



Phytochemical Analysis (LC-MS) of *Azadirachta Indica* Ethanolic Extract, Antioxidant, Anticancer, in Vitro Wound Healing Activity, and Immunomodulatory Effects of *Azadirachta Indica* Ethanolic Extract

Loganathan Thavamani^{1,2}, Palanisamy Selvaraj¹, Muarali R³, Natesan Vijayakumar¹, Aruna Periyasamy², Muddukrishnaiah Kotakonda⁴, Sirajudheen Mukriyan Kallungal⁴

¹Department of Biochemistry and Biotechnology, Annamalai University, Annamalai Nagar 608002. Tamil Nadu, India.

²Department of Botany, Govt, Arts College for Men, Nandanam, Chennai-600036, Tamil Nadu, India.

³Department of Plant Biology and Plant Biotechnology, LN Government College (Autonomous), Ponneri - 601204, Tamil Nadu, India.

⁴Department of Pharmaceutics, Jamia Salafiya Pharmacy College, Pulikkal, Malappuram District -673637..

*Corresponding author's E-mail: Palanisamy Selvaraj

Article History	Abstract
Received: 06 June 2023 Revised: 05 Sept 2023 Accepted: 13 Dec 2023	<p>This study presents a comprehensive investigation of the medicinal attributes of the ethanolic extract obtained from <i>Azadirachta indica</i> (<i>A. indica</i>) leaves collected from the Jamia Salafiya Pharmacy College campus in Malappuram District, Kerala, South India. The plant was meticulously identified by Dr. Samuel Thavamni B, a plant pharmacognosy specialist. The ethanolic extraction process was performed using the Soxhlet method. Phytochemical analysis was performed using liquid chromatography-LC-MS (liquid chromatography-mass and Mass spectroscopy). The <i>in vitro</i> antioxidant, MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) assay, Apoptosis, and Immunomodulatory activity of ethanolic <i>A. indica</i> were evaluated. The extraction resulted in a semi-solid, dark-coloured extract with a yield of 8.67%. Phytochemical analysis using LC-MS identified key constituents such as octadecanoic acid, tetradecanoic acid, caryophyllene, 1,4-Eicosadiene, and triacontanoic acid, methyl ester, known for anti-inflammatory, antioxidant, and antimicrobial properties. <i>In vitro</i> antioxidant assays revealed a concentration-dependent inhibitory effect, with the <i>A. indica</i> ethanolic extract demonstrating substantial antioxidant capacity (77.81% at 100 µg), comparable to that of ascorbic acid (97.22%). However, the MTT assay indicated a concentration-dependent decrease in cell viability, emphasizing the need for caution owing to potential cytotoxic effects. Acridine orange/ethidium bromide staining provided insights into the induction of apoptosis in MCF-7 cells, highlighting the beneficial effects of the extract on cellular morphology. The scratch wound healing assay suggested the potential of the extract to promote fibroblast migration, implicating its role in enhancing wound healing. Immunomodulatory activity assessment revealed concentration-dependent modulation of immune responses, with the extract demonstrating a notable immunomodulatory effect of 77.81% at 100 µg. These findings shed light on the medicinal potential of <i>A. indica</i>, emphasizing the need for further research to elucidate the underlying mechanisms. The diverse chemical composition and multifaceted effects of the extract, from antioxidant and cytotoxic activities to immunomodulation and wound healing promotion, underscore its significance in pharmaceuticals and herbal remedies.</p>
CC License CC-BY-NC-SA 4.0	<p>Keywords: <i>Azadirachta indica</i>, ethanolic extract, antibacterial activity, <i>in vitro</i> anticancer activity, FE-SEM, TEM</p>

1. Introduction

Oxidative stress is a common denominator in many chronic diseases, including cancer.^{1,2} Antioxidants from medicinal plants act as frontline defenders against oxidative damage, providing a foundation for cellular health.³ By mitigating the impact of free radicals, these compounds may

indirectly contribute to reducing the risk of cancer development. The relationship between anticancer and immunomodulatory effects is intricately related to the body's defence mechanisms. Medicinal plants, known for their anticancer properties, often exhibit the ability to stimulate or modulate the immune system. For instance, compounds derived from plants such as echinacea have been shown not only to impede the growth of cancer cells, but also to enhance immune responses.^{4,5} This dual action highlights the potential synergy between the immune system and specific plant-derived compounds in the fight against cancer. Furthermore, the immunomodulatory effects of medicinal plants extend beyond cancer and play a crucial role in the overall health. A robust immune system is essential for preventing and managing various diseases, and medicinal plants contribute to this by regulating immune responses.⁶ As research in this field advances, a holistic understanding of how antioxidants, anticancer agents, and immunomodulators intersect and complement each other emerges. This multidimensional perspective underscores the significance of embracing a diverse array of medicinal plants to promote health, prevent disease, and explore novel therapeutic avenues. Medicinal plants have been integral to traditional healing practices for centuries and offer a rich source of therapeutic compounds. Among their myriad benefits, many possess potent antioxidant properties that help combat oxidative stress within the body. Antioxidants neutralize free radicals, which are unstable molecules that can damage cells and contribute to various diseases. Plants such as green tea, turmeric, and Ginkgo biloba are celebrated for their antioxidant properties, supporting overall health and potentially reducing the risk of chronic conditions. In addition to their antioxidant properties, numerous medicinal plants exhibit anticancer properties. Compounds found in plants such as garlic, ginger, and echinacea have demonstrated promising anticancer effects in various studies. These plants may inhibit the growth of cancer cells, induce apoptosis, and interfere with processes that contribute to tumor development. Although further research is ongoing, the potential of medicinal plants to complement traditional cancer treatments is a compelling avenue of exploration.⁹ Furthermore, many medicinal plants exhibit immunomodulatory effects, meaning that they can modulate the immune system's activity. Plants such as astragalus, mushrooms, and echinacea have been recognized for their ability to enhance immune function, aiding the body in defending against infections and illnesses.¹⁰ These immunomodulatory properties makes medicinal plants valuable allies in supporting overall well-being and maintaining a robust immune system. As the understanding of plant-based medicine evolves, harnessing the power of these botanical wonders may pave the way for innovative approaches to health care and wellness. In this study, we focused on the phytochemical analysis of the ethanolic extract of *A. indica* using Liquid Chromatography-Mass Spectrometry (LC-MS) and evaluated its Antioxidant, Immunomodulatory, anticancer, and anti-inflammatory activities.

2. Materials And Methods

Preparation of the extract from *A indica*

A. indica plant collection and authentication *A. indica* were collected from Jamia salafiya pharmacy college campus, Malappuram District Kerala, South India. The specimen was identified by the plant pharmacognosy specialist Dr. Samuel Thavamni B Professor and Head, Department of Pharmacognosy, Ahalia School of Pharmacy, Palakkad, Kerala, India.

Ethanolic Extraction Of *A Indica* Leaf Extraction By Soxhlet

A. indica leaves were harvested and thoroughly cleaned with sterile water before undergoing sterilization with a 0.1% mercuric chloride solution. The ethanolic extract of *A. indica* was obtained using a modified version of the method described by Shilpa et al. (2020). The extraction process was repeated until drops of the ethanolic solvent flowed through the syphon tube without leaving any residue. The resulting *A. indica* ethanolic extract was then employed for subsequent phytochemical analysis.^{11,12}

Phytochemical Evaluation By LC-MS

The ethanolic extract of the *A. indica* leaves was subjected to phytochemical analysis using LC-MS. The chemical constituents present in the ethanolic extracts of *A. indica* were identified through LC-MS analysis conducted on a Mariner Bio spectrometer equipped with a binary pump. The HPLC system was coupled to a Q-TOF mass spectrometer with an ESI source. The full-scan mode spanning m/z 100–1200 was used, maintaining a source temperature of 140°C. The HPLC column employed for analysis was Phenomenex 5 μ C8, with dimensions of 150 \times 2 mm i.d. The solvent used was methanol with 0.3% formic acid, which was delivered at a total flow rate of 0.1 mL/min through isocratic elution. MS spectra were acquired in the positive ion mode.^{13, 14}

In Vitro Antioxidant Assays -DPPH

The free radical scavenging activity of the ethanolic extract of *A. indica* was assessed by evaluating its ability to donate hydrogen or scavenge radicals using the stable DPPH (2,2-

diphenylpicrylhydrazyl) radical method. 0.1 mM DPPH solution in *A. indica* ethanol was prepared, and 1.0 ml of this solution was combined with 3.0 ml of the extract solution in water at various concentrations. After a 30-minute incubation, absorbance was measured at 517 nm. A lower absorbance of the reaction mixture indicated a higher free radical scavenging activity. The DPPH radical scavenging ability was quantified using the following formula: % of DPPH radical scavenging activity = (Control OD - Sample OD / Control OD) × 100, where the control represents the absorbance of the control reaction, and the test is the absorbance in the presence of extracts. Mean values were derived from triplicate experiments.^{15,16}

MTT Assay of A Indica Leaf Ethanolic Extract

The evaluation of *A. indica* leaf ethanolic extract in MCF-7 cells followed a protocol based on the methods outlined by Ala et al. (2018) and Nemati et al. (2013), with certain modifications. Fresh MCF-7 cells were cultured aseptically in 96-well plates at a concentration of 1×10^4 cells/mL, as determined using a hemocytometer, to maintain a consistent cell volume throughout the experiment. After 24 h, the cells were exposed to *A. indica* ethanolic extract at varying concentrations (0–200 µg/mL) and incubated for an additional 24 h at 37°C, 95% air, and 5% CO₂. Following treatment, the cell culture wells were rinsed with culture media, and MTT dye (5 mg/mL in PBS) was added to discern live and dead cells. Cell viability was assessed using a multi-well plate reader at 540 nm, and the results were expressed as the percentage of viable cells compared with the control. The IC₅₀ values, representing the concentration at which 50% inhibition occurred, were determined using the *A. indica* ethanolic extract dose-response curve (n=3) at various time points. Proliferation inhibition (%) was calculated using the formula: (Ac-At/Ac) X 100.^{17,18} Acridine orange/ethidium bromide (AO/EB) staining was used to measure apoptotic induction.

The investigation of the apoptosis-inducing effects of *A. indica* leaf ethanolic extract on L6 cell lines followed the methodology outlined by Annamalai et al. in 2020, with slight modifications. To examine apoptosis, a microscopic fluorescence analysis was performed. A mixture of AO/EB in a 1:1 ratio was prepared using PBS and applied to L6 cells treated with *A. indica* leaf ethanolic extract (100 µg/mL). After a 5-minute incubation, the cells were observed under a fluorescence microscope at 40X magnification.

In-vitro wound healing activity-scratch assay- *A. indica* leaf ethanolic extract

L6 cells were cultured in sterile six-well plates until they reached confluency. To create controlled injury, a straight-line scrape was made across healthy monolayer cells using a sterile pipette tip. The remaining undetected cells were removed by washing with sterile phosphate buffer solution. *A. indica* leaf ethanolic extract (50 µg) and a 0.2% FBS control were added to the tested cells under aseptic conditions, followed by an incubation period of 48 hr at 37°C in a cell culture incubator. After the 48-hr incubation, the cell growth pattern in the scratched area was observed and recorded using a phase-contrast microscope.^{20, 21}

Immunomodulatory Activity of A Indica Leaf Ethanolic Extract

Leukocyte suspensions were prepared by combining them with 0.5 ml of phosphate-buffered saline (PBS) in various test tubes. A single drop of endotoxin-activated plasma and 0.1 ml of PBS solution were added to the initial two tubes. The remaining tubes were treated with 0.1 ml of *A. indica* leaf ethanolic extract at varying concentrations (5, 10, 20, 40, 60, 80, 100, 500, and 1000 µg/ml). Subsequently, a freshly prepared 0.15% nitroblue tetrazolium (NBT) solution was introduced into each tube, and the mixture was incubated at 37°C for 20 min. Following incubation, the test tubes were centrifuged at 400 rpm for 5 min and the supernatant was discarded. The collected cell pellets were resuspended in 2 ml of PBS solution. A thin layer of the resuspended leukocyte solution was applied to a slide, which was then dried, heat-fixed, and counterstained with diluted carbolfuchsin. The slide underwent washing under tap water, air drying, and focusing under 100× oil immersion.^{22, 23.}

3. Results and Discussion

A. *Indica* Medicinal Plant Collection And Extraction

Authenticated *A. indica* samples were carefully collected without adulterants (Figure 1). Healthy leaf materials were collected with our interest, and shade drying was used to dry and prevent the degradation of bioactive phytoconstituents. After drying, 500 g of *A. indica* leaves were weighed and kept aside. The sample was subjected to successive hot continuous ethanolic extractions using a Soxhlet apparatus (Figure 2).



Figure 1: *A. indica* leaf

The extractive values and physical characteristics of *A. indica* ethanolic solvent provided valuable insights into the properties of the obtained extract. The use of ethanol as the solvent resulted in a semi-solid consistency, indicating the substantial presence of nonvolatile components in the extract. The brown coloration of the extract suggested the presence of diverse phytochemicals, including polyphenols, flavonoids, and other bioactive compounds known for their antioxidant properties. A yield percentage of 8.67% indicates the amount of extract obtained from the initial plant material. While this yield may seem modest, it is important to consider the qualitative aspects of the extract, such as its color and consistency, which suggest a concentration of bioactive compounds. The semi-solid consistency is often associated with the presence of lipids, waxes, or other viscous components, which may contribute to the therapeutic potential of the extract.

The brown coloration could be attributed to the presence of chlorophyll, tannins, or other pigments, indicative of the diversity of the chemical constituents extracted from *A. indica*. The color and consistency of the extract play a crucial role in assessing its potential applications as they are often correlated with the presence of specific phytochemicals with therapeutic benefits. Overall, these physical and chemical characteristics lay the groundwork for further investigation of the medicinal potential of *A. indica* ethanolic extract and guide its potential applications in various industries, including pharmaceuticals and herbal remedies.



Figure 2: *A. indica* leaf ethanolic solvent extraction using a Soxhlet apparatus.

Phytochemical analysis of *A. indica* leaf ethanolic extract by LC-MS

The provisional identification of compounds found in *A. indica* leaf ethanolic extract using LC-MS analysis is outlined. The detection and identification of these compounds relied on their fragmentation patterns, along with references from PubChem and other relevant research articles. Peak area and retention time served as key parameters for compound identification. Phytochemical analysis of *A.*

indica leaf ethanolic extract using LC-MS revealed a diverse array of compounds with potential therapeutic significance. The identified constituents include octadecanoic acid, 2,3-dihydroxypropyl ester (mass: 358), known for its anti-inflammatory and antioxidant properties; tetradecanoic acid (mass: 228), indicative of the involvement of fatty acids in the extract, potentially influencing anti-inflammatory responses; caryophyllene (mass: 204), a sesquiterpene recognized for its anti-inflammatory and analgesic effects; 1,4-Eicosadiene (Mass: 278), a compound with potential bioactive properties influencing cellular processes; and triacontanoic acid, methyl ester (mass: 466), associated with antimicrobial effects. Collectively, these findings highlight the complex chemical composition of *A. indica*, suggesting a multifaceted approach to its medicinal properties, including anti-inflammatory, analgesic, and antimicrobial potential. Further research on these identified compounds may unravel the mechanisms underlying the therapeutic benefits of *A. indica*, supporting its traditional use in herbal medicine and exploring its potential pharmaceutical applications.

***In vitro* antioxidant assays (DPPH) of *A. indica* ethanolic extract**

The concentration-dependent inhibition exhibited by the *A. indica* ethanolic extract (*A. indica* EE) and ascorbic acid is noteworthy and provides insights into their potential antioxidant activities. At the highest concentration (100 µg), *A. indica* EE demonstrated a robust inhibition percentage of 77.81%, demonstrating its substantial antioxidant capacity. This compares favorably with the standard antioxidant ascorbic acid, which exhibits a slightly higher inhibition percentage of 97.22%. As the concentration of *A. indica* EE decreased, the inhibitory effect decreased, with values of 64.65%, 62.53%, 54.12%, and 43.32% at 50, 25, 12.5, and 6.25 µg, respectively. Even at lower concentrations of 3.125 µg and 1.5625 µg, *A. indica* EE displayed notable inhibitory percentages of 30.39% and 24.75%, respectively, indicating its antioxidant potential across a broad concentration range (Table 1 and Figure 3). In comparison, the antioxidant activity of ascorbic acid declined gradually with decreasing concentration. These data suggest that *A. indica* EE possesses concentration-dependent antioxidant properties, demonstrating its potential as a natural source of antioxidants with efficacy comparable to or even exceeding that of the commonly used antioxidant ascorbic acid.

Table 1: *In-vitro* antioxidant assays (DPPH) of *A. indica* ethanolic extract

Concentration (µg)	<i>A. indica</i> EE (% of Inhibition)	Ascorbic acid (% of Inhibition)
100	77.81	97.22
50	64.65	92.54
25	62.53	85.27
12.5	54.12	78.73
6.25	43.32	72.84
3.125	30.39	64.58
1.5625	24.75	57.22
0.78125	9.13	24.02
0.390625	4.32	13.67

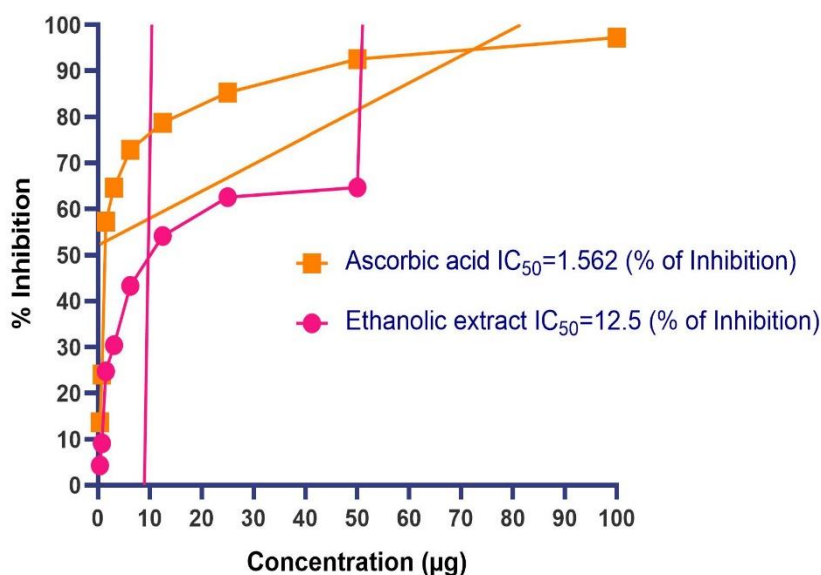


Figure 3: In-vitro antioxidant assays (DPPH) of *A. indica* ethanolic extract

MTT assay of *A. indica* leaf ethanolic extract

The MTT assay results for the *A. indica* leaf ethanolic extract revealed a concentration-dependent effect on cell viability. The ethanolic extract exhibited 80.31% inhibition at 25 µg, indicating a significant reduction in cell viability. As the concentration decreased to 12.5, 6.25, and 3.125 µg, the percentage of inhibition decreased progressively to 70.65%, 65.54%, and 59.12%, respectively. Even at lower concentrations of 1.5625, 0.78125, and 0.390625 µg, the *A. indica* ethanolic extract continued to demonstrate inhibitory effects, with percentages of 47.31%, 36.39%, and 25.75%, respectively (Figure 4 and Table 2). Notably, at the lowest concentrations of 0.1953125 µg and 0.0976 µg, the inhibitory percentages further decreased to 13.13% and 3.32%, respectively, suggesting a milder impact on cell viability at these levels. This concentration-dependent response highlights the potential cytotoxic effects of *A. indica* ethanolic extract, emphasizing the importance of careful consideration of dosage when exploring its therapeutic applications.

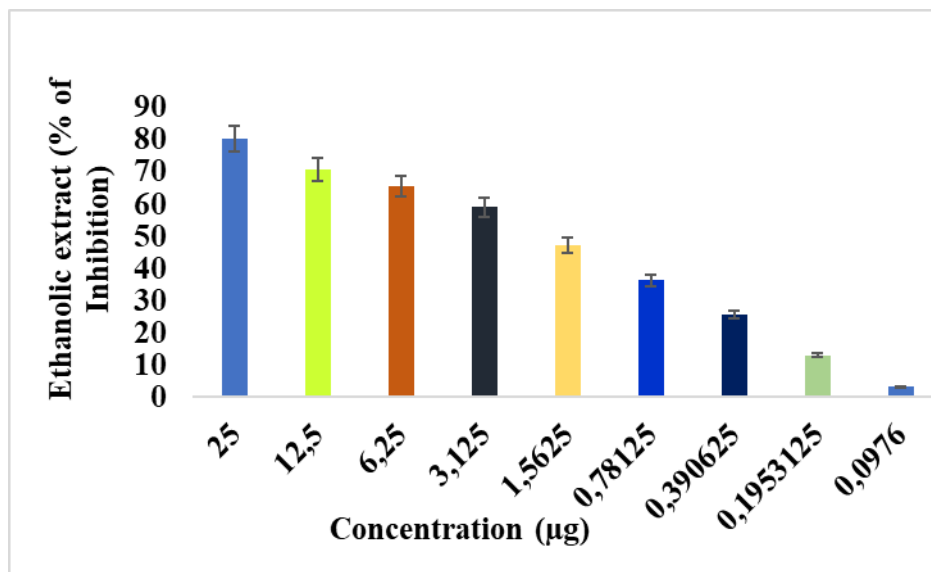


Figure 4: MTT assay of *A. indica* leaf ethanolic extract

Table 2: MTT assay of *A. indica* leaf ethanolic extract

Concentration (µg)	<i>A. indica</i> leaf EE (% of Inhibition)
25	80.31
12.5	70.65
6.25	65.54
3.125	59.12
1.5625	47.31
0.78125	36.39
0.390625	25.75
0.1953125	13.13
0.0976	3.32

Acridine orange/ethidium bromide (AO/EB) staining was used to measure apoptotic induction

A. indica ethanolic extract on MCF-7 cell apoptosis, researchers utilized the acridine orange/ethidium bromide (AO/EB) fluorescence staining technique to investigate cellular morphology. This method revealed distinct fluorescence patterns: viable cells emitted green fluorescence, early apoptotic cells displayed orange-green fluorescence due to nuclear condensation, and late apoptotic cells exhibited shades from orange to red, indicating highly condensed or fragmented chromatin. AO/EB staining not only differentiated between viable and apoptotic cells but also provided a nuanced understanding of the various stages of apoptosis induced by the *A. indica* ethanolic extract in MCF-7 cells, enhancing our understanding of its impact on cellular health (Figure 5).

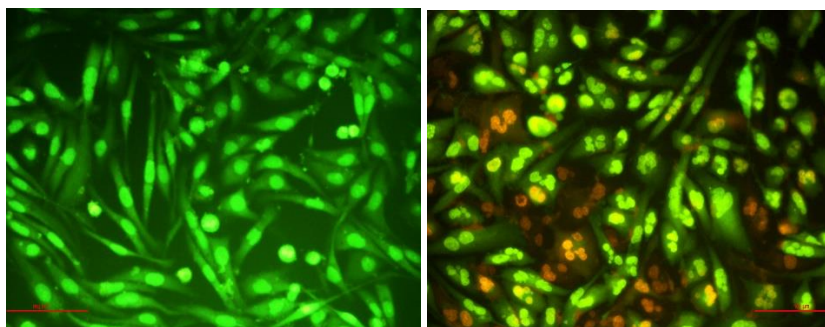


Figure 5: A: MCF-7 control cells; B: *A. indica* ethanolic extract treated MCF-7 cells

***In vitro* inflammatory activity-scratch assay- *A. indica* leaf ethanolic extract**

To assess the potential impact of *A. indica* ethanolic extract on skin fibroblasts, a scratch wound healing assay was conducted. The regrowth progress to close the scratch wound was monitored at 0, 24, and 48 h of incubation in a medium containing 50 μg *A. indica* ethanolic extract. The results revealed that the restoration of full cellular density in fibroblasts occurred more rapidly in the presence of *A. indica* ethanolic extract than in the control group. In simple terms, the extract significantly promoted the migration rate of fibroblasts at each time point. This observation has significant implications for the potential wound healing properties of *A. indica* ethanolic extract. The accelerated migration of fibroblasts suggests a potential role in enhancing the regenerative capabilities of the skin. Fibroblasts play a crucial role in wound healing by producing collagen and other extracellular matrix components. The observed promotion of fibroblast migration by *A. indica* ethanolic extract indicated its potential to positively influence the early stages of the wound healing process (Figure 6).

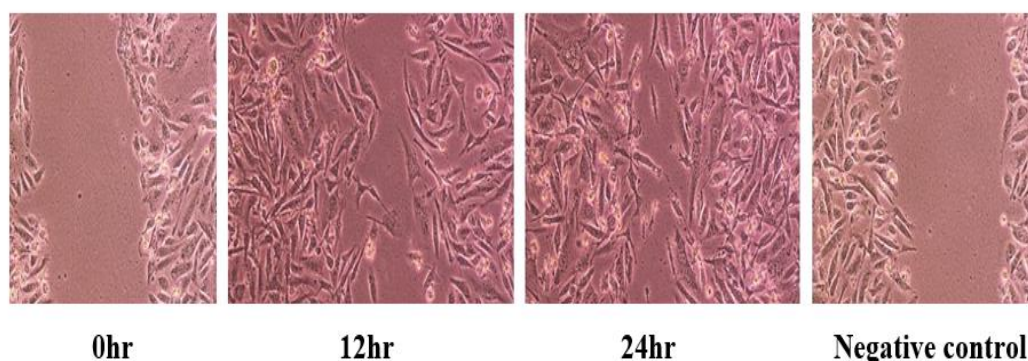


Figure 6: *In vitro* inflammatory activity scratch assay using *A. indica* leaf ethanolic extract.

Immunomodulatory activity of *A. indica* leaf ethanolic extract

The data obtained from the immunomodulatory activity assessment of *A. indica* leaf ethanolic extract, as measured by the percentage of nitroblue tetrazolium (NBT)-positive cells, indicated a concentration-dependent response. At the highest concentration of 100 μg , the extract demonstrated a notable immunomodulatory effect, with 77.81% NBT-positive cells. As the concentration was decreased to 50, 25, and 12.5 μg , the percentage of NBT-positive cells also decreased gradually to 64.65%, 62.53%, and 54.12%, respectively. This pattern continued with further reductions in concentration, reaching 43.32%, 30.39%, 24.75%, 9.13%, and 4.32% at 6.25 μg , 3.125 μg , 1.5625 μg , 0.78125 μg , and 0.390625 μg , respectively (Figure 7 and Table 3). The decrease in the percentage of NBT-positive cells with decreasing concentrations suggests concentration-dependent modulation of immune responses. The ability of the extract to reduce NBT-positive cells may indicate the regulation of reactive oxygen species (ROS) production, reflecting its potential to modulate immune cell activity. Immunomodulation is a crucial aspect of medicinal plants, and these findings suggest that *A. indica* leaf ethanolic extract may possess immunomodulatory properties that can be further explored for potential therapeutic applications in enhancing or regulating immune responses.

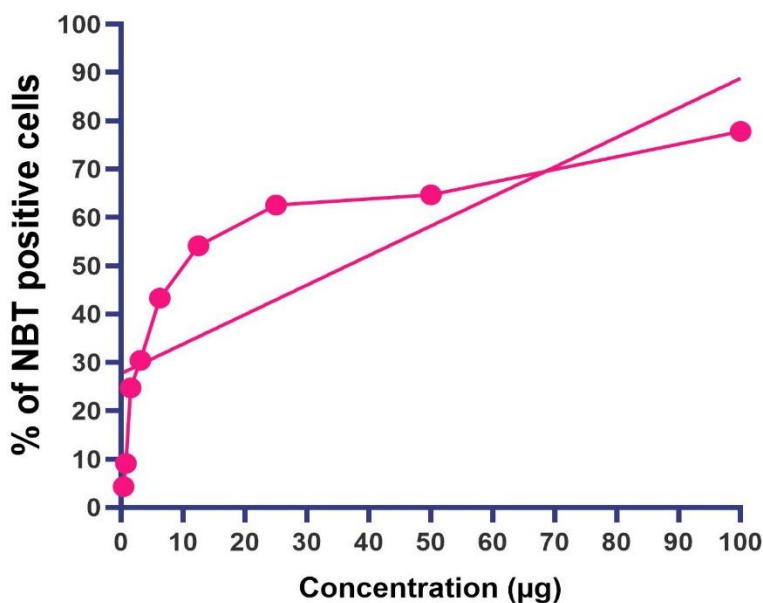


Figure 7: Immunomodulatory activity of *A. indica* leaf ethanolic extract

Table 3: Immunomodulatory activity of *A. indica* leaf ethanolic extract

Concentration (µg)	% of NBT positive cells
100	77.81
50	64.65
25	62.53
12.5	54.12
6.25	43.32
3.125	30.39
1.5625	24.75
0.78125	9.13
0.390625	4.32

4. Conclusion

Investigation of the *A. indica* ethanolic extract has revealed promising medicinal attributes across various parameters. The extract obtained through meticulous collection and extraction processes exhibited a semi-solid consistency and brown coloration, indicative of a rich composition of bioactive compounds. Phytochemical analysis via LC-MS identified constituents such as octadecanoic acid (358), tetradecanoic acid (228), caryophyllene (204), 1,4-Eicosadiene (278), triacontanoic acid, and methyl ester (466), which are known for their anti-inflammatory, antioxidant, and antimicrobial properties. The in vitro antioxidant assays demonstrated a concentration-dependent inhibitory effect, with the *A. indica* ethanolic extract displaying substantial antioxidant capacity, reaching an impressive inhibition percentage of 77.81% at 100 µg, comparable to the standard antioxidant ascorbic acid (97.22%). However, the MTT assay indicated a concentration-dependent effect on cell viability, with inhibition percentages ranging from 80.31% at 25 µg to 3.32% at 0.0976 µg. This underscores the need for cautious dosage considerations owing to potential cytotoxic effects. The acridine orange/ethidium bromide staining technique provided detailed insights into apoptotic induction in MCF-7 cells, with distinct fluorescence patterns indicating the beneficial effects of the extract on cellular morphology. The scratch wound healing assay suggested the potential of the extract to promote fibroblast migration, implicating its role in enhancing the wound healing process. Assessment of immunomodulatory activity revealed concentration-dependent modulation of immune responses, with the extract demonstrating a notable immunomodulatory effect of 77.81% at 100 µg. These findings will help to understand the medicinal use of *A. indica*, and further research is required to unravel the mechanisms underlying the medicinal properties of the extract and optimize its usage in pharmaceuticals and herbal remedies.

Conflict of interest

Nil

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Nil

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