6.1 Introduction of *In-vitro* studies

In-vitro bioassays are the important tools in the pharmacological evaluations of natural products especially the phytoconstituents. The applications of *in-vitro* methods for the screening of botanicals include bioassay guided fractionation, pharmacological and biological characterization, interaction studies, assistance in stability studies and the identification of mode of actions. Overall, the choice of any evaluation tool either *in-vitro* bioassays or *in-vivo* models are governed by the background data available on the phytoconstituents to be evaluated and the goal of the research program.

6.1.1 Cell-viability assay (MTT Assay)

The MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide) is a mono-tetrazolium salt that consists of a positively charged quaternary tetrazole ring core containing four nitrogen atoms surrounded by three aromatic rings including two phenyl moieties and one thiazolyl ring. MTT is cell permeable. The net positive charge of MTT allows it to readily penetrate the intact plasma membranes of viable cells. Other tetrazolium salts, such as MTS, XTT, and WST-1, are negatively charged compounds and unable to cross the plasma membrane. [1] Since MTT is a salt, high levels may be toxic to cells. Should the stock concentration of MTT be too high, cells may be disturbed and reduce viability readings. In such high concentrations, the cytotoxicity of formazan can also pose experimental difficulties when the culture media must be removed from the well-plate. [2]

Reduction of MTT results in disruption of the core tetrazole ring and the formation of a violet-blue water-insoluble molecule called formazan. The MTT reagent can pass through the cell membrane as well as the mitochondrial inner membrane of viable cells presumably due to its positive charge as well as its lipophilic structure and is reduced to formazan by metabolically active cells. The chromogenic nature of this redox chemical reaction provides a colorimetric-based measurement of intracellular formazan production based on which the MTT assay was developed by Mosmann et al. in 1983. [3] Consequently, the assay has extensive utility as a cell metabolic activity assay. However, its utility has increasingly been applied to infer secondary processes or states of cells, such as viability, which is frequently unsubstantiated. The MTT assay is typically performed after a few hours of incubation of cells with MTT. The water-insoluble formazan produced is then solubilized by a solvent such as Dimethyl sulfoxide (DMSO). Subsequently, the lowering of the light transmission by absorbance and other mechanisms by the homogenized MTT-formazan solution is measured by a microplate reader in terms of its optical density (OD) at a wavelength which MTT-derived formazan absorbs the most (around 570 nm). The measured OD values are assumed to be a representation of formazan concentration and consequently the intracellular reduction of MTT. This has been the basis of applying the MTT assay for nearly four decades as a common tool to measure cell proliferation/viability, drug cytotoxicity, and mitochondrial/metabolic activity of cells. [4,5]

6.1.1.1 Principle of MTT Assay

The principle behind the MTT assay is that only viable cells have active metabolism and can convert MTT to purple formazan, which has an absorbance maximum at 570nm. The amount of formazan produced can be measured using a spectrophotometer and is proportional to the number of viable cells. When cells die, they lose their metabolic activity and are unable to convert MTT resulting in no visible color change.

6.1.2 Introduction of Cytokines

6.1.2.1 History of cytokine

In 1957, Alick lsaacs and Jean Lindenmann studied the interference of heat-killing influenza virus on the growth of live virus in chicken chorionic chorioallantoic membrane fragments and found that heat-inactivated influenza virus incubated with membranes produced a substance that interfered with infection and replication of the live virus. This substance (Interferon-alpha) was the first discovered cytokine and was later named interferon because of its interfering effects.[6] After this, other types of interferons and other cytokines have been discovered. Scientists respectively named them according to their different functions, interleukin (IL), interferon (IFN), tumor necrosis factor(TNF), colony stimulating factor (CSF), chemokine, and growth factor (GF).

Cytokines are a class of low molecular weight polypeptide molecules that are responsible for communication between nearby cells, particularly cells of hematopoietic origin. Cytokines from cell proliferation involving inflammation, immunological, migration, fibrosis, and repair each significant biological processes like angiogenesis. [7] Cytokines produced mainly by helper T cells (Th cells) and macrophages. Although they can be transiently induced and secreted by almost all nucleated cells. (Figure. 6.1) [8]

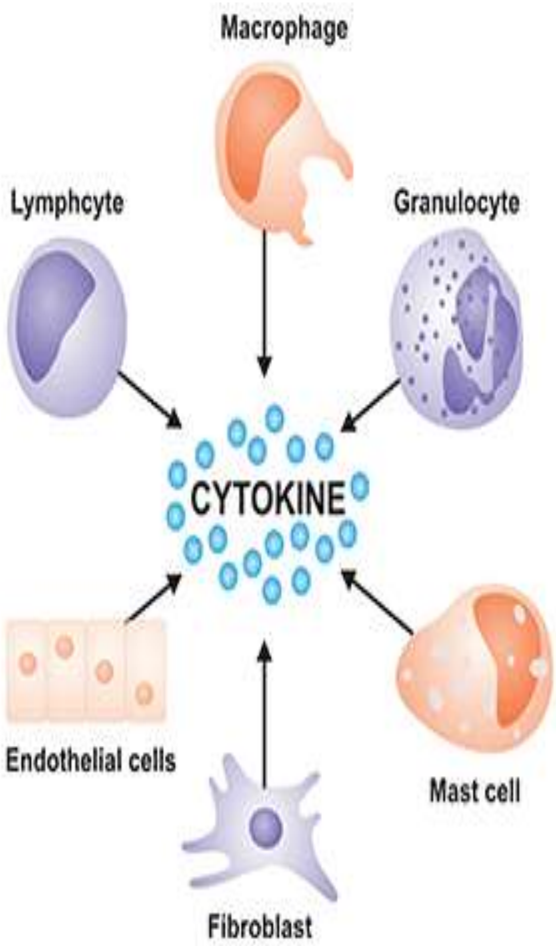


Figure 6.1 Various cells that produce cytokines.

6.1.2.2 Role of Cytokines

Cytokines exert biological effects by binding to corresponding cytokine receptors on the cell surface. The combination of cytokines and their receptors initiates complex intracellular molecular interactions that ultimately cause changes in cellular gene transcription. (Figure 6.2) [9] The role of cytokines is networked. In other words, each cytokine can act on a variety of cells; each cell can be regulated by a variety of cytokines; different cytokines have synergistic or mutually restrictive effects. It thereby constitutes a complex cytokine immune regulation network. [10]

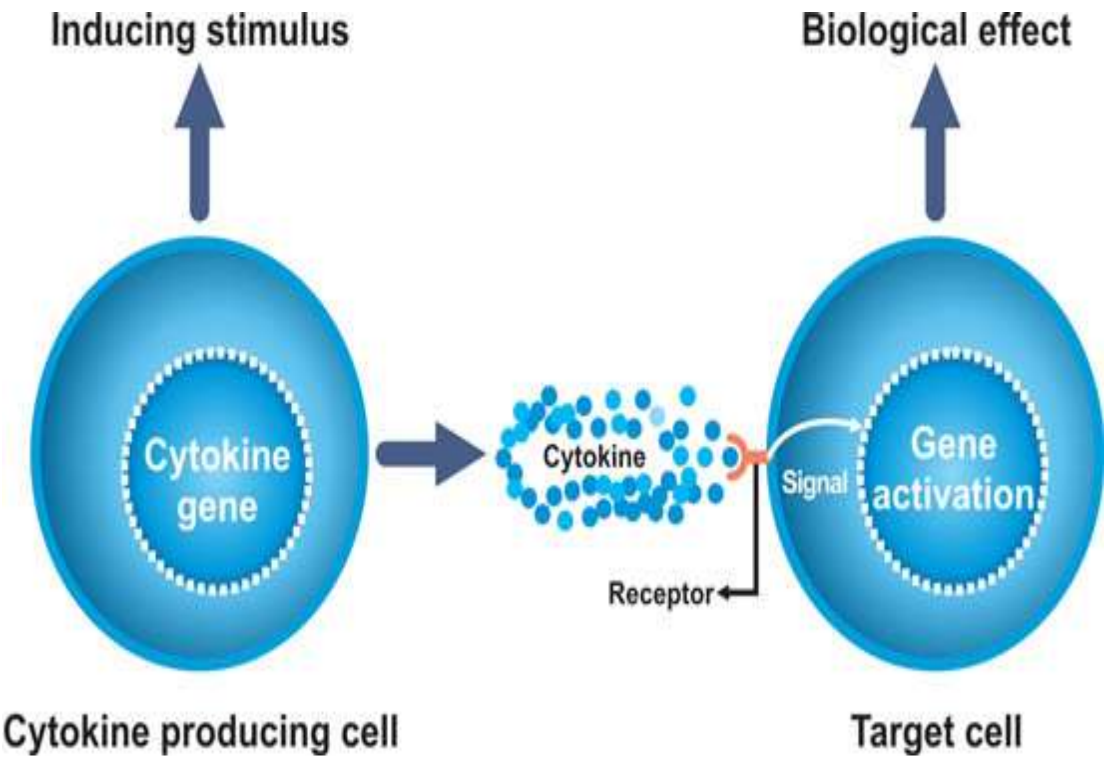


Figure 6.2 The process by which cytokines work [11]

Cytokines are biomarkers of inflammation-based diseases, so almost all types of diseases involve cytokines as potential biomarkers. Elevated cytokine concentrations indicate that activation of the cytokine pathway is associated with inflammation. Therefore, cytokine measurement is very important, it helps to elucidate the molecular level of immune regulation mechanism, disease prevention, diagnosis, and treatment. [12] For example, interferon-γ may be useful in the treatment of chronic granulomatous disease and bone sclerosis disease and interferon-α for the treatment of hepatitis C and Juvenile laryngeal papillomatosis. Interferon beta is applied to treat multiple sclerosis, and interleukin-2 (IL-2) can treat a variety of metastatic cancer, in particular the use of genetic engineering techniques producing a recombinant cytokine, which has been used to treat tumors, infection, inflammation, and other hematopoietic disorders. And these therapies receive good effect and are prospective in a very broad application.

6.1.2.3 Cytokine Detection Methods

1. Real-time Quantitative Polymerase Chain Reaction Assay
2. Enzyme-linked Immunosorbent Assay
3. Enzyme-linked Immunosorbent Site Enzyme-linked Aggregate Exposure
4. Cytokine Bead Array (CBA)
5. Immunohistochemistry

❖ **Enzyme-linked Immunosorbent Assay (ELISA)**

Enzyme-linked immunosorbent assay (ELISA) is a method that utilizes the specific binding of an antibody molecule to an antigen molecule and separates the free heteroprotein from the target protein bound to the solid phase carrier and uses a special marker to qualitatively or quantitatively analyse it. It allows the detection of cytokine secretion at the protein level. It uses an enzymatic reaction that produces colour. The intensity of the colour is related to the amount of protein present. ELISA platforms are widely used to quantify cytokines. When multiple cytokines in a single sample and measuring a need to develop a variety of ELISA. This is time-consuming and labour intensive and requires a relatively large number of tissue samples.

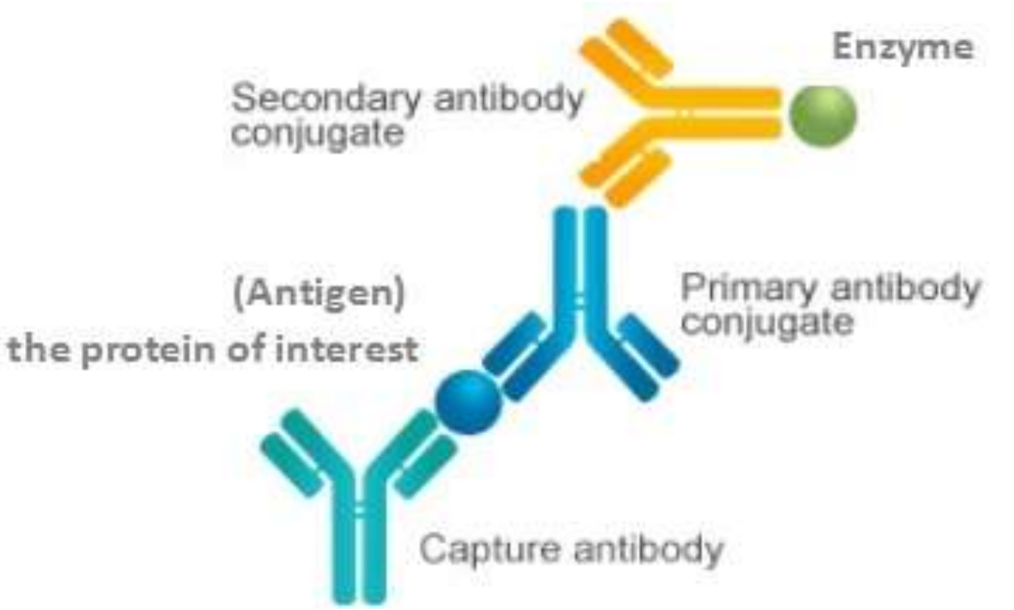


Figure 6.3 Sandwich ELISA

Due to the amplifying potential of enzyme labels, immunoassays that use enzyme-conjugated antibodies have become increasingly popular because of their high specificity and sensitivity. In 1971, Engvall and Perlmann coined the term "enzyme-linked immunosorbent assay" which is perhaps better known by the acronym, "ELISA", to describe an enzyme-based immunoassay method which is useful for measuring antigen concentrations. (Figure 6.3) [13]

Cytokine sandwich ELISA is a highly sensitive enzyme immunoassay designed to specifically detect and measure the concentration of soluble cytokines and chemokines. The process begins with the use of highly purified anti-cytokine antibodies, known as capture antibodies, which are noncovalently adsorbed onto plastic microwell plates, mainly through hydrophobic interactions. After washing the plates to remove any unbound antibodies, these immobilized antibodies capture soluble cytokine proteins from the samples applied to the plate. Unbound material is then washed away, and the captured cytokines are detected using biotin-conjugated anti-cytokine antibodies, known as detection antibodies, followed by an enzyme-labeled avidin or streptavidin stage. A chromogenic substrate is added, and the resulting color change, generated by the enzyme-linked detection reagents, is measured using a spectrophotometric ELISA plate reader at the appropriate optical density (OD). When the plate reader is connected to a computer, it simplifies data storage and reanalysis.

In a sandwich ELISA assay, a standard curve is created by preparing serial dilutions of a cytokine protein solution with a known concentration. These standard curves, also known as calibration curves, are typically plotted with the standard cytokine protein concentration (expressed in ng or pg of cytokine per ml) on the x-axis and the corresponding average OD value of replicate samples on the y-axis. The cytokine concentrations in the test samples are then determined by interpolating their OD values from this standard curve. This process is made easier by using an ELISA computer software program. Generally, it is useful to perform a dilution series of the unknown samples to be assured that the OD will fall within the linear portion of the standard curve. Depending on the nature of the ELISA reagents used, investigators may choose to apply different curve fit analysis to their data, including either linear-log, log-log, or four-parameter transformations. [14]

Although opinions differ, one convention for determining the ELISA sensitivity is to choose the lowest cytokine concentration that gives a signal which is at least two or three standard deviations above the mean background signal value. Because of the enzyme-mediated amplification of the detection antibody signal, the sandwich ELISA can measure physiologically relevant (ie, > 5-10 pg/ml) concentrations of specific cytokine and chemokine proteins, which are present in mixed cytokine milieus, e.g., from stimulated lymphocyte culture supernatants. Although many different types of enzymes have been used, horseradish peroxidase (HRP) and alkaline phosphatase (AKP) are the enzymes that are often employed in ELISA methods. [15]

In inflammation, macrophage plays an important role by producing reactive oxygen species (ROS), reactive nitrogen species (RNS), cytokines such as interleukin-1β (IL-1ß), IL-6, tumor necrosis factor-α (TNF-α), IL-4, IL-10 and inflammatory mediator nitric oxide (NO) and prostaglandin (PGE). Exposure of bacterial lipopolysaccharides (LPS) has been found to increase the mRNA expression of those inflammatory cytokines and mediators. LPS is a bacterial endotoxin which stimulates innate immunity by regulating inflammatory mediator such as TNF-α, IL-6, and IL-4. The suppression of inflammatory mediator synthesis has been known to be one of the useful therapeutic strategies in the treatment of inflammatory diseases. Lipopolysaccharides (LPS) is the major outer surface membrane component of Gram-negative bacteria and a biologically active component. Thus, LPS-mediated inflammatory response is a major inflammation source from exposure to gram-negative bacterial infection. During this inflammatory response, macrophages first help in endocytosis of bacterium debris, followed by generation of inflammatory cytokines and expansion of the local inflammatory response. [16,17]

In the present study, in order to investigate the mechanisms of LPS induced-inflammatory response in macrophage cells, the effects of LPS exposure on the expression of interleukin (IL)-6, IL-4, IL-10, tumor necrosis factor (TNF)-α were examined in human monocytes THP-1 cells. Subsequently, inflammation-related transcription factors and intracellular signaling pathways that may be involved in LPS-induced pro-inflammation cytokine production were explored. The aim of this study was to investigate the effects of aqueous and methanolic extracts of *Adhatoda vasica, Calotropis procera* and *Rosa indica* extracts on inflammation using an in vitro model LPS-stimulated human monocytic THP-1 cells and monitoring of the production of inflammatory cytokines, IL-6, IL-4, TNF- α, and IL-10.

6.2 Materials and methods

Rationale for selecting THP-1 cells

The rationale for selecting THP-1 cells for inflammation research revolves around their human monocytic origin, ability to differentiate into macrophage-like cells, responsiveness to inflammatory stimuli, and consistent, reproducible behaviour in in vitro settings. THP-1 cells are capable of producing a range of cytokines and other inflammatory mediators upon activation (e.g., TNF-α, IL-6, IL-10, IL-4).

6.2.1 Cell culture

THP-1 human monocytic cells were obtained from the National Center for Cell Sciences (Pune, India). The cells were maintained in RPMI-1640 (Himedia, Mumbai India) supplemented with 10% fetal calf serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 1x Antibiotic, Antimycotic Solution, and incubated at 37°C with 5% CO_2. All experiments were performed with exponentially growing cells. For differentiation to a macrophage phenotype, THP-1 cells were adjusted to the desired concentrations for each experiment and incubated with 50 µM phorbol myristate acetate (PMA) diluted in complete culture medium for 24 h. Then, the cells were washed with serum-free RPMI-1640 medium prior to each experiment.

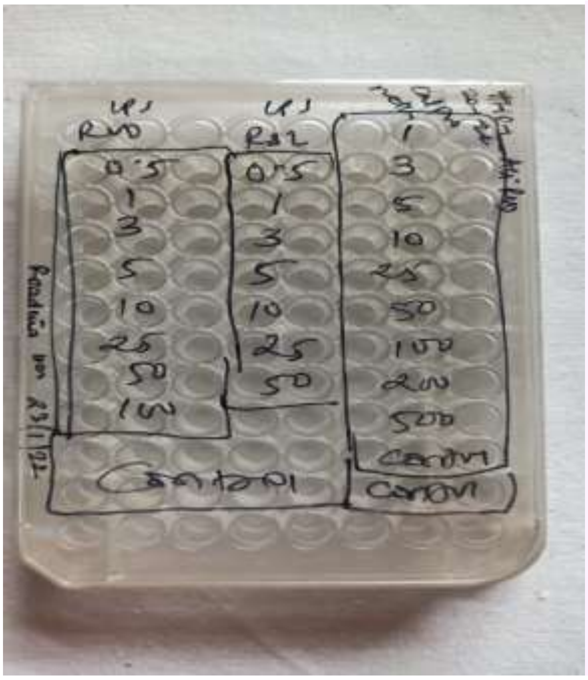
6.2.2 Cell viability assay by MTT assay

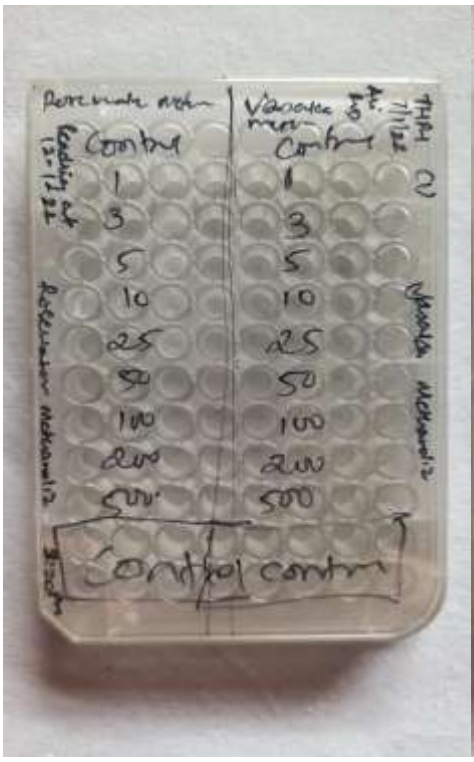
PMA (Phorbol myristate acetate) was used to convert the cells from monocytes to macrophages and LPS (Lipopolysaccharides) was used to induce inflammation in the cells. THP-1 cells (1×10^4 cells/well, pretreated with 20 µM PMA (Phorbol myristate acetate- to convert the cells from monocytes to macrophages) were plated in 96-well plates for 24 h. The cells were then stimulated with the indicated concentration of LPS (Lipopolysaccharides- to induce inflammation in the cells) or in combination with aqueous and methanolic extracts of *Adhatoda vasica, Calotropis procera* and *Rosa indica* for 24h. Normal incomplete RPMI-1640 media without LPS was used as a negative control. Subsequently, MTT solution was added for 4 h, following which the supernatants were removed and 150 µl dimethyl sulfoxide was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm.

6.2.3 Evaluation of anti-inflammatory activity of extracts by ELISA Test

THP-1 cells (1×10^5 cells/well, pretreated with 50 µM PMA) were seeded in triplicate into 24-well tissue culture plates for 24 h. The cells were then stimulated with LPS (1 µg/ml) for 24 h.

The levels of human cytokines were measured in the collected supernatants with human IL-6 (Cat. No.-KLU0007), IL-10 (Cat. No.-KLU0006), IL-4(Cat. No.- KB1066) and TNF-α (Cat. No.-KLU0003), ELISA kits (Krishgen Biosciences, India), according to the manufacturer's protocols.



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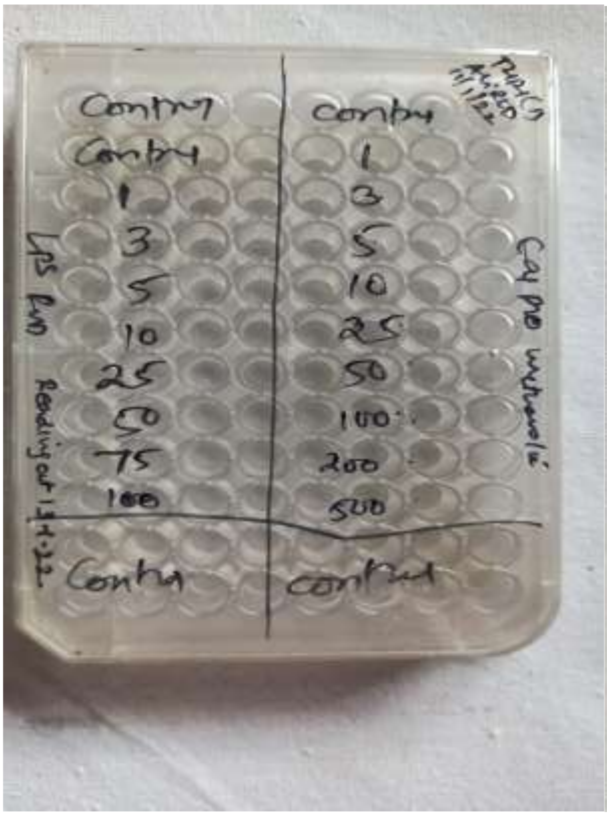
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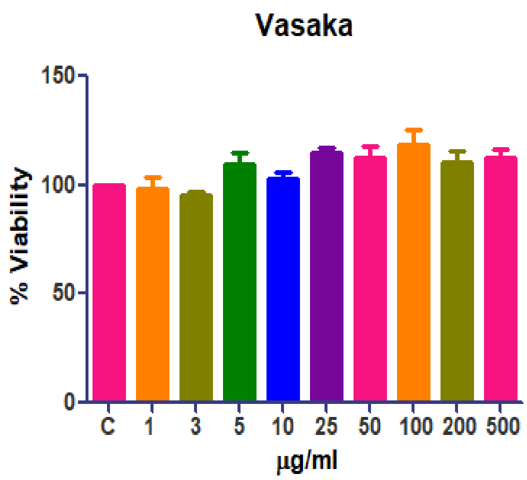
6.3 Results and Discussion

6.3.1 Cell viability assay

The results of MTT assay were graphically reported for eight samples including three aqueous extracts of each plant, three methanolic extracts of each plant, mixture of three aqueous extracts and mixture of three methanolic extracts. The graphs depicted the % viability on the y-axis and the concentration (1 µg/ml to 500 µg/ml) of the extracts on x-axis.



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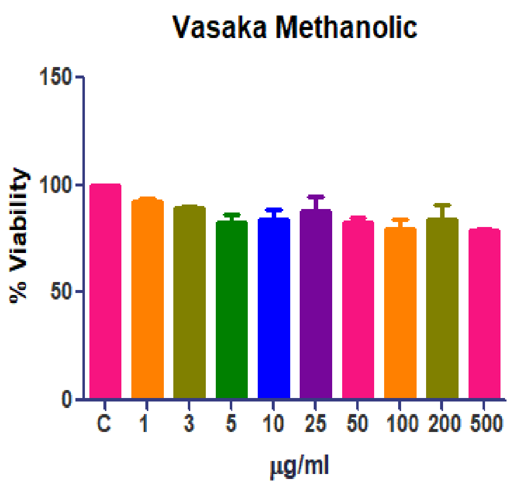

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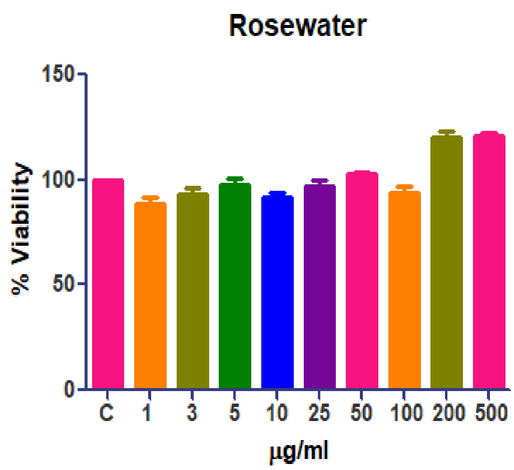

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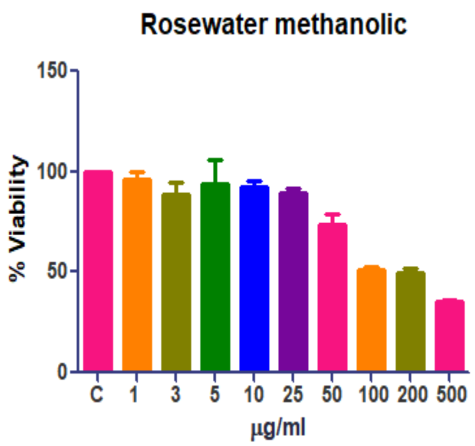

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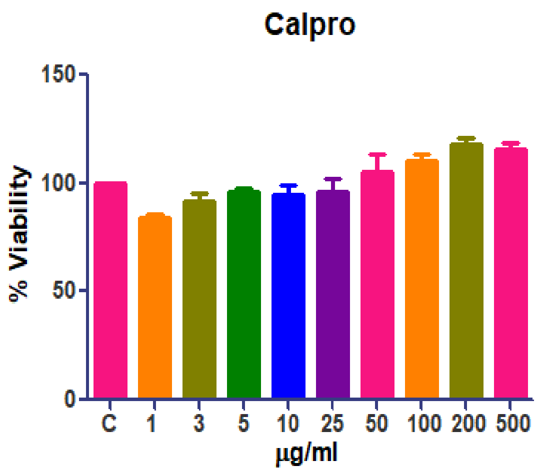

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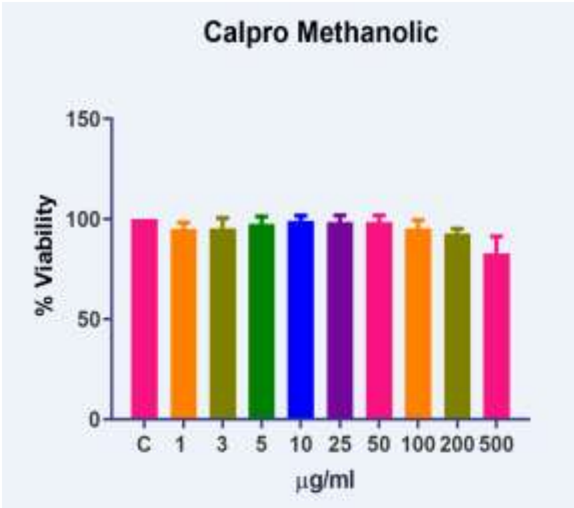

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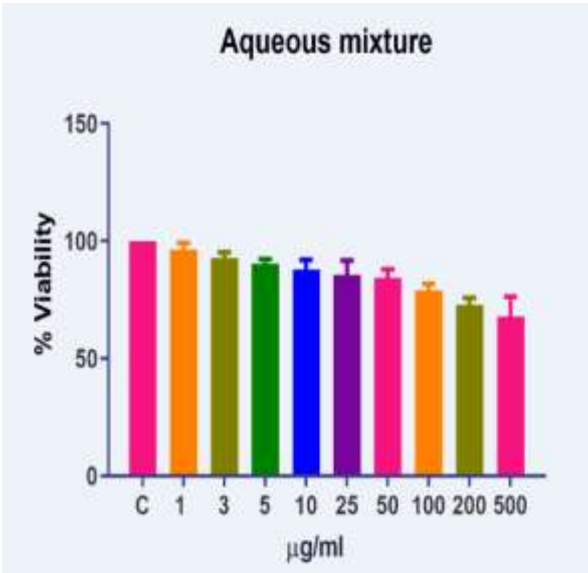

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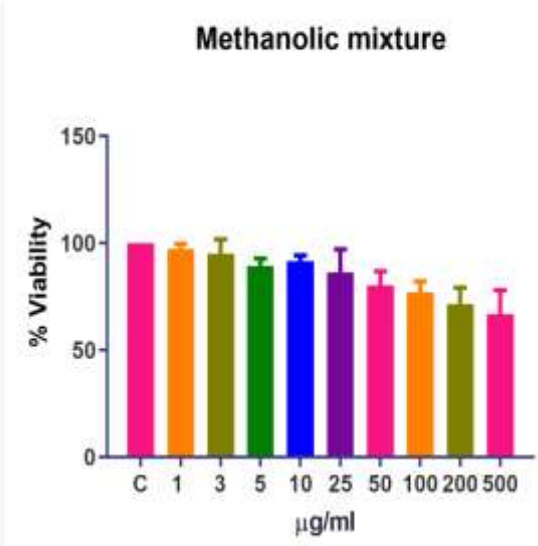


H

Figure 6.4. Effects of various plant extracts on THP-1 cell viability.

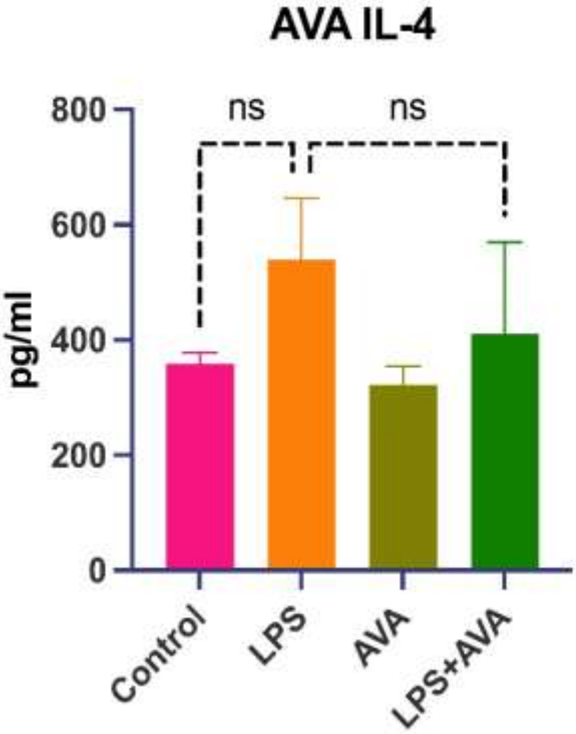
THP-1 cells were treated with different extracts and mixtures for 24 h and cell viability was assessed by the MTT assay. The extracts tested were: (A) Aqueous extract of *Adhatoda vasica*, (B) Methanolic extract of *Adhatoda vasica*, (C) Aqueous extract of *Rosa indica*, (D) Methanolic extract of *Rosa indica*, (E) Aqueous extract of *Calotropis procera*, (F) Methanolic extract of *Calotropis procera*, (G) Aqueous mixture of extracts of *Calotropis procera, Rosa indica,* and *Adhatoda vasica*, and (H) Methanolic mixture of the three. Data are presented as mean ± SEM from at least three independent experiments. Statistical significance compared to the control was determined using one-way ANOVA.

6.3.2 Conclusion of MTT assay

The cell viability assay, conducted across a concentration range of 1 µg/ml to 500 µg/ml, revealed that none of the individual plant extracts or the mixture of all three plant extracts affected cell viability, as measured by the MTT assay. (Figure 6.4) These findings suggest the absence of lethality or local toxicity following administration of the highest dose (500 µg/ml). However, it is noteworthy that lethality was observed with the Rosa indica methanolic extract at doses exceeding 100 µg/ml. Further investigation into the underlying mechanisms of this lethality and potential dose-dependent effects is warranted to ensure the safety and efficacy of the plant extract. The MTT assay showed that the aqueous extracts are better than the methanolic extract. So, only water extracts were chosen for further study.

6.3.3 Conclusion of Evaluation of anti-inflammatory activity of extracts by ELISA Test

The results of the ELISA test were graphically reported for each cytokine individually with six extracts. (Three aqueous extracts of each plant and three methanolic extracts of each plant). The graphs depicted the concentration of each cytokine (such as TNF-alpha, IL-4, IL-10) on the y-axis and the concentration of the control cells, cells with LPS, only plant extract and LPS with extracts on the x-axis. Each extract was evaluated separately, illustrating its effect on the production or inhibition of the specific cytokine. The graphical representation allowed for a visual assessment of the dose-response relationship between the plant extracts and cytokine levels, providing valuable insights into their anti-inflammatory activity and potential therapeutic effects.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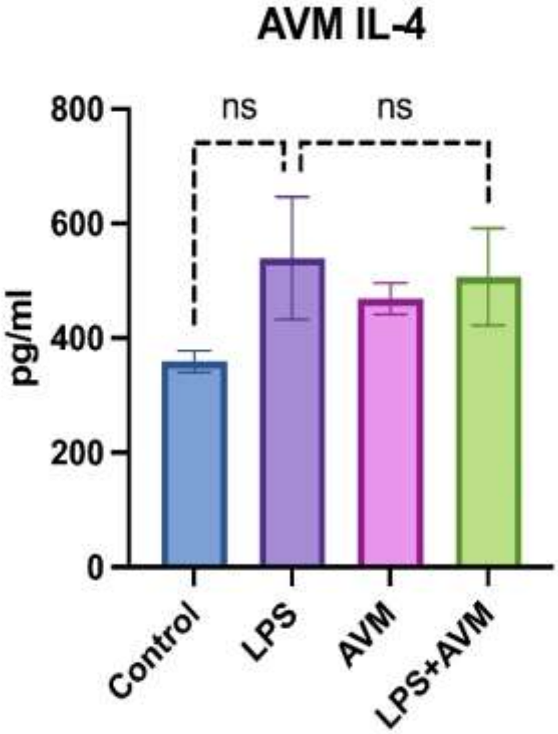
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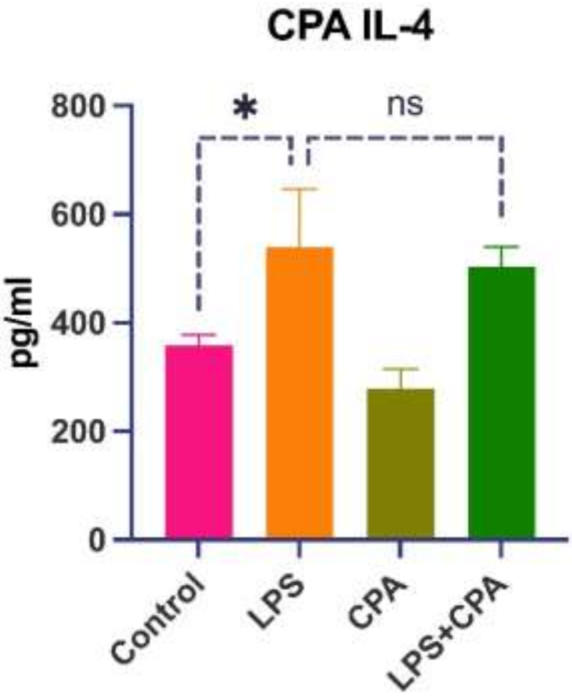
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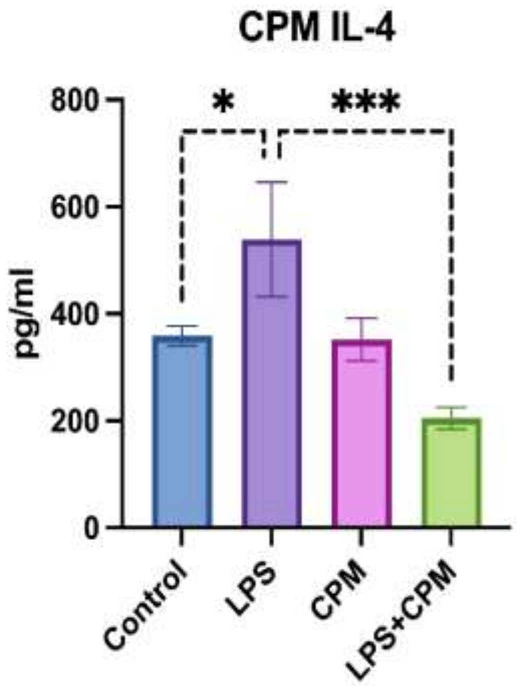
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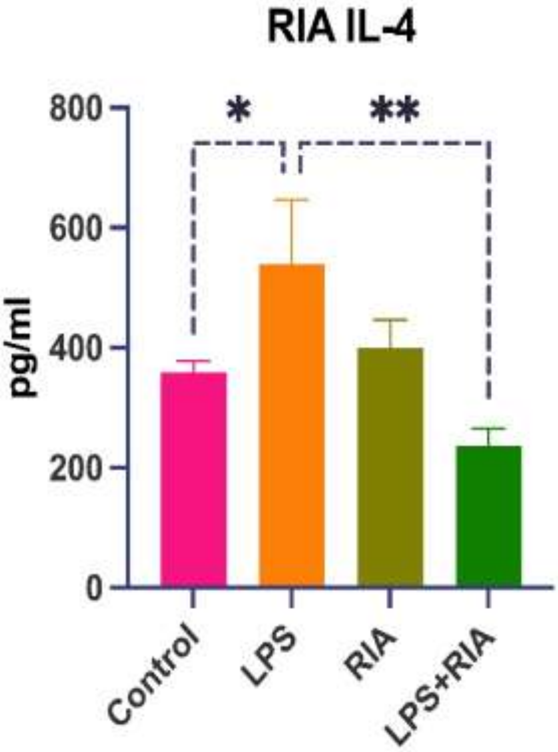

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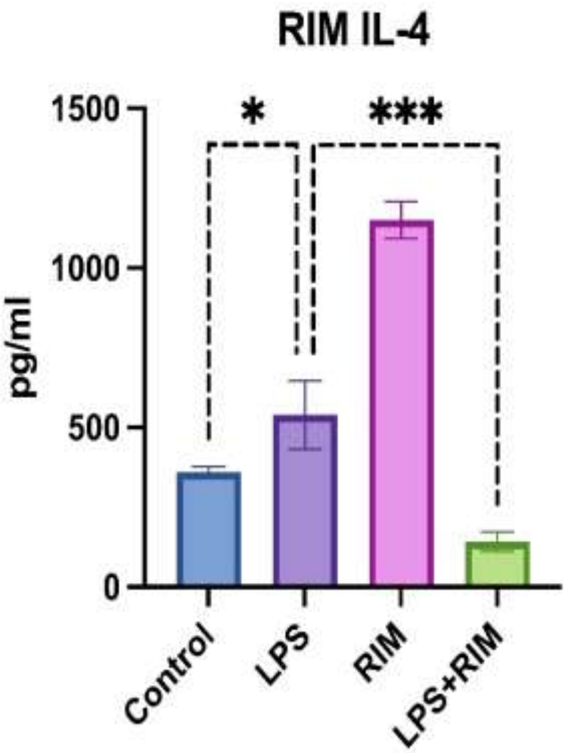

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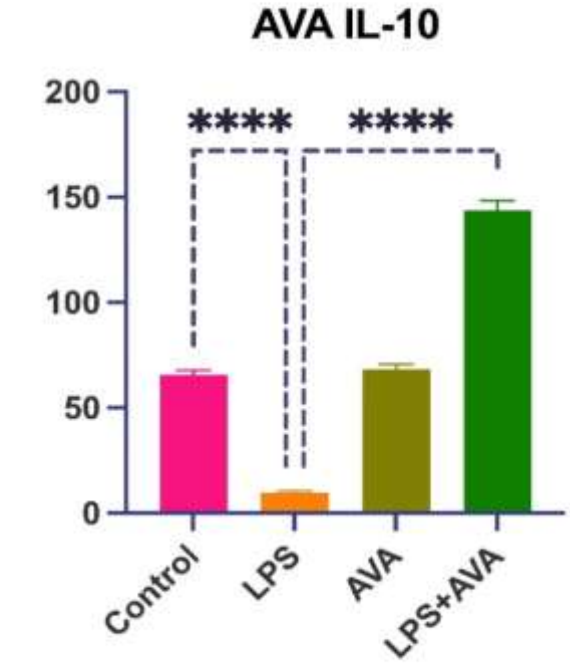
Figure 6.5. Pro-inflammatory IL-4 production in response to various plant extracts.

IL-4 levels were quantified in THP-1 cells in presence/absence of LPS with different plant extracts: (a) Aqueous extract of *Adhatoda vasica*, (b) Methanolic extract of *Adhatoda vasica*, (c) Aqueous extract of *Rosa indica*, (d) Methanolic extract of *Rosa indica*, (e) Aqueous extract of *Calotropis procera*, (f) Methanolic extract of *Calotropis procera*. Data represent the mean ± SEM from at least three independent experiments. Statistical significance relative to the untreated control was determined using one-way ANOVA. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

6.3.3.1 Discussion on IL-4 assay

Based on graphs, (Figure 6.5) *Rosa indica* aqueous extract treatment showed higher IL-4 inhibition activity compared to the other extracts. It can be seen that all other aqueous extracts were able to inhibit IL-4 production in LPS-induced cells. The LPS induction was successfully increase the IL-4 concentration, showed by significantly high IL-4 level in positive control (LPS-induced cells without treatment) compared to the negative control (normal cells without LPS induction).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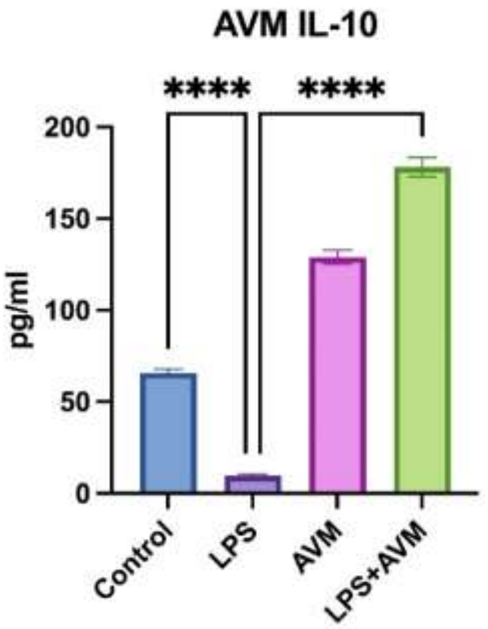
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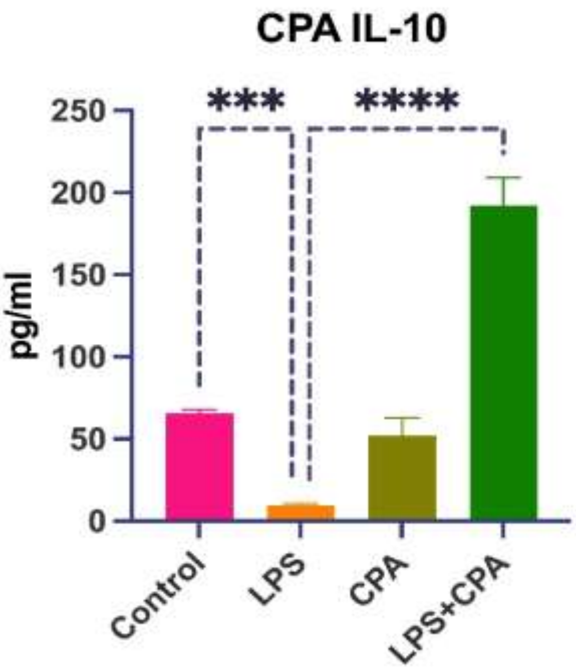
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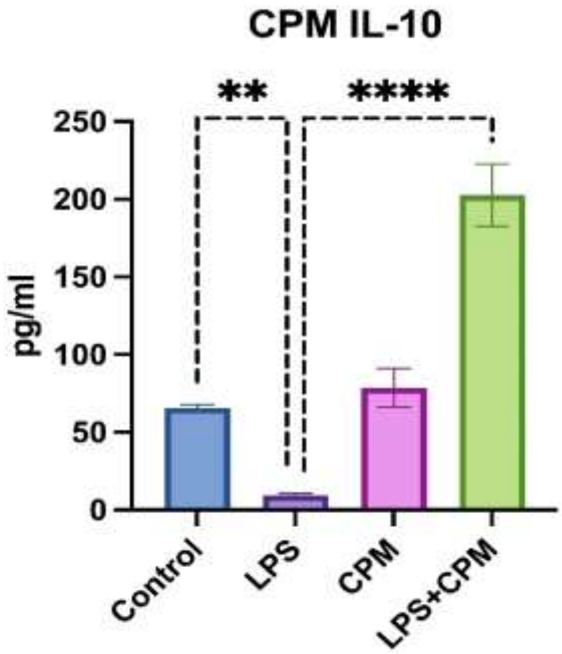
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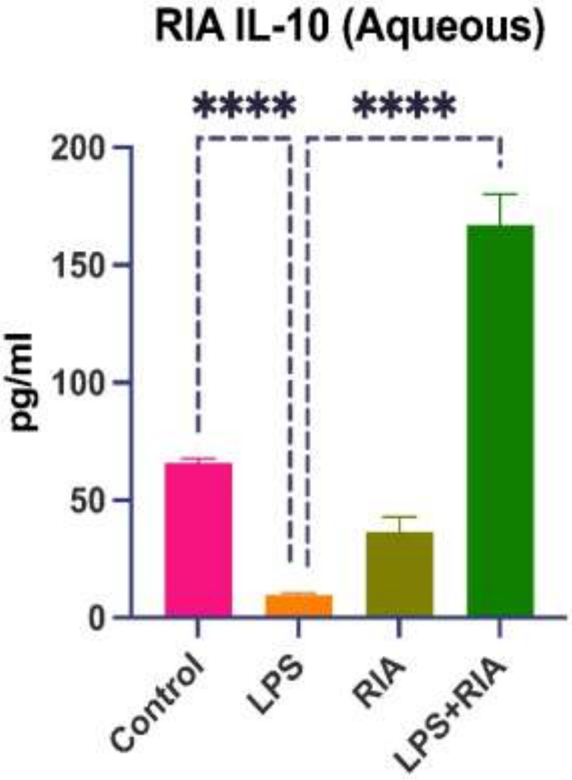

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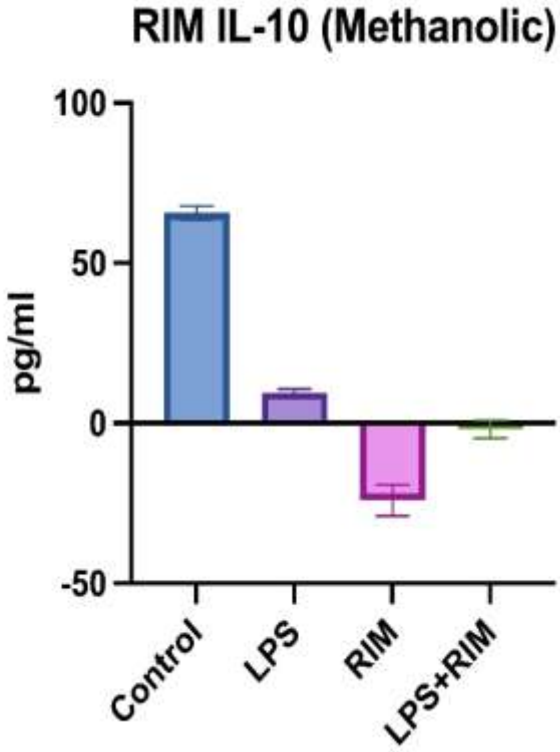

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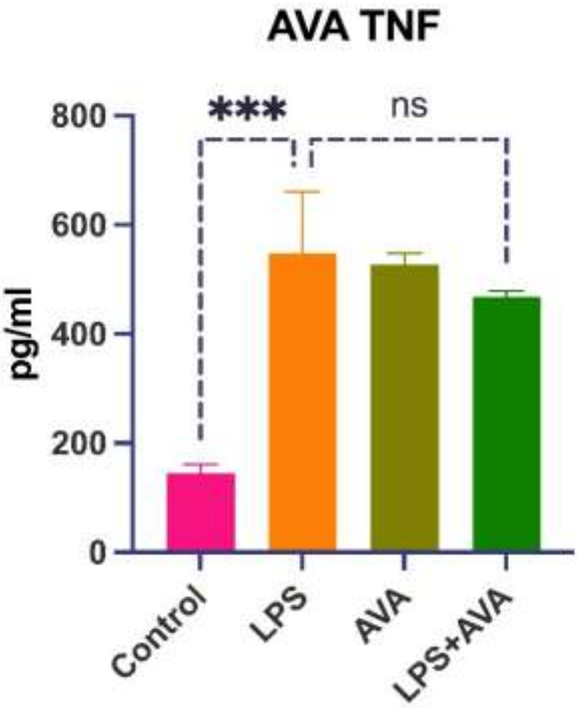
Figure 6.6. Anti-inflammatory IL-10 production in response to various plant extracts. IL-10 levels were quantified in THP-1 cells in presence/absence of LPS with different plant extracts: (a) Aqueous extract of *Adhatoda vasica*, (b) Methanolic extract of *Adhatoda vasica*, (c) Aqueous extract of *Rosa indica*, (d) Methanolic extract of *Rosa indica*, (e) Aqueous extract of *Calotropis procera*, (f) Methanolic extract of *Calotropis procera*. Data represent the mean ± SEM from at least three independent experiments. Statistical significance relative to the untreated control was determined using one-way ANOVA. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

6.3.3.2 Discussion on IL-10 assay

IL-10 is having anti-inflammatory property. LPS cause inflammation. So, when the cells were induced with LPS, it decreases the concentration of IL-10. Based on graphs, (Figure 6.6) *Calotropis procera* aqueous extract treatment showed higher IL-10 concentration as compared to the other extracts. So, it indicates best anti-inflammatory activity than other extracts. It can be seen that other aqueous extracts were able to increase IL-10 production in LPS-induced cells. The LPS induction was successfully decrease the IL-10 concentration, showed by significantly low IL-10 level in positive control (LPS-induced cells without treatment) compared to the negative control (normal cells without LPS induction).



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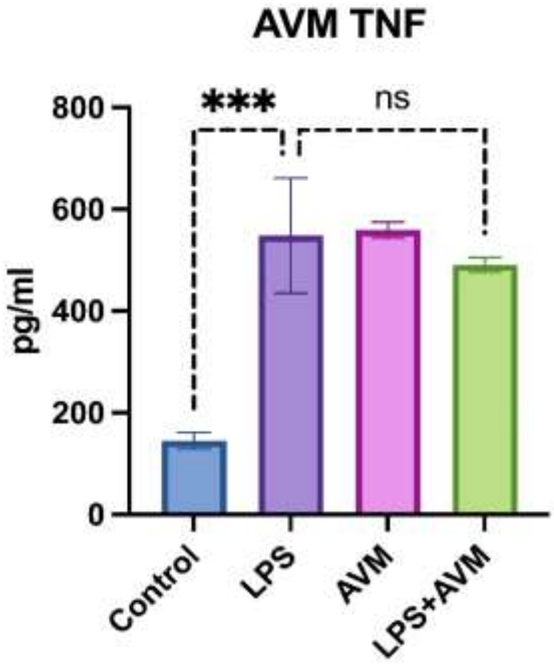
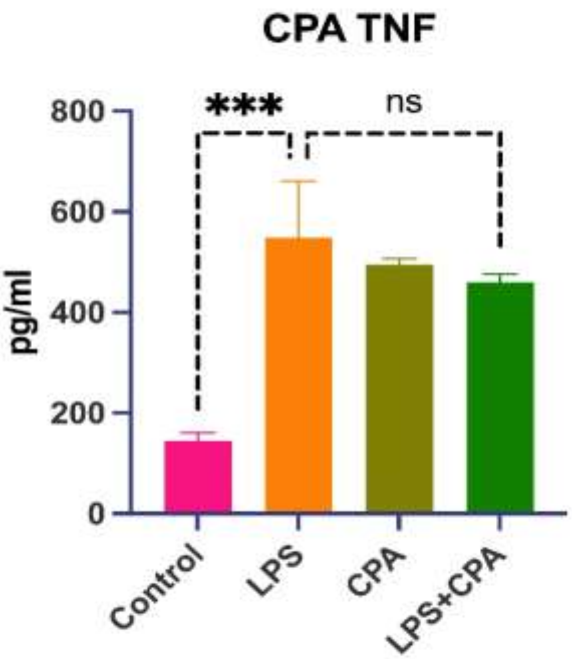


B



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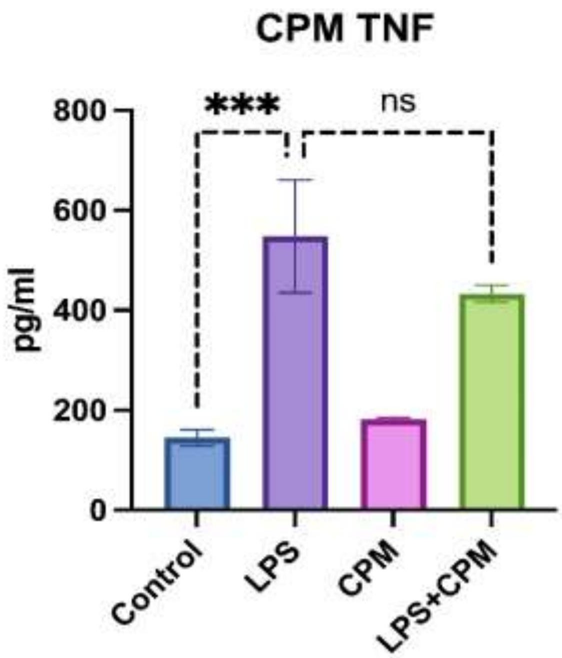

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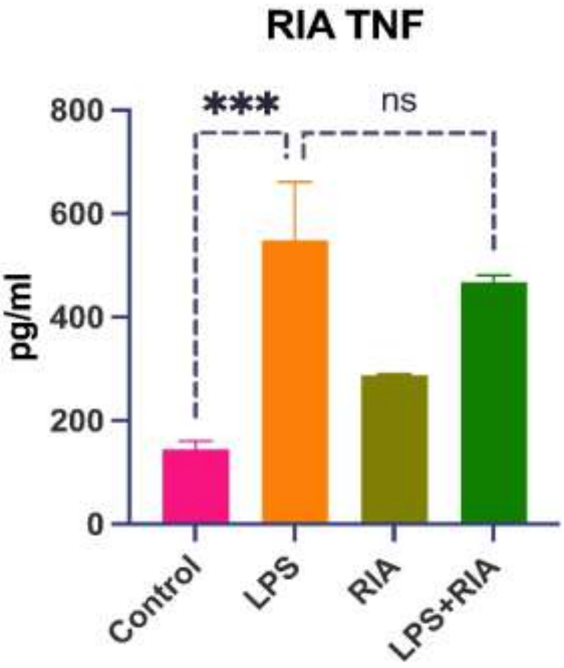
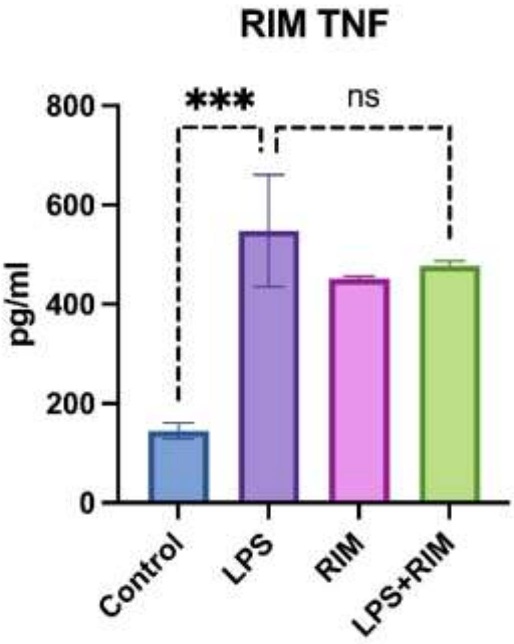


E



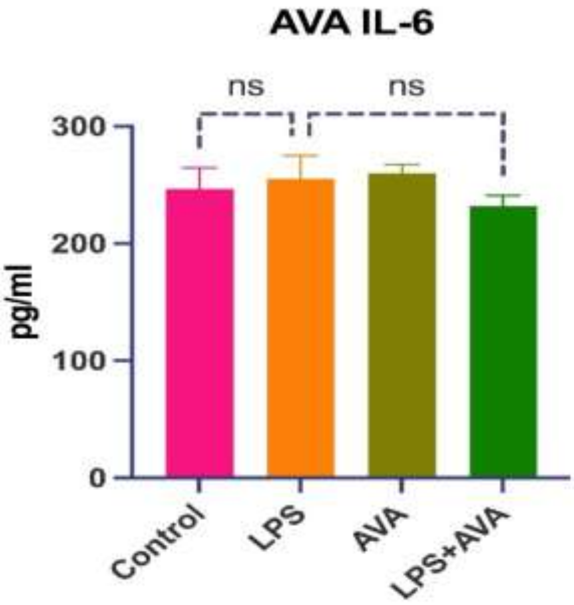
F

Figure 6.7 **Pro-inflammatory TNF-α production in response to various plant extracts.**

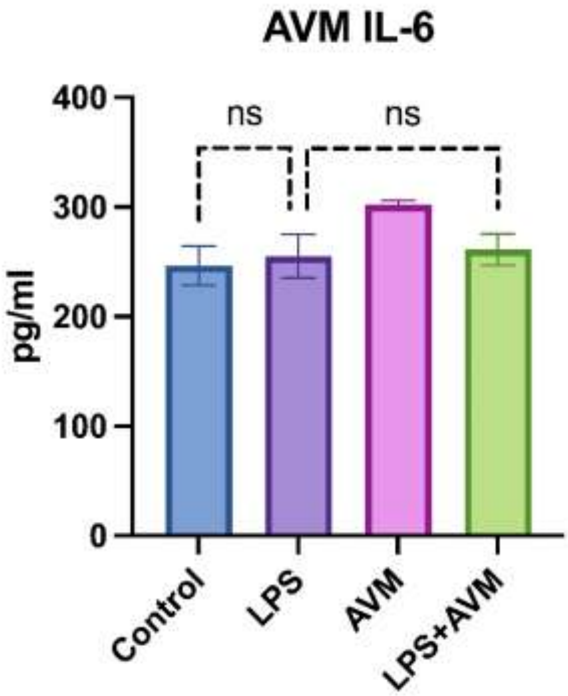
TNF-α levels were quantified in THP-1 cells in presence/absence of LPS with different plant extracts: (A) Aqueous extract of *Adhatoda vasica*, (B) Methanolic extract of *Adhatoda vasica*, (C) Aqueous extract of *Rosa indica*, (D) Methanolic extract of *Rosa indica*, (E) Aqueous extract of *Calotropis procera*, (F) Methanolic extract of *Calotropis procera*, Data represent the mean ± SEM from at least three independent experiments. Statistical significance relative to the untreated control was determined using one-way ANOVA. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

6.3.3.3 Discussion on TNF-α Assay

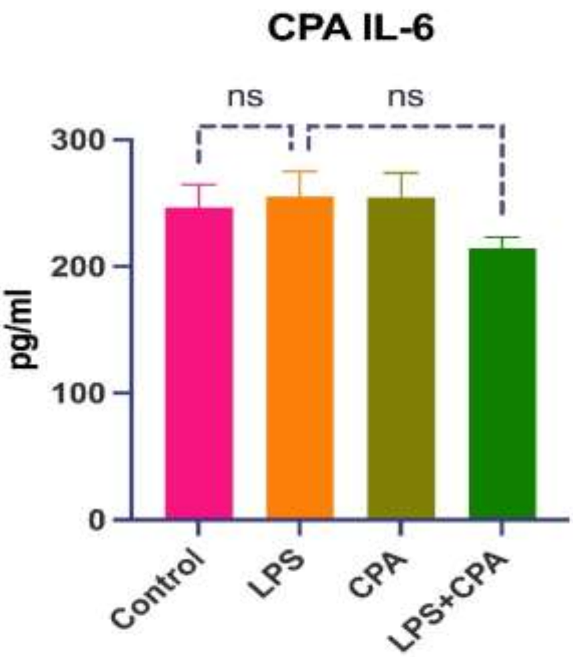
Based on graphs (Figure 6.7), we could not conclude that the concentration of TNF-α was significantly decrease or increase after the treatment of the extracts. Inflammation involves a complex interplay of various cytokines, chemokines, and cellular responses. TNF-α might be just one of many factors involved in the inflammatory process, and its effects may be modulated by other signalling molecules or pathways. The methodologies employed in the studies, including assay sensitivity, detection methods, and data analysis techniques, could influence the interpretation of results. Issues such as assay variability, sample contamination, or experimental artifacts could contribute to inconsistent findings.


AVA IL-6
ns
ns
300
200
100
0
pg/ml
Control
LPS
AVA
LPS+AVA

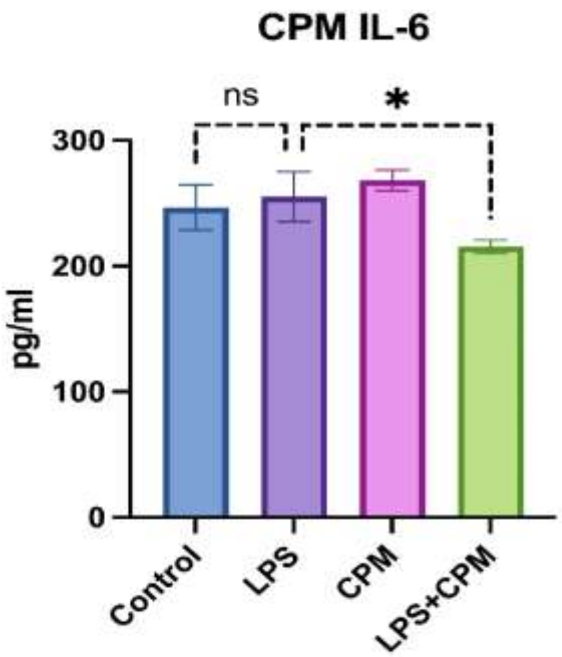
A


AVM IL-6
400
300
200
100
0
ns
ns
pg/ml
Control
LPS
AVM
LPS+AVM

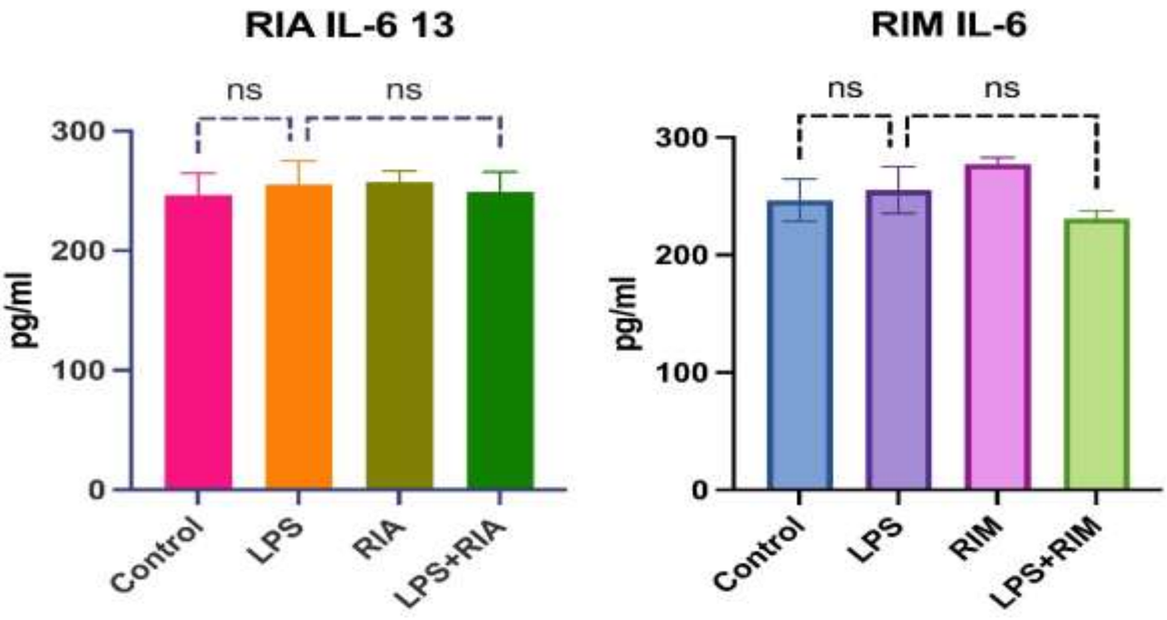
B


CPA IL-6
ns
ns
300
200
100
0
pg/ml
Control
LPS
CPA
LPS+CPA

C


CPM IL-6
ns
*
300
200
100
0
pg/ml
Control
LPS
CPM
LPS+CPM

D



E F

Figure 6.8 (**Anti-inflammatory IL-6 production in response to various plant extracts.**

IL-6 levels were quantified in THP-1 cells in presence/absence of LPS with different plant extracts: (A) Aqueous extract of *Adhatoda vasica*, (B) Methanolic extract of *Adhatoda vasica*, (C) Aqueous extract of *Rosa indica*, (D) Methanolic extract of *Rosa indica*, (E) Aqueous extract of *Calotropis procera*, (F) Methanolic extract of *Calotropis procera*, Data represent the mean ± SEM from at least three independent experiments. Statistical significance relative to the untreated control was determined using one-way ANOVA. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

6.3.3.4 Discussion on IL-6 Assay

Based on the graphical analysis, it appears that the concentration of IL-6 in the control and the other three samples remained consistent. This indicates that neither lipopolysaccharide (LPS) nor the extracts had a significant effect on the concentration of IL-6. Therefore, the behavior of IL-6 cannot be reliably predicted from the graphs alone. Further investigation and additional experimental data may be necessary to elucidate the role of IL-6 in the context of the experimental conditions and the effects of LPS and the extracts on its production.

6.4 Conclusion of *in-vitro* assay

The aqueous extracts of *Rosa indica*, *Adhatoda vasica*, and *Calotropis procera* have demonstrated significant anti-inflammatory activity by reducing the production of pro-inflammatory cytokines IL-4 and TNF-α in activated THP-1 cells. We propose that the mechanism of action of these extracts involves the inhibition of the overproduction of inflammatory mediators, including TNF-α and IL-4. Additionally, these extracts have been observed to increase the levels of IL-10, which possesses anti-inflammatory properties. This modulation of cytokine levels suggests the potential of these plant extracts as therapeutic agents for managing inflammatory conditions.

6.5 References

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