OVERVIEW OF IN VITRO METHODS FOR EVALUATING ANTICANCER ACTIVITY AND THE ANTICANCER POTENTIAL OF INDIAN MEDICINAL PLANTS

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Abstract

This study aims to assess the anticancer potential of Indian medicinal plants using in vitro methods. Various plant extracts and bioactive compounds have shown promising anticancer activity against different cancer cell lines. Indian medicinal plants such as Curcuma longa (turmeric), Withania somnifera (ashwagandha), Azadirachta indica (neem), Ocimum sanctum (holy basil), Zingiber officinale (ginger), Momordica charantia (bitter melon), Moringa oleifera (moringa), and Aegle marmelos (bael) have demonstrated significant anticancer effects. These plants exhibit mechanisms like induction of apoptosis, inhibition of cell proliferation, suppression of inflammation, and prevention of metastasis. Active compounds, including curcumin, withanolides, and azadirachtin, have shown potential in targeting various cancer types, including breast, lung, and colon cancers. In vitro evaluations of Indian medicinal plants provide promising evidence of their anticancer potential. The bioactive compounds from these plants exhibit multiple mechanisms of action, such as apoptosis induction and cell cycle regulation. These findings underscore the need for further in vivo studies and clinical trials to validate the therapeutic potential of these plant-derived compounds for cancer treatment.

Keywords: Anticancer activity, Indian medicinal plants, In vitro assays, Bioactive compounds, Cancer therapy.

Background

Cancer remains a leading cause of mortality worldwide, presenting significant challenges due to its complex biology and diverse manifestations. The global incidence of cancer continues to rise, highlighting the urgent need for the discovery of novel and more effective therapeutic agents.[1] Despite advancements in synthetic drug development, issues such as drug resistance, adverse side effects, and high treatment costs underscore the necessity for alternative therapeutic strategies.[2] Traditional medicine, particularly plant-based remedies, has gained considerable attention as a promising area of research in the search for anticancer agents. Indian medicinal plants, rich in bioactive compounds, have emerged as valuable resources due to their accessibility, affordability, and historical usage in treating various ailments, including cancer.[3]

Indian Medicinal Plants and Their Anticancer Potential

India, with its vast biodiversity and traditional medical systems such as Ayurveda, Siddha, and Unani, offers a rich repository of medicinal plants with potential anticancer properties.[4] Historically, plants like *Curcuma longa* (turmeric), *Withania somnifera* (ashwagandha), and *Azadirachta indica* (neem) have been extensively utilized for their therapeutic benefits. Phytochemicals derived from these plants, including flavonoids, alkaloids, tannins, and

terpenoids, exhibit significant biological activities, such as antioxidant, anti-inflammatory, and anticancer effects.[3,4] These natural compounds often act through multiple mechanisms, such as inducing apoptosis, inhibiting cell proliferation, and suppressing angiogenesis, making them valuable candidates for anticancer therapy.

Advances in In Vitro Methodologies for Evaluating Anticancer Potential

The advent of in vitro methodologies has revolutionized the evaluation of the anticancer potential of medicinal plants. These methods provide a controlled environment to assess the efficacy of plant extracts and isolated compounds against various cancer cell lines. Key in vitro techniques include cytotoxicity assays like MTT and Trypan Blue, apoptosis detection methods such as Annexin V/PI staining, and gene expression studies using RT-PCR. These techniques are crucial for identifying promising candidates and elucidating their mechanisms of action at the cellular and molecular levels.[6]

Significance of Indian Medicinal Plants in Anticancer Research

Indian medicinal plants hold a unique advantage due to their extensive historical use in traditional medicine systems, which provide a rich foundation of ethnopharmacological knowledge for scientific exploration.[7] For example, **turmeric** (*Curcuma longa*), commonly known as "Haldi," has been a staple in Indian households for centuries. Turmeric contains **curcumin**, a polyphenolic compound extensively studied for its anticancer properties.[8] Curcumin has been shown to modulate multiple signaling pathways, including NF-κB, PI3K/Akt, and MAPK, thereby inhibiting cancer progression and metastasis.[8]

Another prominent example is ***Withania somnifera***, commonly known as ashwagandha.[9] Traditionally used in Ayurveda as a rejuvenator and adaptogen, modern research has identified **withanolides** as the active compounds in ashwagandha. These withanolides exhibit potent anticancer properties by inducing apoptosis and inhibiting angiogenesis in cancer cells.[9]

Similarly, ***Azadirachta indica***, commonly known as neem, has been extensively used in traditional medicine. Extracts from neem have demonstrated cytotoxicity against a wide range of cancer cell lines, primarily through the induction of oxidative stress and mitochondrial dysfunction.[10]

Mechanisms of Anticancer Action

Indian medicinal plants exhibit a variety of anticancer properties through diverse mechanisms, often targeting multiple pathways simultaneously. One of the primary mechanisms is the induction of apoptosis, a programmed cell death process that is essential for eliminating cancer cells. Many plant-derived compounds activate intrinsic pathways, which involve mitochondrial signaling, or extrinsic pathways, which involve death receptor signaling. These pathways lead to the activation of caspases, key enzymes that dismantle the cellular components of cancer cells, effectively halting their proliferation and survival.[11]

Another critical mechanism is the arrest of the cell cycle at specific phases, preventing cancer cells from dividing and propagating. Phytochemicals such as flavonoids and alkaloids can interfere with regulatory proteins like cyclins and cyclin-dependent kinases (CDKs), disrupting the cell cycle at crucial checkpoints such as the G1/S or G2/M phase. This inhibition impairs the ability of cancer cells to replicate, thereby suppressing tumor growth.[12]

Additionally, Indian medicinal plants have been shown to inhibit angiogenesis, the process by which new blood vessels are formed to supply nutrients and oxygen to tumors. By targeting angiogenic factors such as vascular endothelial growth factor (VEGF), these plants

effectively starve the tumor of essential resources, leading to reduced growth and metastasis. For instance, compounds from Tinospora cordifolia have demonstrated significant anti-angiogenic activity, further emphasizing the therapeutic potential of these plants.[11]

Suppression of metastasis is another vital mechanism exhibited by plant extracts. Metastasis, the spread of cancer cells to distant organs, is a major cause of cancer-related mortality. Certain plant-derived compounds inhibit enzymes like matrix metalloproteinases (MMPs), which are involved in the degradation of the extracellular matrix, a crucial step in cancer cell migration and invasion. This inhibition reduces the likelihood of metastatic spread, enhancing the overall efficacy of anticancer therapy.[12]

In Vitro Methods

Trypan Blue Dye Exclusion Assay

The trypan blue dye exclusion assay is frequently used to assess cell viability. The procedure begins with washing cells using Hank's Buffered Salt Solution (HBSS) and centrifuging them for 10–15 minutes at 10,000 rpm. This washing and centrifugation step is repeated three times to ensure purity. Following this, cells are resuspended in a known volume of HBSS, adjusting the concentration to 2×10^6 cells/mL.[13]

The prepared cell suspension is aliquoted into Eppendorf tubes in 0.1 mL portions, each containing approximately 200,000 cells. These cells are then treated with various drug dilutions and incubated at 37°C for 3 hours. After incubation, an equal volume of 0.4% trypan blue dye is added to the treated cells and mixed thoroughly. After 1 minute of staining, the cells are loaded onto a hemocytometer. Viable cells exclude the dye and remain unstained, while non-viable cells absorb the dye and appear colored. Viable cell counts should be promptly recorded, as live cells may eventually take up the dye if left standing too long.[13]

$$\text{Growth inhibition (\%)} = 100 - \frac{\text{(Total cells-Dead cells)}}{\text{Total cells}} \times 100$$

LDH (Lactic Dehydrogenase) Assay

The LDH assay is a spectrophotometric method that measures the activity of lactate dehydrogenase, an enzyme released from damaged cells. This assay quantifies the reduction of NADH during the enzymatic conversion of pyruvate to lactate.[14]

Cells are lysed using a buffer containing 50 mM Tris-HCl (pH 7.4), 20 mM EDTA, and 0.5% Sodium Dodecyl Sulfate (SDS). The lysate undergoes sonication and is then centrifuged at 13,000 × g for 15 minutes. For enzymatic analysis, an assay mixture with a final volume of 1 mL is prepared, which includes 33 µL of the sample in 48 mM PBS (pH 7.5), 1 mM pyruvate, and 0.2 mM NADH. The reaction's progress is monitored at 340 nm, and the percentage of LDH release is calculated based on the total LDH, determined by combining the activity in the cellular lysate and culture medium.[14]

MTT Assay

The MTT assay evaluates cell viability through the reduction of yellow tetrazolium salt (MTT) to purple formazan crystals by metabolically active cells. Cells are seeded in 96-well plates at a density of 2×10^5 cells/mL in 100 µL of RPMI 1640 medium and incubated in a CO_2 incubator (37°C, 5% CO_2) for 24 hours. After this period, the medium is replaced with

fresh medium containing varying concentrations of the test sample and incubated for another 24–48 hours.[15]

Next, 20 µL of MTT stock solution (5 mg/mL in PBS) is added to each well, followed by an additional 5-hour incubation. After carefully removing the medium, 200 µL of DMSO is added to dissolve the formazan crystals. The plate is then shaken at 150 rpm for 5 minutes, and optical density is measured at 560 nm. Untreated cells serve as controls, with their viability set to 100%. Results are expressed as a percentage of viability relative to the control.[15]

XTT Assay

The XTT assay is a colorimetric technique to measure cell proliferation and viability. It depends on the conversion of XTT (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) into an orange formazan dye by mitochondrial dehydrogenases in viable cells.[16]

Cells are cultured in a medium supplemented with 10% FBS in 96-well plates until they achieve 70–80% confluence. Post-treatment with the test compound for 24 hours, 50 µL of the XTT labeling mixture is added to each well, followed by a 4-hour incubation at 37°C. The resultant formazan dye is soluble in aqueous solutions, and its optical density is measured at 450 nm, using 650 nm as the reference wavelength. Analysis is performed using a spectrophotometer or ELISA reader.[16]

Sulforhodamine B (SRB) Assay

The sulforhodamine B assay employs a bright pink aminoxanthene dye that binds to basic amino acids in mildly acidic conditions and dissociates under basic conditions. Cells are plated in 96-well flat-bottom plates at a density of 5,000–10,000 cells per well, adjusting for differences in cell line growth rates.[16]

After overnight adhesion, serial dilutions of the test compound are added to triplicate wells, while control wells receive a 1:10 dilution of water in the medium. Plates are incubated at 37°C in a 5% CO_2 atmosphere for 3 days. Post-incubation, cells are fixed with cold 50% trichloroacetic acid to a final concentration of 10% and incubated at 4°C for 1 hour. The plates are washed five times with deionized water and stained with 0.4% SRB dissolved in 1% acetic acid for 15–30 minutes. Excess dye is removed by washing with 1% acetic acid.

Air-dried plates are solubilized with 10 mM Tris base, and absorbance is measured at 595 nm using a microplate reader. This assay is widely utilized to evaluate growth inhibition in response to drug treatments.[16]

$$\text{The percent growth inhibition is calculated as} = \frac{\text{Control-Sample}}{\text{Control}} \times 100$$

Anticancer Potential of Indian Medicinal Plants:

Natural compounds derived from Indian medicinal plants have shown significant anticancer properties, attracting considerable global research interest in the quest for effective cancer treatments. Nature remains an invaluable resource for discovering bioactive compounds with the potential to inhibit cancer development. Plant-derived natural products, such as flavonoids, terpenoids, and steroids, are particularly noteworthy due to their diverse pharmacological effects, including cytotoxic and chemopreventive activities.[17]

A landmark in the use of plant-based anticancer agents was the discovery of vinca

alkaloids—vinblastine and vincristine—from *Catharanthus roseus* (Madagascar periwinkle). These compounds marked a new era in cancer treatment, being the first plant-derived agents to achieve clinical success.[17]

Medicinal plants are abundant in antioxidants, including vitamins (A, C, E, and K), carotenoids, flavonoids (such as flavones, isoflavones, and anthocyanins), polyphenols (such as ellagic acid, gallic acid, and tannins), saponins, enzymes, and essential minerals (such as selenium, zinc, and manganese). These compounds significantly contribute to the therapeutic potential of medicinal plants.[18]

This review highlights 50 Indian medicinal plants from 35 botanical families with reported anticancer activity. Detailed information is provided on the plant parts used, extract types, cancer models tested, and specific cancer cell lines studied. These plants have shown efficacy against a variety of tumors, including sarcomas, lymphomas, carcinomas, and leukemias, in both experimental and clinical settings.[18]

Several medicinal plants have demonstrated significant anticancer activity in both in vivo and in vitro models. For example:

- ***Abrus precatorius*** has shown efficacy against Yoshida sarcoma and Dalton's lymphoma.
- ***Alstonia scholaris*** has exhibited activity against Ehrlich ascites carcinoma.
- ***Cymbopogon flexuosus*** has been evaluated in leukemia and sarcoma 180 models.
- ***Ocimum gratissimum*** has shown effects in breast cancer studies.
- ***Phellinus rimosus*** and ***Punica granatum*** have been studied for lymphoma and prostate cancer, respectively.
- Other notable examples include ***Moringa oleifera*** (skin cancer, multiple myeloma), ***Allium sativum*** (sarcoma 180), ***Asparagus racemosus*** (liver cancer), and ***Catharanthus roseus*** (P-1534 leukemia).

Plants such as ***Withania somnifera***, ***Azadirachta indica***, ***Curcuma longa***, ***Nigella sativa***, and ***Terminalia chebula*** have also demonstrated promising results against various malignancies. Despite the abundance of in vitro studies, further in vivo investigations are required to validate these findings and pave the way for clinical applications.[9,10]

Significance of In Vitro Studies in Scientific Research

In vitro studies, conducted in controlled laboratory environments outside living organisms, offer several significant advantages across various scientific disciplines, particularly in pharmacology, toxicology, and molecular biology. These studies allow researchers to manipulate experimental conditions, such as temperature, pH, and nutrient availability, ensuring precision and reproducibility. By isolating specific variables, in vitro studies reduce interference from systemic factors, leading to more accurate results.

Furthermore, in vitro experiments are cost-effective compared to in vivo studies, requiring fewer resources, less sophisticated equipment, and minimal regulatory oversight. These studies also align with ethical considerations by reducing reliance on animal testing, supporting the principles of the 3Rs (Replacement, Reduction, and Refinement). Using human cell lines and organoid models in these studies provides more directly translatable data, minimizing ethical concerns associated with animal use.[19]

Applications of In Vitro Studies

In vitro studies are integral to high-throughput screening, enabling rapid testing of thousands of compounds for biological activity or toxicity, thereby accelerating drug discovery and development. These studies offer valuable insights into cellular processes and mechanisms, allowing direct observation of molecular interactions, such as receptor-ligand binding or enzyme inhibition. The flexibility of in vitro systems permits customization to mimic specific biological environments, such as tumor microenvironments, and facilitates diverse experimental designs, including dosage and combination therapy testing.[18,19]

These studies are essential for safety assessment, widely used for evaluating the toxicity and safety profiles of drugs and chemicals before advancing to in vivo testing. Human-relevant models provide early warnings about potential adverse effects, reducing risks in later stages of development.

Advantages of In Vitro Studies

In vitro studies reduce variability by using standardized cell lines, ensuring uniformity and reproducibility across different research facilities. They are particularly suited for long-term studies, allowing the investigation of chronic exposure effects without the logistical or ethical complexities of extended animal studies.[19] In the realm of personalized medicine, in vitro techniques using patient-derived cells or tissues enable the study of disease progression and individualized treatment responses.[20]

While in vitro studies cannot entirely replace in vivo or clinical studies, their advantages make them a cornerstone in early-stage research, screening, and mechanistic investigations. With advancements like organ-on-chip systems and 3D bioprinting, in vitro methodologies continue to revolutionize scientific discovery and translational medicine.

Conclusion

The in vitro evaluations of Indian medicinal plants have shown promising evidence of their anticancer potential, establishing a solid foundation for the development of natural cancer therapies. These plants, rich in bioactive compounds, have demonstrated a range of mechanisms of action, including the induction of apoptosis, inhibition of cell proliferation, suppression of inflammatory pathways, and modulation of key cell cycle regulators. Compounds such as curcumin from *Curcuma longa*, withanolides from *Withania somnifera*, and azadirachtin from *Azadirachta indica* have shown cytotoxic effects on various cancer cell lines, suggesting their potential to target multiple pathways involved in tumor growth and metastasis.[9]

The anticancer activity of these plants is multifaceted. Many of these compounds can selectively induce apoptosis in cancer cells while sparing normal cells, thus minimizing potential side effects. They also regulate various molecular targets, including oncogenes, tumor suppressor genes, and apoptotic markers, which are crucial in tumor progression and treatment resistance. The ability of these compounds to modulate pathways such as NF-kB, MAPK, and PI3K/Akt further enhances their therapeutic promise.[21]

Moreover, some medicinal plants exhibit synergistic activity when combined with conventional therapies, potentially enhancing the overall effectiveness of cancer treatment. Despite the promising in vitro findings, the transition from laboratory-based studies to clinical applications requires rigorous in vivo testing to confirm the efficacy and safety of these plant-derived compounds.[21]

Further research is essential to validate the therapeutic potential of these compounds in preclinical and clinical settings. The development of standardized extraction methods, dose optimization, and the exploration of combination therapies with existing anticancer drugs are

crucial next steps. Indian medicinal plants represent a valuable source of novel, bioactive compounds that could significantly contribute to the advancement of cancer treatment, offering an alternative or adjunct to current chemotherapy regimens.

Conflict of Interest

None

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