OPEFB FA and Fertilizer FA produces TNFα with the same trend for each concentration (Figure 2).

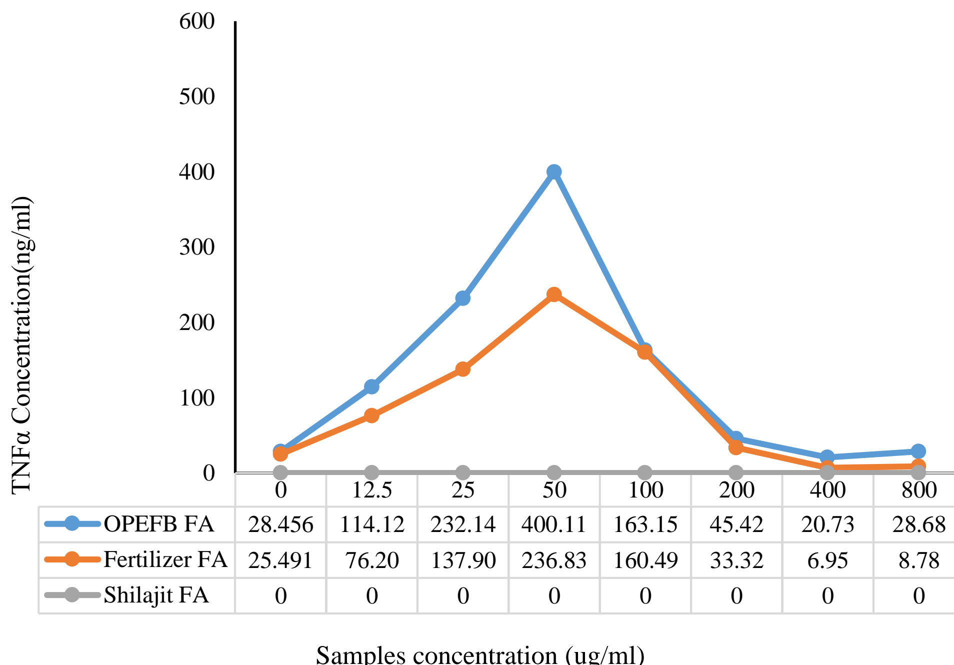


Fig. 2. The effect of FA addition on the TNF-α concentration, implying that OPEFB FA treatment at the optimum concentration of 50 µg/mL showed potential in the immune response to inflammation.

The OPEFB FA treated cell showed higher TNF-α produced than Fertilizer FA, with an optimum concentration at 50 µg/mL. The addition of Shilajit FA at any concentration did not affect TNF-α production. These results can be obtained due to the low FA content in Shilajit, in accordance with a study by Sasikala & Deeptha [23] found only 0.96% FA in Oriens Shilajit. This result implies that treatment of OPEFB FA at a concentration of 50 µg/mL showed its optimum concentration potential in the immune response to inflammation.

Table 1. Summary of statistical descriptive analysis of TNF-α affected by samples

Groups	Count	Sum	Average	Variance
OPEFB FA	8	1032,809	129,101	17772,530
Fertiliser FA	8	685,946	85,743	7105,258
Shilajit FA	8	0	0	0

According to Table 1, the highest TNF-α was produced by OPEFB FA treatment compared to other samples, with an average of 129,101 ng/ml. The mean values of the samples were significantly different according to the ANOVA results, as indicated by a *p-value* of 0.029 (below α=0.05) (Table 2).

Table 2. Summary of ANOVA analysis of TNF-α affected by samples.

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	69063,761	2	34531,881	4,164	0,029	3,466
Within Groups	174144,522	21	8292,596			
Total	243208,284	23				

This result indicated that OPEFB FA treatment can affect TNF-α production with a significantly higher average than Fertilizer FA and Shilajit FA. It can be made a point to suggest that the potential of OPEFB as a raw material for FA extracts is better than the source of FA from Fertilizer and Shilajit.

3.2.2 Interferon-gamma (IFN-δ) analysis

Interferon-gamma (IFN-δ) is an essential cytokine in host resistance against viral infections and pathogenic microbes. IFNδ induces various significant physiological responses that contribute to immunity [20]. The OPEFB FA treatment gave the highest IFNδ concentration at 12.5 µg/ml and Fertilizer FA at 50µg/ml (Figure 3).

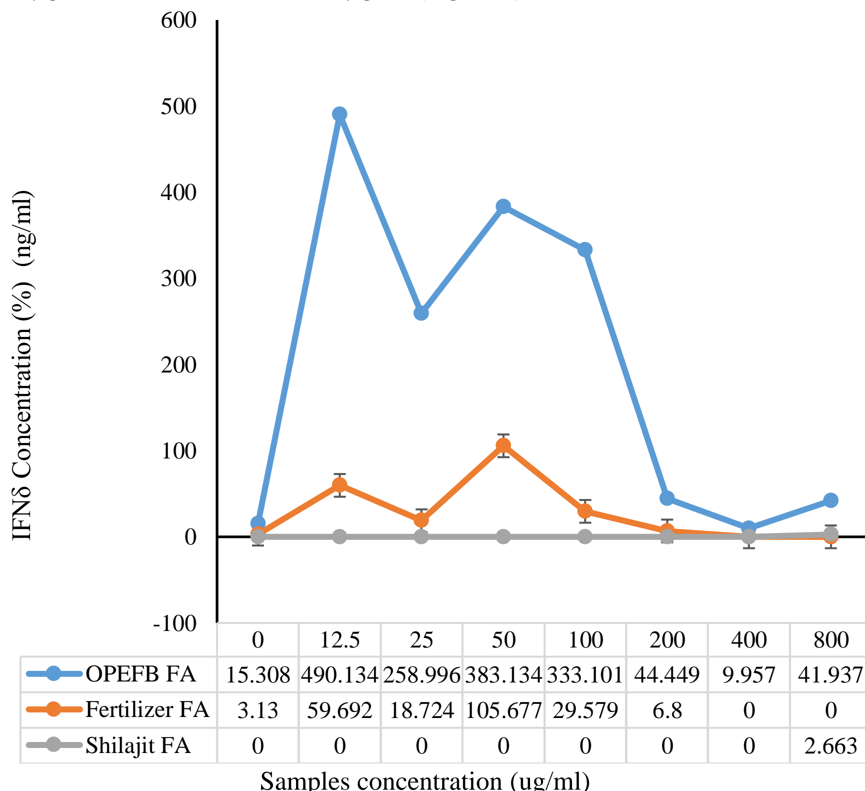


Fig. 3. The effect of FA addition on the IFN-δ concentration, addition of OPEFB and Fertilizer FA produced IFN-δ, indicates the potential of the FA to defend against infection and pathogens.

The Shilajit FA treatment at any concentration did not produce IFNδ. Similar to the TNF-α result, the concentration of FA in Shilajit may be in insignificant quantity to affect the production of IFN-δ [23]. The addition of OPEFB FA produced higher IFN-δ than Fertilizer

FA. The application of the OPEFB FA at a concentration of 12.5 ug/ml produced the highest IFNδ, which is thought to have the potential to defend against infection and pathogenic microbes.

Table 3. Summary of statistical descriptive analysis of IFN-δ affected by samples

Groups	Count	Sum	Average	Variance
OPEFB FA	8	1577,016	197,127	36893,381
Fertiliser FA	8	223,602	27,950	1394,653
Shilajit FA	8	2,663	0,332	0,886

According to Table 3, the highest IFN-δ was detected by OPEFB FA treatment than other samples with an average of 197,127 ng/ml. The mean values of the samples were significantly different according to the ANOVA analysis results, as indicated by a p-value of 0.004 (below $\alpha=0.05$) (Table 4).

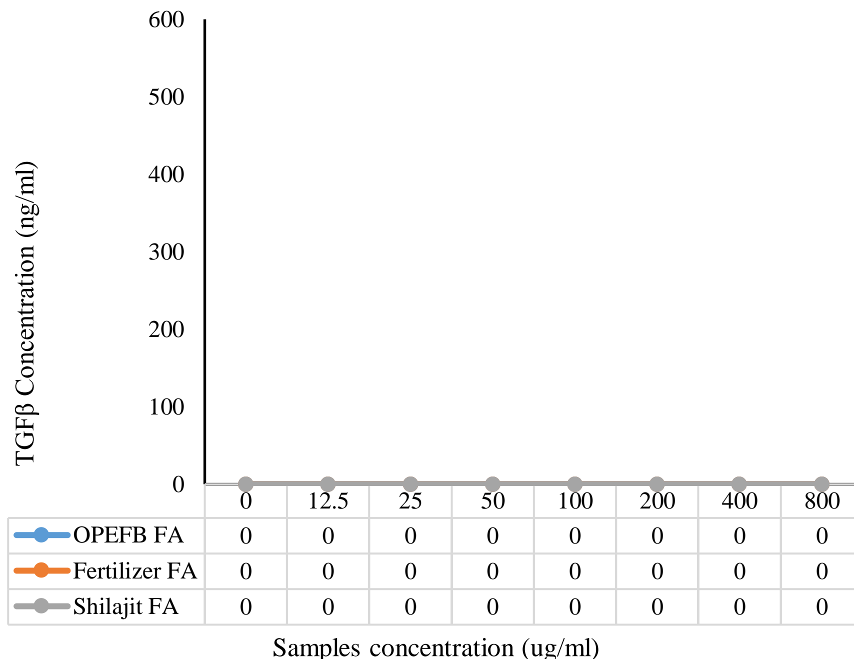
Table 4. Summary of ANOVA analysis of IFN-δ affected by samples

Source of Variation	SS	df	MS	F	P-value	F crit
Between Groups	181630,45	2	90815,226	7,115	0,004	3,466
Within Groups	268022,45	21	12762,973			
Total	449652,90	23				

The result indicated that OPEFB FA treatment could affect IFN-δ production with a significantly higher average than Fertilizer FA and Shilajit FA. It can be made a point to suggest that the potential of OPEFB as a raw material for FA extracts is better than the source of FA from Fertilizer and Shilajit.

3.2.3 Transforming Growth Factor beta (TGF-β) analysis

Transforming Growth Factor beta (TGF-β) is a multifunctional growth factor that affects a wide range of biological processes, including cell proliferation, differentiation, modulation of immune responses, and formation of fibrous tissue [20]. The addition of OPEFB FA, Fertilizer FA and Shilajit FA did not produce TGF-β (Figure 4).



	0	12.5	25	50	100	200	400	800
OPEFB FA	0	0	0	0	0	0	0	0
Fertilizer FA	0	0	0	0	0	0	0	0
Shilajit FA	0	0	0	0	0	0	0	0

Fig. 4. The effect of FA addition on the TGF-β concentration, addition of all FA samples at any concentrations did not produce TGF-β, indicating that the samples could not stimulate the production of detectable TGF-β.

Presumably, the concentration could not stimulate the production of TGF-β that can be detected using the ELISA. Further testing is needed by detecting TGF-β using other techniques, such as flow cytometry, to see the population of cells that produce TGF-β.

3.2.4 Interleukin-2 (IL-2) Analysis

Interleukin-2 (IL-2) is a protein produced in the human body that promotes the multiplication and maturation of cells that fight infection [20]. The addition of OPEFB FA and Fertilizer FA samples stimulates IL-2 production at concentrations of 12.5 µg/ml, 25 µg/ml, and 50 µg/ml. Fertiliser FA showed higher results at these concentrations than OPEFB FA (Figure 5).

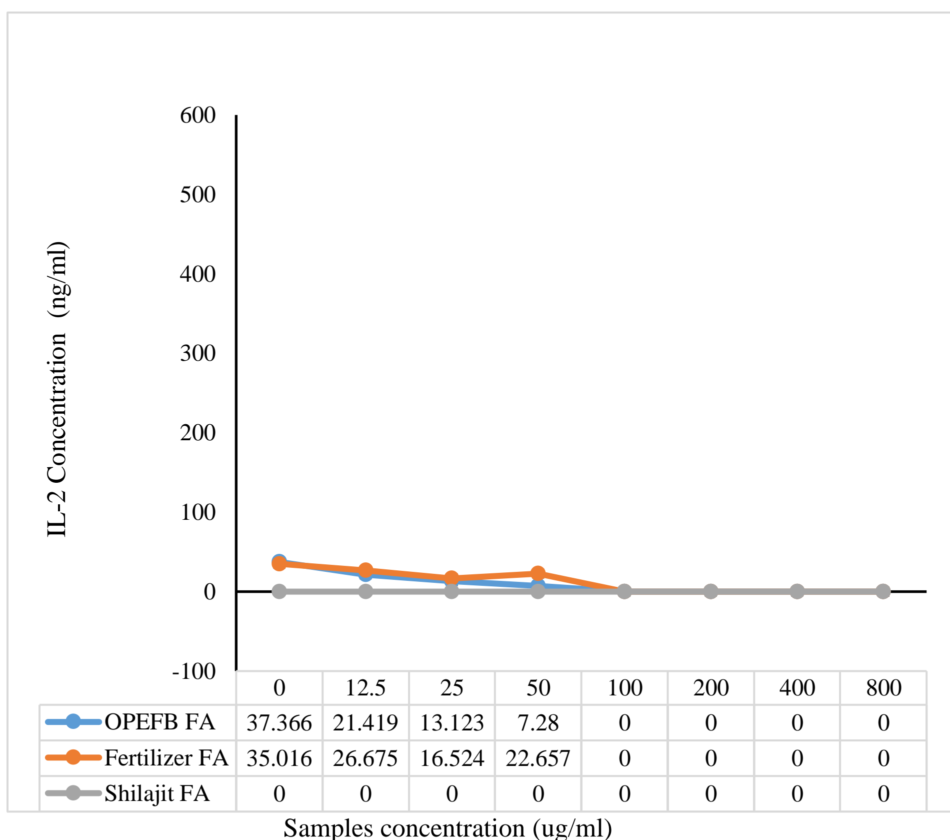


Fig. 5. The effect of FA addition on the IL-2 concentration, OPEFB, and FA Fertilizer stimulated IL-2 production, indicating the potential of the samples in immune cell activation and proliferation, Shilajit sample showed no effect on detectable IL-2 production.

Results showed that Fertiliser and OPEFB FA samples stimulated IL-2 production, indicating the potential of the samples in immune cell activation and proliferation. On the other hand, Shilajit did not affect the production of IL-2 at any given concentration.

Table 5. Summary of statistical descriptive analysis of IL-2 affected by samples.

Groups	Count	Sum	Average	Variance
OPEFB FA	8	79,188	9,8985	185,194375
Fertiliser FA	8	100,872	12,609	207,451865
Shilajit FA	8	0	0	0

According to Table 5, the highest IL-2 was produced by OPEFB FA treatment than other samples, with an average of 79,188 ng/ml. The mean values of the samples were found not to be significantly different according to the ANOVA analysis results demonstrated by a p-value of 0.090 (above $\alpha = 0.05$) (Table 6).

Table 6. Summary of ANOVA analysis of IL-2 affected by samples

Source of Variation	SS	df	MS	F	p-value	F crit
Between Groups	704,83731	2	352,418658	2,69264255	0,09099212	3,46680011
Within Groups	2748,5236	21	130,88208			
Total	3453,361	23				

The result indicated that OPEFB FA treatment can affect IL-2 production, but the effect of OPEFB FA was not significantly different with Fertilizer FA and Shilajit FA. Further analysis is needed to detect CD4 cell populations that can stimulate IL-2 in the event of infection. Further testing can be done using other techniques, such as flow cytometry, to see the population of CD4 cells that can stimulate IL-2.

3.2.5 Cytokine measurement with the addition of OPEFB FA

Measurement of cytokine levels has generated useful insight into pathological processes. For instance, in some conditions such as Crohn's disease and rheumatoid arthritis [24]. Cytokines function in the body as proteins that are attached to soluble receptors, carrier proteins, or inhibitors, which can cover up their simple detection by ELISA assay or radioimmunoassay. This study compared the proteins Tumor Necrosis Factor-alpha (TNF-α) and interferon-gamma (IFNδ) of cells treated with OPEFB FA at different concentrations.

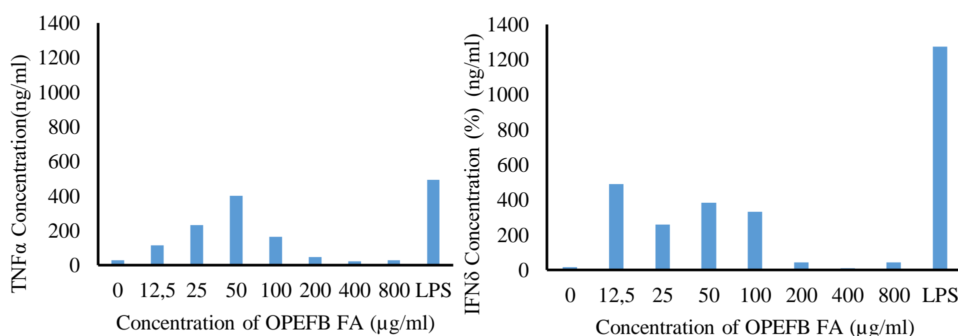


Fig. 6. Application of OPEFB FA at a concentration of 50 µg/ml showed its potential in the immune response to inflammation and defence against infection.

The cytokine measurements conducted on cells treated with OPEFB FA demonstrate a capacity to modulate inflammation and bolster infection defence, as highlighted by the TNFα and IFNδ expression levels at a concentration of 50 µg/mL.

3.2.6 Biological significance of the ELISA assay findings

The observed differences in cytokine production among the FA samples, particularly the superior activity of FA derived from Oil Palm Empty Fruit Bunches (OPEFB), underscore its significant immunomodulatory potential. OPEFB FA consistently elicited higher levels of key pro-inflammatory and adaptive cytokines, such as Tumor Necrosis Factor-alpha (TNF-α) and Interferon gamma (IFN-δ), compared to other samples like Fertilizer FA and Shilajit FA. These findings highlight the efficacy of OPEFB FA in promoting immune responses, with TNFα playing a pivotal role in acute inflammation and IFN-δ enhancing resistance to viral and microbial infections. Interestingly, the cytokine profiles also revealed that Shilajit FA, likely due to its lower FA content, exhibited negligible impact on TNF-α and IFN-δ production, aligning with prior studies indicating limited bioactive FA in some Shilajit formulations [23].

The biological significance of these results lies in the potential application of OPEFB FA as a natural immunomodulator in therapeutic contexts. Its ability to stimulate TNF-α and IFN-δ suggests utility in conditions requiring enhanced immune activation, such as infection

control and inflammation regulation. Furthermore, the cytokine analysis revealed variable concentrations for adaptive immunity markers, with OPEFB FA also showing promising, albeit less conclusive, effects on Interleukin-2 (IL-2), which is crucial for T-cell proliferation and immune cell coordination. These distinctions highlight the value of OPEFB FA as a renewable source of biologically active compounds for immune-related applications.

3.3 Potential limitations

Despite the promising findings, the study is subject to several limitations that warrant consideration. First, the absence of in vivo validation represents a significant gap, as the immunomodulatory effects observed in vitro may not directly translate to complex physiological environments. In vivo studies would be essential to confirm the safety, efficacy, and functional mechanisms of OPEFB FA, particularly under conditions mimicking disease or immune stress.

Second, the restricted scope of cytokine profiling limits the breadth of the conclusions. While the study successfully quantified TNF-α, IFN-δ, TGF-β, and IL-2, the results for TGF-β were inconclusive across all samples, likely due to low sensitivity or unsuitable detection conditions. Similarly, the IL-2 data, although suggestive of immune activation, lacked robust confirmation of downstream effects, such as T-cell proliferation or regulatory feedback. Incorporating additional cytokines, such as IL-6 or IL-10, and employing advanced methods like flow cytometry could provide a more comprehensive understanding of the immunomodulatory profile of FA samples.

Finally, variability in the FA content and chemical composition among the samples highlights the need for standardised extraction and characterisation protocols. Differences in raw materials, processing methods, and environmental conditions may significantly influence the bioactivity of FA, necessitating further studies to optimise and validate the production process. Addressing these limitations will be crucial for advancing OPEFB FA as a viable candidate for therapeutic applications.

4 Conclusion

OPEFB-derived Fulvic Acid (FA) stimulated splenocyte growth, with a higher percentage of cell viability than Fertilizer and Shilajit FA. The analysis of cytokine levels in cells exposed to OPEFB FA reveals its potential to regulate inflammatory responses and enhance defence mechanisms against infections, as indicated by the expression of TNF-α and IFN-δ at a concentration of 50 μg/mL. Further testing should still be done on TGF-β and IL-2 using other methods, such as flow cytometry.

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