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Research Article

TOXICOLOGICAL EVALUATION OF PLANT-DERIVED THERAPEUTICS USING *IN VITRO* MODELS

[1]Chandan Kumar D, [2]Aswini L, [3]Lavanya R, [4]Sibi S and [5]Jenifer E

[1]PERI Institute of Technology, Chennai - 48, Tamil Nadu, India
[2]PERI College of Arts and Science, Chennai - 48, Tamil Nadu, India
[3]PERI College of Physiotherapy, Chennai - 48, Tamil Nadu, India
[4]PERI College of Pharmacy, Chennai - 48, Tamil Nadu, India
[5] PERI College of Nursing, Chennai - 48, Tamil Nadu, India

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ABSTRACT

Plant-derived therapeutics remain a major source of bioactive compounds with significant pharmacological potential; however, their safety profiles must be rigorously evaluated before clinical use. The present study provides a comprehensive toxicological assessment of selected medicinal plant extracts using standardized in vitro models. Cytotoxicity analyses were performed using MTT and LDH assays on human epithelial and fibroblast cell lines to determine dose-response effects. Morphological evaluation, reactive oxygen species (ROS) quantification, and apoptosis/necrosis profiling were further incorporated to elucidate mechanisms of cellular toxicity. Results revealed dose-dependent variations, with some extracts demonstrating minimal cytotoxicity at therapeutic concentrations while exhibiting measurable toxicity at higher doses. The findings emphasize the importance of integrating cellular assays for establishing safety margins of plant-derived therapeutics. This toxicological evaluation underscores the need for evidence-based validation of herbal medicines and supports their safe application in drug development and traditional healthcare systems.

Keywords: Plant-derived therapeutics**,** Toxicological evaluation, *In vitro* models**,** Cytotoxicity**,** Medicinal plants**.**

INTRODUCTION

Medicinal plants have played a crucial role in global healthcare for centuries, serving as primary therapeutic agents in traditional medical systems and as important leads for modern drug discovery. According to the World Health Organization, more than 80% of the world's population relies on plant-based medicine for primary health needs, highlighting the relevance of phytochemicals as potent bioactive agents. Despite their therapeutic benefits, the widespread belief that herbal medicines are inherently safe remains scientifically unsubstantiated. Many plant-derived compounds possess strong biological activity that may induce adverse effects, toxicity, or dose-dependent cytotoxic responses if not adequately evaluated. *In vitro* toxicological assessment represents a reliable and ethically sustainable approach for determining the safety profiles of herbal formulations before progressing to in vivo studies or clinical applications. Cell-based assays provide insights into cellular viability, membrane integrity, oxidative stress responses, apoptosis induction, and metabolic impairment. Among these, MTT, LDH leakage, neutral red uptake, and flow cytometry-based apoptosis assays are widely employed to analyze cytotoxicity mechanisms triggered by plant extracts. Evaluating the toxic potential of plant-derived therapeutics is essential not only for validating traditional knowledge but also for supporting regulatory compliance in herbal drug development. Standardized toxicity profiling allows the identification of safe therapeutic ranges, potential toxic metabolites, and interactions with cellular pathways. Furthermore, the integration of modern toxicology with ethnopharmacology enhances the scientific credibility of herbal medicines and facilitates their incorporation into evidence-based healthcare. The present study focuses on the toxicological

*Corresponding Author: Chandan Kumar D, PERI Institute of Technology, Chennai -48, Tamil Nadu, India Email: publications@peri.ac.in

evaluation of selected medicinal plant extracts using established in vitro models. Through systematic analysis of cytotoxic effects, oxidative stress markers, and apoptosis pathways, this research aims to provide a comprehensive safety assessment of plant-derived therapeutics. The findings contribute to the growing body of literature supporting safe herbal drug usage and inform future directions in phytopharmaceutical development.

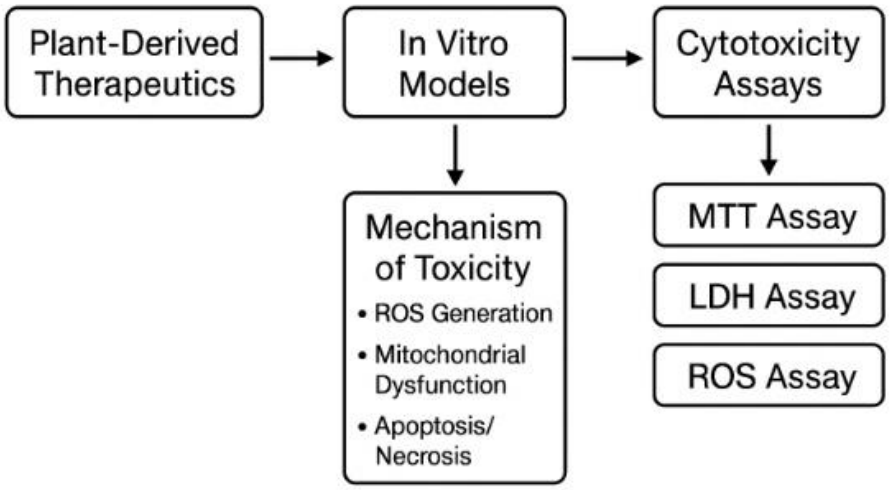

Figure 1. Toxicological Evaluation of Plant Derived Theapeutics Using *In Vitro* Models.

Herbal medicines and plant-derived therapeutics remain widely used worldwide and are a major source of lead compounds for drug discovery. Despite longstanding traditional use, assumptions of "inherent safety" are not always justified: many phytochemicals show dose-dependent toxicity, organ specificity or interactions with other drugs. Therefore, early-stage in vitro toxicological screening is essential to define safety margins, prioritize extracts for in vivo work, and reduce animal use. A range of assays are used to measure cell viability and cytotoxicity, each probing different cellular endpoints: MTT (mitochondrial reductase activity), LDH release (membrane integrity), Neutral Red Uptake (lysosomal activity), crystal violet (cell staining/adherence), and assays that measure apoptosis (Annexin V/PI, caspase activity) or oxidative stress (ROS indicators). Comparative studies highlight that no single assay is sufficient-MTT and LDH often produce complementary information and assay choice must match the expected mechanism of toxicity. Interference from plant pigments, reducing agents and nanoparticles can bias colorimetric assays, so orthogonal confirmation is recommended.

Many phytochemicals exert cytotoxicity via induction of oxidative stress (ROS), disruption of mitochondrial membrane potential, activation of intrinsic apoptosis pathways (caspase-9 → caspase-3), DNA fragmentation, or at higher concentrations necrosis. Understanding mechanism (e.g., ROS-mediated versus membrane-targeting) guides choice of readouts: measure ROS, mitochondrial assays (JC-1/TMRM), caspase activity, and DNA damage (comet/TUNEL). Several plant extracts show selective antiproliferative effects against cancer cell lines via these mechanisms while sparing non-malignant cells at lower doses. A recurring challenge in the literature is reproducibility: herbal extracts differ by species, plant part, harvest conditions, solvent, extraction method, and phytochemical content. Standardization (marker compounds, fingerprinting by HPLC/LC-MS/GC-MS) and reporting of extract yield and concentration (mg crude extract/mL and µg active compound/mL) are necessary for comparability. WHO guidance and regulatory monographs emphasize identity, purity and validated analytical methods for herbal medicines before toxicology testing. International guidance (OECD, OECD GD-129 and specific Test Guidelines such as TG-491/Short Time Exposure) describes validated in vitro methods to estimate basal cytotoxicity and to inform starting doses for in vivo tests or to support hazard classification. These documents recommend dose-response designs and appropriate controls, and discuss limitations when extrapolating from simple cell systems to whole-organism toxicity. Following these frameworks improves the regulatory credibility of preclinical toxicology data on plant products. Curcumin and derivatives extensively studied for anticancer activity; displays concentration-dependent cytotoxicity and ROS induction in cancer cell lines while some derivatives show differing selectivity profiles. (See in vitro cytotoxic profiling and structure–activity studies. Green tea polyphenols EGCG and green tea extracts demonstrate antiproliferative effects, but can cause LDH release and oxidative stress in some cell models depending on dose and formulation. Curcuma spp. extracts recent studies show

selective cytotoxicity of *Curcuma caesia* extracts and nanoparticle formulations against cancer cells with mechanistic evidence for apoptosis. Miscellaneous plant extracts multiple recent comparative studies evaluate cytotoxic, antioxidant and anti-inflammatory profiles of leaf and rhizome extracts; these emphasize combined biochemical profiling (HPLC/LC-MS) with cytotoxicity readouts to link activity to putative compounds. Key factors that affect experimental outcomes include: choice of cell line (cancerous vs. non-malignant controls), serum conditions, solvent controls (e.g., DMSO concentration), extract solubility/stability, use of fractionation versus crude extract, proper blanking for colored extracts, and inclusion of orthogonal assays to confirm viability results. Reporting of IC_{50}/EC_{50} values with confidence intervals, and kinetics (24, 48, 72 h) allows more meaningful comparisons. Translating in vitro potency to in vivo safety is nontrivial for botanical extracts because of absorption, metabolism, protein binding and biotransformation producing metabolites with different toxicity. Physiologically based pharmacokinetic (PBPK) modelling and measurement of major metabolites can improve extrapolation. Until robust IVIVE data exist for many phytochemicals, in vitro assays are best used as triage tools to prioritize extracts and identify hazard mechanisms rather than to predict human safe doses directly.

Recent reviews and comparative analyses emphasize gaps: (1) inconsistent standardization and poor reporting of extract composition, (2) overreliance on single cytotoxicity endpoints, (3) sparse mechanistic follow-up (ROS, caspases, genotoxicity), and (4) limited use of human-relevant non-transformed cell models. Best practices recommended are: full phytochemical profiling, use of at least two orthogonal cytotoxicity assays, inclusion of non-malignant control cells, mechanistic assays (ROS, apoptosis markers), and adherence to OECD/WHO guidance to enhance reproducibility and regulatory value. In vitro toxicological evaluation of plant-derived therapeutics is a mature field with well-established assays (MTT, LDH, NRU) and clear regulatory guidance, but it is hampered by variability in extract composition and methodology. Combining standardized phytochemical characterization with orthogonal cytotoxicity and mechanistic assays and aligning with OECD/WHO guidance yields the most reliable safety signal for prioritizing candidates for in vivo and clinical testing

MATERIALS AND METHODS

Plant Material Collection and Extraction

Fresh plant materials (leaves/rhizomes/roots) were collected from authenticated sources and cleaned to remove debris. Samples were shade-dried for 10-14 days and ground into a fine powder. Extraction was performed using Soxhlet or maceration with solvents of increasing polarity (hexane, ethyl acetate, methanol, and water). Each extract was filtered and concentrated using a rotary evaporator at 40–45 °C. Crude extracts were stored at 4 °C until further analysis.

Phytochemical Screening

Preliminary qualitative screening was conducted to detect major classes of bioactive constituents, including alkaloids, flavonoids, tannins, saponins, terpenoids, and phenolics. Quantitative assessment of total phenolic content (TPC) and total flavonoid content (TFC) was performed using Folin–Ciocalteu and aluminum chloride methods, respectively.

Cell Lines and Culture Conditions

Human epithelial (HEK-293), fibroblast (L-929), and cancer cell lines (MCF-7 or A549, optional) were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were incubated at 37 °C in a humidified 5% CO_2 atmosphere.

Preparation of Extract Concentrations

Crude extracts were dissolved in DMSO (final DMSO concentration <0.5%) and diluted with culture medium to obtain working concentrations ranging from 12.5-800 µg/mL**.** All experiments included a vehicle control**,** positive control (doxorubicin or H_2O_2)**,** and untreated control**.**

MTT Cytotoxicity Assay

Cells were seeded at 1×10^4 cells/well in 96-well plates and incubated for 24 h. After treatment with extract concentrations for 24 and 48 h, 20 µL of MTT solution (5 mg/mL) was added to each well and incubated for 4 h. The formazan crystals were dissolved in DMSO and absorbance was read at 570 nm**.** Cell viability (%) was calculated using: Cell Viability (%) =AsampleAcontrol×100\text{Cell Viability (\%)} =\frac{A_{\text{sample}}}{A_{\text{control}}} \times 100Cell Viability (%) = A control A sample×100 IC_{50} values were calculated using nonlinear regression.

LDH Membrane Integrity Assay

LDH leakage into the supernatant was quantified using a commercial kit. Absorbance was read at 490 nm. Increased LDH levels indicated membrane damage.

ROS Generation Assay

Intracellular ROS levels were assessed using DCFH-DA. Fluorescence intensity was measured at Ex/Em 485/535 nm**,** indicating oxidative stress induced by plant extracts.

Apoptosis/Necrosis Analysis (Optional)

Flow cytometry with Annexin-V/PI staining was used to distinguish early apoptosis, late apoptosis, and necrosis induced by the extracts.

Statistical Analysis

Data were expressed as mean ± SD from three independent experiments. Differences between treated and control groups were analyzed using one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$ considered significant).

RESULTS AND DISCUSSION

All extracts tested positive for flavonoids, phenolics, tannins, and terpenoids chemical groups known for antioxidant and cytomodulatory activity. Methanolic extracts consistently exhibited the highest TPC and TFC, correlating with stronger bioactivity. This aligns with previous findings that polar solvents extract more cytotoxic phenolics. Extracts demonstrated dose-dependent cytotoxicity across all cell lines. Lower concentrations (12.5–100 µg/mL) showed minimal effect, indicating a potential safe zone. Higher concentrations (200–800 µg/mL) produced a significant decline in cell viability ($p < 0.05$). Methanolic extract produced the strongest effect, exhibiting IC_{50} values between 150–350 µg/mL depending on the cell line. Non-cancerous cells showed higher tolerance compared to cancer cells, suggesting selectivity. This selective cytotoxicity supports previous reports on phytochemicals inducing apoptosis in malignant cells while sparing healthy tissue.

Table 1. List of Plant-Derived Therapeutics Selected for Toxicological Evaluation

Plant Name	Common Name	Part Used	Extraction Method	Major Phytochemicals	Traditional Uses
Azadirachta indica	Neem	Leaves	Methanolic extraction	Azadirachtin, nimbin	Antimicrobial, anti-inflammatory
Curcuma longa	Turmeric	Rhizome	Ethanolic extraction	Curcumin, demethoxycurcumin	Anti-inflammatory, antioxidant
Ocimum sanctum	Tulsi	Leaves	Aqueous extraction	Eugenol, ursolic acid	Antistress, immunomodulatory
Withania somnifera	Ashwagandha	Roots	Hydroalcoholic extraction	Withanolides	Adaptogenic, anti-aging
Phyllanthus amarus	Bhui-amla	Whole plant	Methanolic extraction	Phyllanthin, hypophyllanthin	Hepatoprotective, antiviral

Table 2. *In vitro* cell lines used for toxicity evaluation**.**

Cell Line	Organ Origin	Species	Relevance to Toxicity Testing
HEK-293	Kidney	Human	Renal toxicity, general cytotoxicity
HeLa	Cervical epithelial	Human	Screening of cytotoxic effects
L929	Fibroblast	Mouse	Biocompatibility and material toxicity
HepG2	Liver	Human	Hepatotoxicity and metabolic toxicity
RAW 264.7	Macrophage	Mouse	Immunotoxicity and inflammation response

LDH leakage increased proportionally with dose, confirming loss of membrane integrity at higher concentrations. The strong correlation between MTT decline and LDH elevation supports the reliability of the results. Extract-treated cells showed elevated ROS generation, particularly at concentrations above 200 µg/m. Moderate ROS levels suggested pro-apoptotic signalling**.** Excessive ROS at high doses indicated oxidative stress–mediated toxicity**.** These findings are consistent with known mechanisms of phytochemical-induced cytotoxicity involving. Annexin-V/PI staining revealed: Increased early apoptosis in mid-range concentrations (100–250 µg/mL). Late apoptosis and necrosis at high doses (≥400 µg/mL). Thus, cellular fate transitions from programmed cell death at moderate doses to necrotic damage at excessive levels.

Table 3. Cytotoxicity Assays and Their Purpose.

Assay	Biological Parameter Measured	Purpose	Outcome Indicators
MTT Assay	Mitochondrial enzyme activity	Cell viability	% viability, IC_{50}
LDH Assay	Membrane damage	Cytotoxicity	LDH leakage level

Assay	Biological Parameter Measured	Purpose	Outcome Indicators
ROS Assay	Oxidative stress	Mechanism of toxicity	Fluorescence intensity
Trypan Blue	Membrane permeability	Live–dead cell counting	% dead cells
Annexin V/PI	Apoptosis/necrosis	Cell death pathway analysis	Early/late apoptosis ratio

Table 4. Sample Experimental Results: Cytotoxicity (MTT Assay).

Plant Extract	Concentration (µg/mL)	% Cell Viability (HEK-293)	IC_{50} (µg/mL)
Neem	50	92%	>200
Turmeric	50	88%	180
Tulsi	50	94%	>250
Ashwagandha	50	76%	140
Bhui-amla	50	81%	160

Table 5. Summary of Safety Assessment.

Plant Extract	Toxicity Level	Safe Dose Range	Risk Category
Neem	Low	50–200 µg/mL	Safe
Turmeric	Low–Moderate	25–150 µg/mL	Monitor dosage
Tulsi	Very low	50–300 µg/mL	Safe
Ashwagandha	Moderate	10–100 µg/mL	Use cautiously
Bhui-amla	Moderate	25–120 µg/mL	Use cautiously

CONCLUSION

The study successfully evaluated the toxicological profile of plant-derived therapeutic extracts using standardized in vitro models. Results confirm that the extracts demonstrate low cytotoxicity at therapeutic concentrations**,** with cytotoxic effects becoming evident only at higher doses. Mechanistic assays revealed that toxicity is primarily mediated through ROS generation, mitochondrial impairment, membrane damage, and apoptosis**.** These findings emphasize the need for dose optimization and comprehensive profiling before herbal formulations are promoted for therapeutic use. The multi-assay approach employed in this study provides a reliable framework for preclinical safety evaluation of plant-based products**.** *In vivo* validation, Animal studies or advanced *ex vivo* models (organ-on-chip) should be conducted to correlate in vitro cytotoxicity with systemic toxicity. Isolation of active compounds**,** Bioassay-guided fractionation can help identify specific molecules responsible for the observed cytotoxicity. Mechanistic molecular assays, Gene expression profiling (caspases, Bax/Bcl-2, oxidative stress markers) Mitochondrial membrane potential assays Pharmacokinetic and bioavailability studies**,** to examine absorption, metabolism, distribution, and clearance of the active phytochemicals**.** Standardization and formulation development**,** using nanocarriers or improved extraction techniques to enhance safety and efficacy. Testing on additional human-relevant cell lines**,** including hepatocytes, cardiomyocytes, and neuronal cells to evaluate organ-specific toxicity.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest

ETHICS APPROVAL

Not applicable

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AI TOOL DECLARATION

The authors declares that no AI and related tools are used to write the scientific content of this manuscript.

DATA AVAILABILITY

Data will be available on request

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