RESEARCH PAPER

Separation of the Active Ingredient Andrographolide from the Plant Andrographis Paniculata and Evaluation of Its Immunomodulatory Effects

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ABSTRACT
Andrographis paniculata is a medicinal plant widely recognized for its diverse pharmacological activities, including immunomodulatory, anti-inflammatory, and antioxidant effects. Andrographolide, the principal bioactive diterpenoid lactone of A. paniculata, has been extensively investigated for its ability to modulate immune and inflammatory responses. However, effective isolation and characterization of Andrographolide are essential for establishing its biological activity and potential therapeutic applications. This study aimed to isolate and characterize Andrographolide from Andrographis paniculata and evaluate its immunomodulatory activity using appropriate in vitro models. We subjected the dried plant material to solvent extraction, followed by chromatographic separation and purification, to obtain an andrographolide-enriched fraction. The isolated compound was characterized using appropriate chromatographic and spectroscopic analytical techniques. We then evaluated the immunomodulatory potential of the isolated andrographolide using in vitro immune-cell-based assays, assessing relevant parameters including macrophage functional activity and cytokine production. We included appropriate positive and negative controls to assess the immunomodulatory effects of the isolated compound. Chromatographic purification yielded a fraction corresponding to Andrographolide, which demonstrated an analytical profile consistent with the target compound. The isolated Andrographolide showed promising immunomodulatory activity, as indicated by changes in immune cell responses and cytokine production compared with the respective controls. These findings suggest that Andrographolide can modulate cellular immune responses and may influence molecular pathways involved in immune regulation.

Keywords *: Andrographis paniculata*; Andrographolide; Chromatographic separation; Characterization; Immunomodulation; Macrophages; Cytokines..
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1. INTRODUCTION

The immune system is a complex, highly coordinated biological network that protects the body against invading pathogens, foreign substances, and abnormal cells while maintaining tolerance **to** self-antigens. It comprises immune cells such as macrophages, monocytes, dendritic cells, natural killer cells, B lymphocytes, and T lymphocytes, along with soluble mediators including cytokines, chemokines, antibodies, and complement proteins. These components interact through multiple signaling pathways to initiate, regulate, and terminate immune responses. An effective immune response requires a delicate balance between immune activation and immune suppression. Excessive or prolonged activation can contribute to chronic inflammation, autoimmune disorders, and tissue injury, whereas inadequate immune responses may increase susceptibility to infections and impair immune surveillance. Consequently, identifying bioactive compounds that regulate immune responses has become an important area of pharmacological and biomedical research.

Immunomodulation refers to altering immune system activity by stimulating, suppressing, or regulating specific immune mechanisms. Immunomodulatory agents may enhance host defense when immune function is inadequate or attenuate excessive inflammatory responses associated with pathological conditions. Conventional immunomodulatory drugs, including corticosteroids, calcineurin inhibitors, and other immunosuppressive agents, are widely used in clinical practice. However, their long-term administration may be associated with adverse effects, including increased susceptibility to infections, metabolic disturbances, organ toxicity, and other treatment-related complications. These limitations have encouraged researchers to investigate naturally occurring compounds as potential sources of immunomodulatory agents. Plant-derived phytochemicals are particularly interesting because of their structural diversity and ability to interact with multiple cellular and molecular targets.

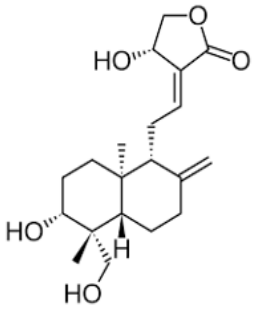
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Nevertheless, their pharmacological activity, safety, reproducibility, and mechanisms of action must be established through systematic experimental evaluation. Medicinal plants have historically contributed to the discovery of therapeutic agents and remain valuable starting materials for pharmacological research. Numerous plant-derived compounds exhibit anti-inflammatory, antioxidant, antimicrobial, and immunoregulatory activities. However, the therapeutic potential of a medicinal plant cannot be attributed uniformly to all its constituents. A plant extract's biological effects depend on its chemical composition, extraction procedure, active-constituent concentration, and interactions among its phytochemicals. Therefore, extracting, separating, purifying, and identifying individual bioactive compounds are essential steps in natural-product research. These investigations enable researchers to distinguish a specific compound's pharmacological effects from those of the crude extract and to establish a more reproducible relationship between chemical composition and biological activity. Among medicinal plants investigated for their pharmacological properties, Andrographis paniculata (Burm. f.) Nees has received considerable scientific attention because of its diverse diterpenoid constituents and reported anti-inflammatory, antioxidant, antimicrobial, and immunomodulatory properties. Andrographolide, its major bioactive labdane-type diterpenoid lactone, is one of the most extensively studied constituents of this plant. Its reported biological activities and interactions with inflammatory signaling pathways make it a promising candidate for further investigation into immune response regulation. Accordingly, separating andrographolide from A. paniculata, followed by chemical characterization and experimental assessment of immunomodulatory activity, represents a rational approach to investigating its potential pharmacological significance. Andrographis paniculata (Burm. f.) Nees belongs to the family Acanthaceae and is commonly known as Kalmegh in India and Chuanxinlian in traditional Chinese medicine. It is an annual herb found across tropical and subtropical Asia, including India, Sri Lanka, China, Thailand, and other Southeast Asian countries. In India, the plant has a long history of use in traditional medicine systems. Researchers have investigated its aerial parts, particularly the leaves and stems, for potential therapeutic applications in inflammatory conditions, infections, and liver-related disorders. Its traditional use has stimulated considerable scientific interest in identifying the chemical constituents responsible for its reported biological activities [1,2,3].

The pharmacological properties of A. paniculata are associated with a diverse range of secondary metabolites, including diterpenoid lactones, flavonoids, polyphenols, and other phytoconstituents. Among these, diterpenoids constitute an important class of bioactive compounds. Andrographolide is the principal and extensively investigated diterpenoid, while other related compounds include 14-deoxyandrographolide, 14-deoxy-11,12-didehydroandrographolide, and neoandrographolide. Although these compounds share structural similarities, their biological activities differ. Structural differences can influence their interactions with cellular targets and, consequently, their pharmacological effects [4,5,6].

The presence of multiple structurally related compounds poses a major challenge for standardizing plant extracts. A crude extract may contain Andrographolide together with other diterpenoids, flavonoids, pigments, and compounds that may contribute independently or synergistically to its biological activity. Consequently, the activity of an extract cannot automatically be attributed exclusively to Andrographolide. Therefore, separating and characterizing individual compounds is necessary to establish their specific biological contributions and improve the reproducibility of subsequent pharmacological experiments

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Andrographis Paniculata Plant

Fig. 1: Andrographolide

Chemical Characteristics and Pharmacological Significance

Andrographolide is a naturally occurring labdane-type diterpenoid lactone and is generally recognized as the major bioactive constituent of A. paniculata. Its molecular

structure contains a diterpenoid carbon skeleton, a lactone ring, unsaturated bonds, and hydroxyl groups. These structural features influence its physicochemical characteristics and interactions with biological systems. The compound has attracted substantial research interest because of its reported anti-inflammatory, antioxidant, antimicrobial, and immunomodulatory properties . [7,8,9]

Although researchers have investigated andrographolide extensively, its pharmacological effects depend on several experimental factors, including concentration, exposure duration, formulation, cell type, and the physiological or pathological context. For example, inflammatory signaling in macrophages may differ from effects observed in activated lymphocytes. Likewise, a concentration well tolerated by one cell type may reduce another cell type's viability. These factors highlight the importance of determining an appropriate concentration range before evaluating an isolated compound's immunological effects.

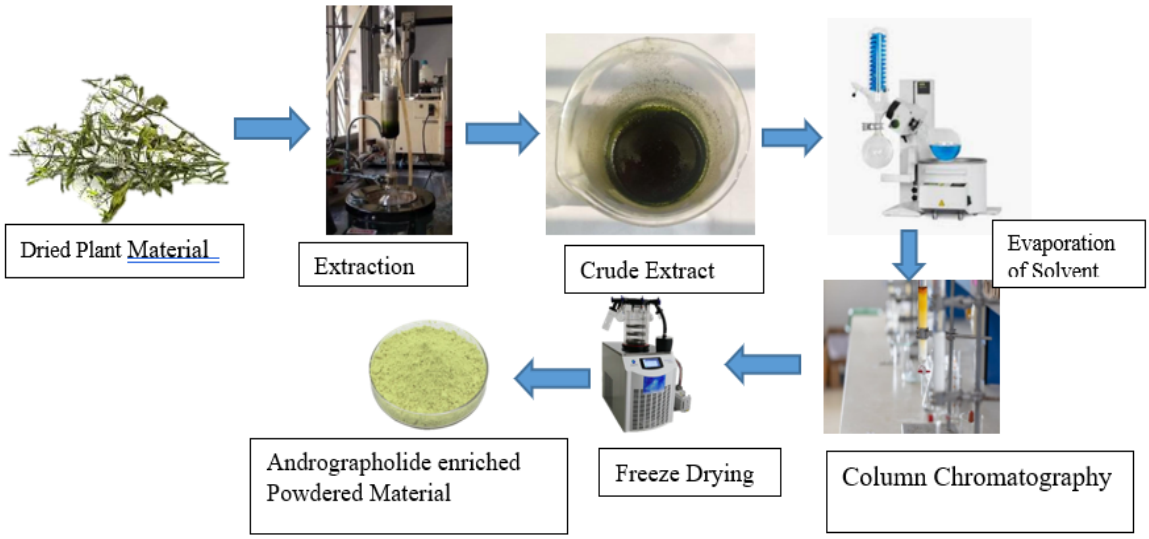
Andrographolide also has physicochemical limitations, particularly poor aqueous solubility, which can complicate delivery and interpretation of biological experiments. Solubility and formulation can affect how much compound is available to interact with cells and tissues. Consequently, reliable isolation, appropriate analytical characterization, and careful preparation of test solutions are important for evaluating its biological activity. Further research is needed to determine how these properties affect reproducibility, bioavailability, and potential therapeutic application.[10,11,12]

Extraction, separation, and purification of Andrographolide

Extracting and isolating bioactive compounds from medicinal plants are fundamental steps in natural-product research. The efficiency of these processes depends on several factors, including the selected plant part, the condition and particle size of the dried material, the solvent system, extraction temperature, extraction duration, and the target compound's polarity. A suitable extraction method should recover the target constituent while minimizing degradation and the co-extraction of unwanted substances.

Soxhlet extraction is one method used to recover plant constituents through repeated contact between the plant material and a heated solvent. In this method, solvent vapor condenses and passes through the sample, allowing soluble compounds to be extracted over repeated cycles. Choose a solvent that recovers andrographolide while limiting the extraction of unwanted matrix components. Also control the extraction conditions because prolonged heating may affect certain plant constituents. The resulting extract is concentrated and can then be separated and purified. Following extraction, the crude material generally contains a mixture of phytochemicals rather than a single compound. Preliminary phytochemical screening can indicate the broad classes of constituents present, but it cannot establish Andrographolide's identity or purity. Further separation is therefore required. Chromatographic techniques, particularly thin-layer chromatography (TLC) and column chromatography, help monitor and separate plant-derived compounds. TLC allows comparison of an extract's migration pattern with that of an authentic reference standard under defined chromatographic conditions. The retention factor (Rf) can be calculated as the distance travelled by the compound divided by the distance traveled by the solvent front. Similar Rf values provide preliminary evidence of similarity but are insufficient on their own to confirm chemical identity.[13,14,15]

Column chromatography separates constituents based on their differential interactions with a stationary phase and a mobile phase. In normal-phase silica gel chromatography, compounds interact differently with the polar stationary phase, resulting in different elution profiles as the mobile-phase composition is adjusted. Appropriate solvent selection, fraction collection, and TLC monitoring can help identify fractions containing Andrographolide. Fractions with similar chromatographic profiles can then be pooled and subjected to further purification, if required. Purification is important because residual plant constituents may influence cell viability, inflammatory responses, and other biological endpoints. If the isolated material contains substantial amounts of other bioactive compounds, the observed activity cannot be confidently attributed to Andrographolide alone. Therefore, assess chemical identity and purity using complementary analytical techniques rather than relying on a single chromatographic observation. [16,17,18]



%Yield of crude extract :
Extraction yield % = Weight of dry extract / Weight of dry plant material x 100 %

% Yield of extract = 6.96

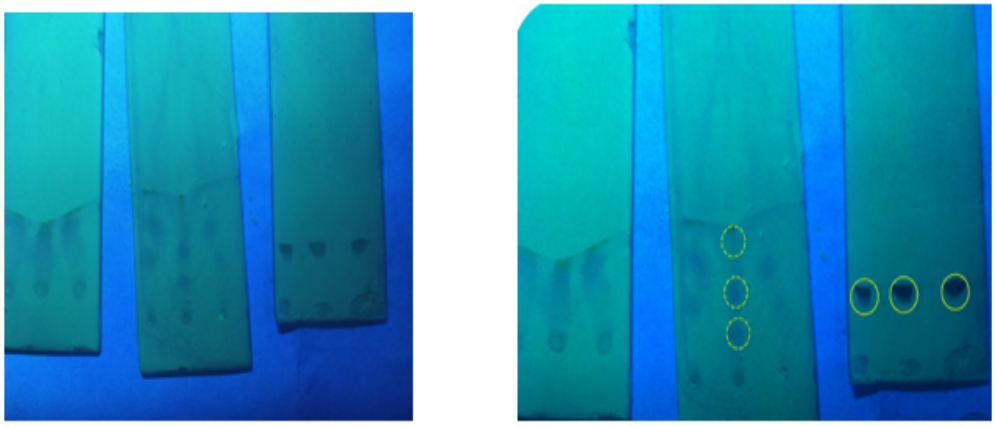


Fig 2: Rf (retention factor) = Distance travelled by compound (spot)/ Distance traveled by solvent front . Rf values = 0.03-0.40. All three spots on the right plate appear at similar heights, so they likely have the same Rf 0.5-0.6 indicates same compound .

ANALYTICAL IDENTIFICATION AND CHARACTERIZATION :

Characterizing isolated Andrographolide is essential to establish the reliability of the extraction and purification process. Different analytical techniques provide complementary information about the compound's chromatographic behaviour, optical properties, molecular mass, and chemical structure. UV–visible spectrophotometry can record the absorption spectrum of the isolated compound under defined solvent and instrumental conditions. The resulting spectrum provides information about its UV absorption characteristics. However, UV absorption alone is not specific enough to establish the identity or purity of Andrographolide because other plant constituents may have overlapping absorption patterns. [19,20,21]

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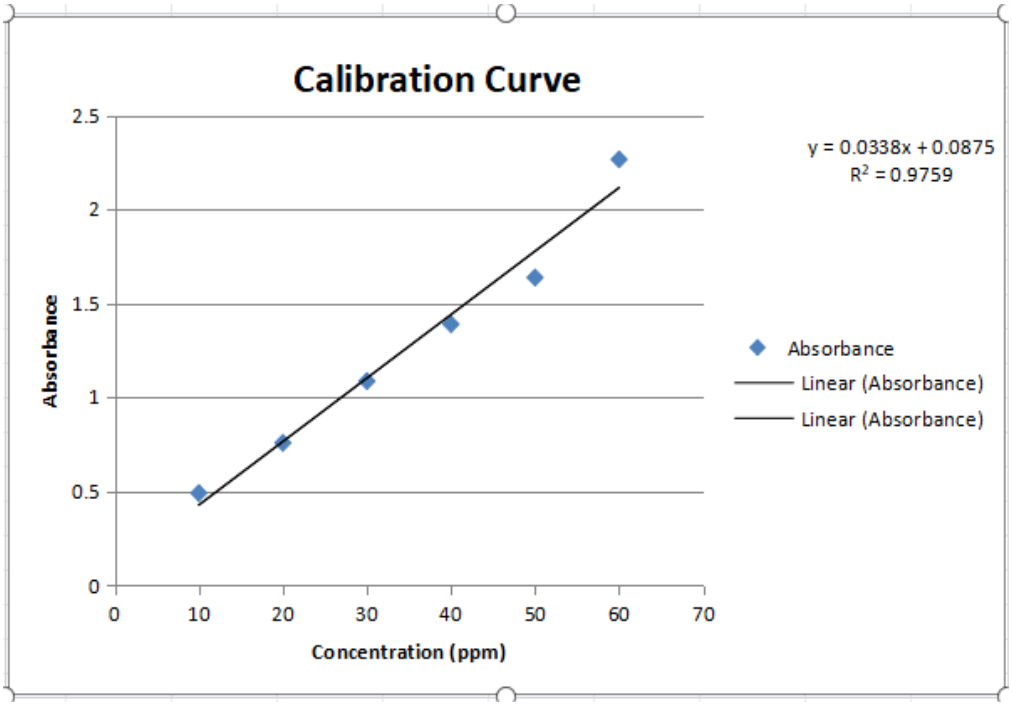
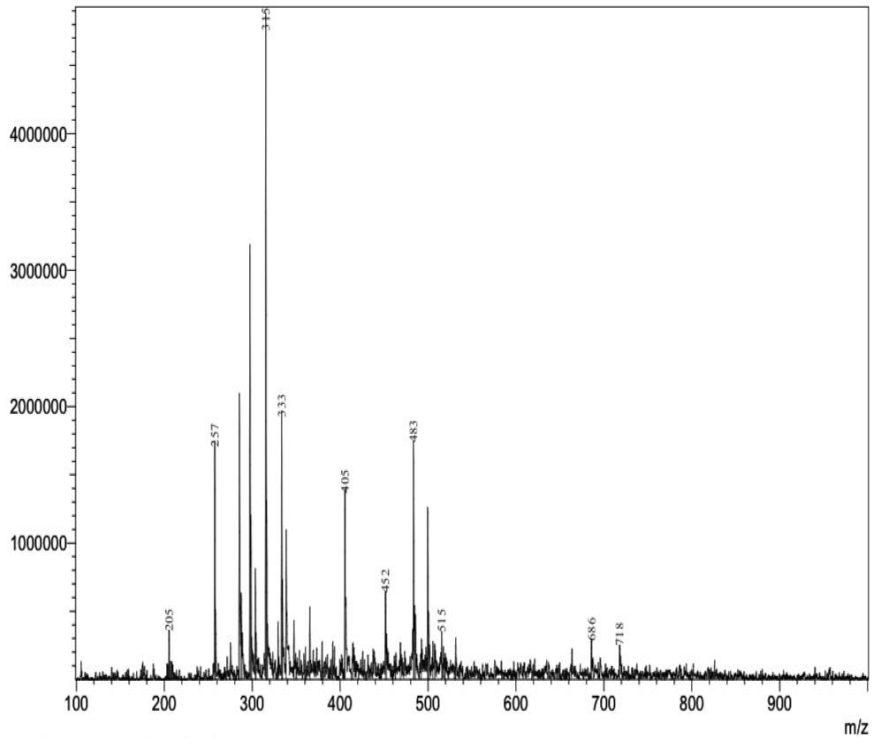


Fig 3: The UV-Vis spectrum of Andrographolide was recorded at 225 nm.

Mass spectrometry provides information about the mass-to-charge ratio (m/z) of detected ions and can support Andrographolide identification by comparing it with the expected molecular mass, ionization behavior, and reference data. The molecular formula of Andrographolide is C20H30O5 , with a molecular weight of approximately 350.45 g/mol. The observed ion depends on the ionization method; for example, electrospray ionization in positive mode may yield a protonated molecule, whereas negative mode may yield a deprotonated molecule. Therefore, the interpretation must consider the ionization mode and the type of adduct detected.



The fragmentation pattern matches labdane diterpene lactones, especially Andrographolide 14-Deoxyandrographolide

Neoandrographolide
14-Deoxy-11,12-didehydroandrographolide

Immunomodulatory potential of Andrographolide

Andrographolide's immunomodulatory activity is particularly interesting because immune regulation involves multiple cell populations and signaling pathways. Unlike a compound that acts exclusively as an immune stimulant or immunosuppressant, Andrographolide has been reported to produce different effects depending on the experimental model and the immune response being investigated. Published studies describe effects on macrophages, lymphocytes, cytokine production, antibody responses, and inflammatory signaling. These findings support its investigation as an immunoregulatory compound, although they do not establish that it produces beneficial immune stimulation in every biological context . [22,23,24]

DPPH Radical Scavenging Assay of Andrographolide

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay is a widely used in vitro method for evaluating the free-radical scavenging capacity of natural products, phytochemicals, and isolated bioactive compounds. In the present study, we used the DPPH assay to investigate the antioxidant potential of Andrographolide isolated from Andrographis paniculata. DPPH is a stable nitrogen-centred free radical that exhibits a deep violet colour in solution and has a characteristic absorption maximum at approximately 517 nm. In the presence of an antioxidant, DPPH accepts an electron or a hydrogen atom from the antioxidant molecule and is reduced to a non-radical form. This reduction decreases the intensity of the violet color, which can be measured spectrophotometrically. The extent of the decrease in absorbance is proportional to the radical-scavenging activity of the tested compound under the specified experimental conditions. A greater reduction in absorbance generally indicates greater DPPH radical-scavenging activity. This method can be used to evaluate andrographolide, a labdane-type diterpenoid lactone isolated from A. paniculata, to determine whether it exhibits measurable radical-scavenging activity at the tested concentrations. However, the presence of hydroxyl groups in its structure does not, by itself, establish strong antioxidant activity. Its actual activity must be determined experimentally and compared with a suitable reference antioxidant. [25,26,27]

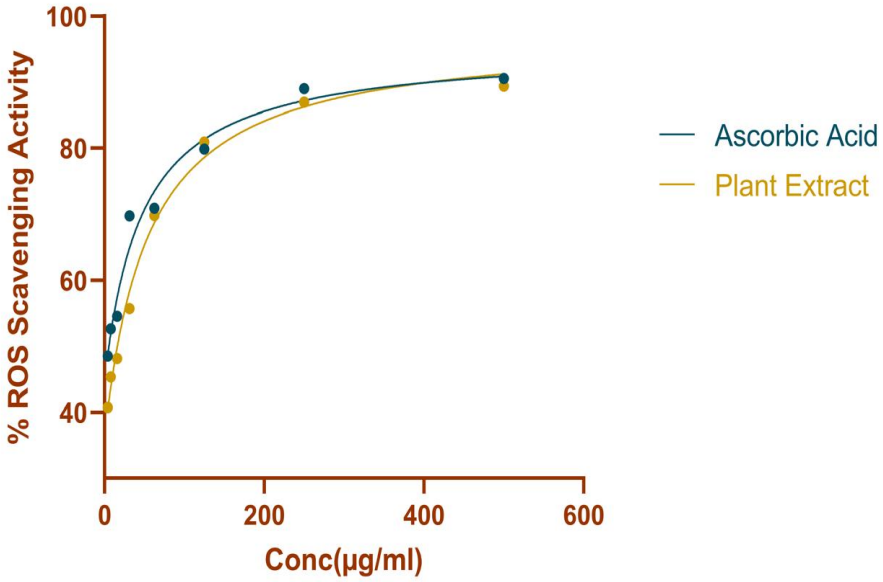


Fig. 4: . DPPH free radical scavenging activity of Andrographolide plant extract and ascorbic acid. Both samples showed concentration-dependent radical scavenging activity over the 3.9–500 µg/mL range. Ascorbic acid showed an IC_{50} value of 47.35 µg/mL, whereas Andrographolide exhibited an IC_{50} value of 54.78 µg/mL, indicating strong antioxidant activity comparable to the standard.

Effects on innate immunity and macrophage function

Innate immunity provides an early defense against pathogens and tissue injury. Macrophages are key components of this system because they recognize foreign material, engulf particles and microorganisms, release cytokines, and support tissue repair. Depending on the microenvironment, macrophages can exhibit different functional states associated with inflammatory responses, antimicrobial defense, and tissue homeostasis. Experimental studies report that Andrographolide can regulate macrophage activation and inflammatory mediator production. In murine RAW 264.7 macrophage models,

studies of A. paniculata extracts and isolated constituents have demonstrated inhibition of inflammatory responses induced by stimuli such as lipopolysaccharide (LPS), including changes in the production of tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), nitric oxide (NO), and prostaglandin E2 (PGE2). These findings indicate that the plant and some of its constituents can influence macrophage-mediated inflammatory processes [17,18] .

The anti-inflammatory potential of Andrographolide (A1) was evaluated by assessing its ability to suppress LPS-induced nitric oxide (NO) production in RAW 264.7 macrophages using the Griess assay. LPS stimulation markedly induced NO production in RAW 264.7 cells, whereas pretreatment with Andrographolide (A1) resulted in a concentration-dependent reduction in NO production. As illustrated in **Figure 5**, Andrographolide (A1) progressively decreased the percentage of NO production with increasing concentrations, indicating its inhibitory effect on LPS-induced NO generation. The concentration–response analysis revealed an IC_{50} of 68.30 µg/mL for Andrographolide (A1), indicating that this concentration was required to achieve 50% inhibition of LPS-induced NO production. The reference drug dexamethasone showed greater inhibitory potency, with an IC_{50} of 36.99 µg/mL. Although Andrographolide (A1) showed lower potency than dexamethasone, its ability to substantially suppress LPS-induced NO production demonstrates a notable anti-inflammatory potential. Collectively, these findings suggest that Andrographolide (A1) may effectively prevent excessive NO generation in activated RAW 264.7 macrophages and support its potential as an anti-inflammatory extract. [28,29,30]

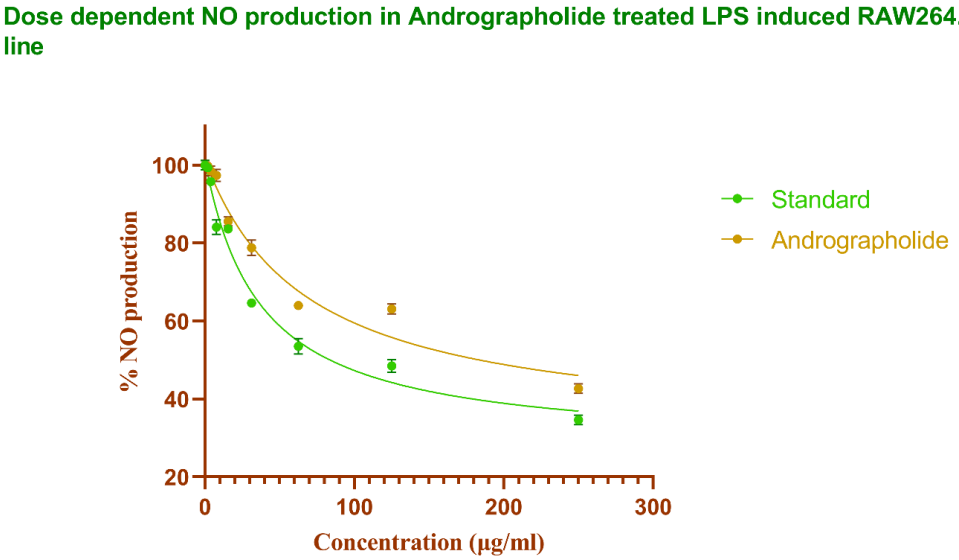


Figure 5 : . Effect of Andrographolide (A1) on LPS-induced nitric oxide production in RAW 264.7 macrophages. Data are presented as mean ± SD from (n=3).

Interpreting these effects requires care. Reduced cytokine production may indicate regulation of excessive inflammatory activation, but it does not necessarily demonstrate enhancement of protective immunity. Similarly, changes in macrophage viability can reduce measured inflammatory mediator release without reflecting a specific immunomodulatory mechanism. Therefore, interpret cytokine measurements alongside cell viability and other relevant functional endpoints. Macrophage polarization is another relevant aspect of immunomodulation. Classically activated, pro-inflammatory macrophages are commonly described as M1-like, whereas alternatively activated, tissue-repair-associated macrophages are often described as M2-like. This classification simplifies a continuous, context-dependent range of macrophage states. Markers such as inducible nitric oxide synthase (iNOS) and selected inflammatory cytokines can characterize inflammatory responses, whereas arginase-1 (Arg1), CD206, and IL-10 can be assessed in selected M2-associated experimental models. No single marker is sufficient to establish a complete polarization state.[31,32,33]

The reported influence of Andrographolide on macrophage activation and inflammatory signaling provides a rationale for investigating its effects on selected macrophage-associated endpoints. However, whether the isolated compound promotes, suppresses, or redirects a particular macrophage response must be established experimentally under the specified condition.

[34,35,36]

Regulation of inflammatory signaling pathways

Andrographolide's biological effects have been associated with modulating several intracellular signaling pathways,

particularly nuclear factor kappa B (NF-κB), mitogen-activated protein kinase (MAPK), and pathways involved in T-cell activation, including nuclear factor of activated T cells (NFAT). These pathways regulate the expression of genes involved in inflammatory responses, cytokine production, cellular activation, and immune homeostasis .[37,38, 39]

NF-κB is a transcriptional regulator involved in the expression of numerous inflammatory mediators. In resting cells, NF-κB is commonly retained in the cytoplasm through interactions with inhibitory proteins, including IκB. After inflammatory signals activate the IκB kinase complex, it promotes IκB degradation and facilitates NF-κB translocation to the nucleus. Nuclear NF-κB then regulates the transcription of target genes involved in inflammatory and immune responses. Experimental evidence that Andrographolide influences NF-κB-associated signaling provides a mechanistic basis for investigating its effects on inflammatory cytokine production. MAPK signaling, including the ERK, JNK, and p38 pathways, regulates cellular responses to extracellular stimuli. These pathways can influence cytokine production, cell survival, differentiation, and activation.[40,41,43] Modulation of MAPK signaling has been reported in studies of Andrographolide, although the direction and extent of the effects can vary according to the model and experimental conditions .[44, 45,46]

NFAT is particularly relevant to T-cell activation and cytokine production. Interference with NFAT-dependent signaling may contribute to the inhibitory effects of Andrographolide observed in certain T-cell models. Taken together, the available literature suggests that Andrographolide may influence immune responses through multiple signaling mechanisms rather than through a single universal pathway. Further mechanistic studies are needed to link these signaling changes to specific functional outcomes. [47]

Importance of in-vitro evaluation and cytotoxicity assessment

To evaluate the cytotoxicity of the A1, cell viability of RAW 264.7 macrophages was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, following previously reported protocols with slight modifications. RAW 264.7 cells were cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution under humidified conditions at 37 °C with 5% CO_2. Once confluent, cells were seeded into 96-well plates at 8 × 10^3 cells per well and incubated for 24 h to allow attachment. Cells were then treated with MKE at concentrations ranging from 3.9 to 250 µg/mL for 24 h. After treatment, add 5 mg/mL MTT solution to each well and incubate for 4 h at 37 °C in a 5% CO_2 atmosphere. The culture medium was subsequently removed, and the resulting formazan crystals were dissolved in 150 µL of DMSO. We measured absorbance at 570 nm using a UV–visible microplate spectrophotometer. Cell viability was expressed as a percentage relative to the untreated control group using the following equation.[48]

$$Cell\ viability\ (\%) = \left(\frac{ODsample}{ODcontrol}\right) x\ 100$$

The cytotoxic effect of Andrographolide on RAW 264.7 macrophages was evaluated using the MTT assay over a concentration range of 3.9–250 µg/mL. Andrographolide did not significantly reduce cell viability across the tested concentrations, indicating minimal cytotoxicity in RAW 264.7 cells (Fig. 6). Even at the highest concentration (250 µg/mL), cell viability remained relatively high. Consequently, the half-maximal inhibitory concentration (IC_{50}) of Andrographolide was estimated to be greater than 250 µg/mL. These findings suggest that Andrographolide exhibits low cytotoxicity and good cellular compatibility in RAW 264.7 macrophages, supporting its suitability for subsequent biological and pharmacological investigations .[49]

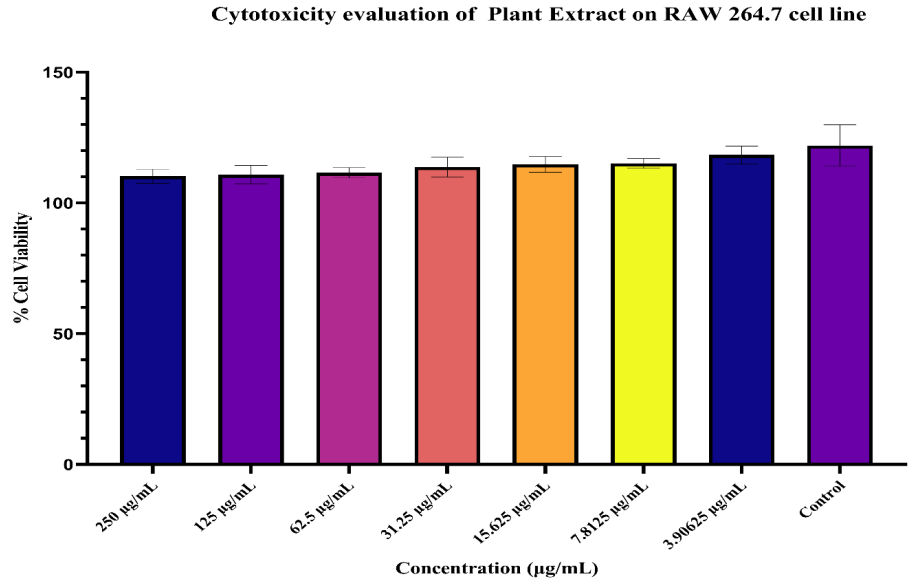


Fig 6 : Cytotoxicity evaluation of Andrographolide in RAW 264.7 macrophages using the MTT assay. Data are presented as mean ± SD from (n=3).

When HEK cells and RAW 264.7 macrophages are used, their roles in the experimental design should be clearly distinguished. HEK cells can provide preliminary cytotoxicity data in a non-immune cellular model, whereas RAW 264.7 cells can be used to investigate macrophage-associated responses. Neither cell line alone establishes systemic safety or overall immunomodulatory efficacy. For macrophage experiments, selecting non-cytotoxic concentrations is especially important. A compound that reduces cell viability may also reduce measured inflammatory cytokine production, creating the appearance of an anti-inflammatory effect. Consequently, integrate cell viability assessment with functional assays such as cytokine quantification, nitric oxide measurement, phagocytosis assays, or other relevant immune endpoints. A concentration-dependent assessment of cell viability therefore provides a useful foundation for subsequent immunomodulatory experiments. Nevertheless, interpret the MTT assay as a preliminary cellular viability assessment rather than a direct measure of immune stimulation or suppression. [50]

We evaluated the cytotoxicity of Plant Extract (Andrographolide) on HEK293 cells using the MTT assay to assess its safety profile in normal mammalian cells. Treatment with Plant Extract across the tested concentration range (3.906–250 µg/mL) showed a concentration-dependent cytotoxic trend . However, the IC50 of Plant Extract exceeded 250 µg/mL, indicating that even at the highest tested concentration, cell viability remained comparatively high and the toxic impact on HEK293 cells was minimal. These results highlight the favourable in vitro safety margin of Plant Extract (Andrographolide) and support its pharmacological investigation.

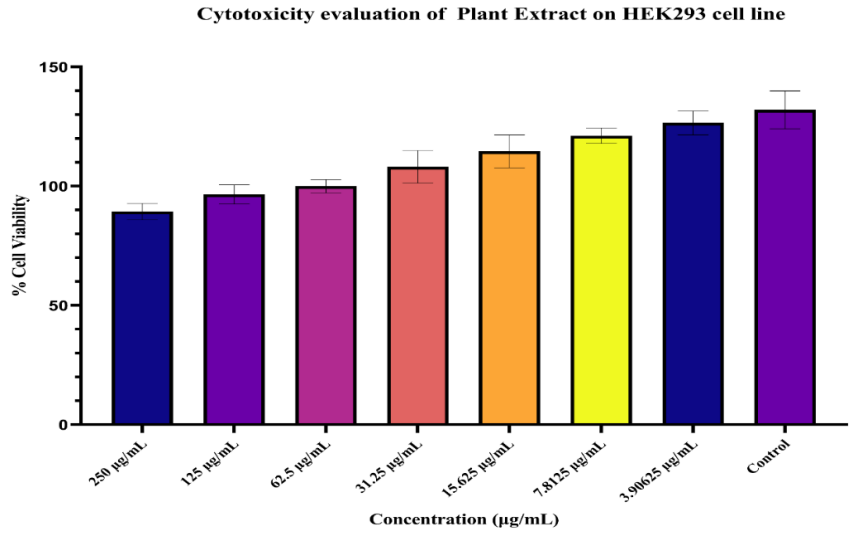


Fig 7 : Cytotoxicity evaluation of Andrographolide in HEK Cell line using the MTT assay. Data are presented as mean ± SD (n=3).

CONCLUSION

The present study demonstrated the successful separation and preliminary characterization of Andrographolide, a major bioactive diterpenoid of Andrographis paniculata, using appropriate extraction, chromatographic, and analytical techniques. We further evaluated the isolated compound for biological properties, including antioxidant activity and concentration-dependent effects on cellular viability. The findings provide preliminary evidence supporting the biological relevance of isolated Andrographolide and its potential to modulate immune-related cellular responses. Overall, the study establishes a systematic approach linking phytochemical separation with pharmacological evaluation and provides a basis for further mechanistic and in vivo investigations to elucidate the immunomodulatory potential and therapeutic significance of Andrographolide.

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