RESEARCH PAPER

# In-Vitro Cytotoxicity, Apoptosis Induction and Cell Cycle Arrest by Hydro-Alcoholic Leaf Extract of Withania somnifera (L.) on Human Leukemia Cell Lines

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*ABSTRACT*

**Background**
Leukemia remains a significant hematological malignancy with considerable treatment-related toxicity and limited curative options, necessitating the exploration of safer therapeutic alternatives. Withania somnifera (L.), a well-established Rasayana in Ayurveda, contains withanolides with documented anticancer potential, warranting systematic evaluation against leukemia cell lines.

**Objectives**
To evaluate the cytotoxic, apoptosis-inducing, and cell cycle-modulatory effects of hydro-alcoholic leaf extract of Withania somnifera (L.), on three human leukemia cell lines: K562 (chronic myeloid leukemia), HL-60 (acute promyelocytic leukemia), and THP-1 (acute monocytic leukemia).

**Materials and Methods**
Hydro-alcoholic extract of Withania somnifera (L.) leaves (70:30 ethanol:water) was evaluated at concentrations of 10–100 µg/mL against K562, HL-60, and THP-1 cell lines. Cytotoxicity was assessed by MTT assay after 24 h treatment. Apoptosis was quantified using Annexin V-FITC/Propidium Iodide (PI) dual staining and flow cytometry. Cell cycle distribution was analyzed by PI staining. Doxorubicin served as positive control.

**Results**
The extract demonstrated dose-dependent cytotoxicity across all three cell lines, with HL-60 exhibiting the highest sensitivity (26.7 ± 0.12% viability at 100 µg/mL). Annexin V-FITC/PI staining confirmed predominantly apoptotic cell death, with HL-60 showing 34.7 ± 1.5% early apoptosis and 29.3 ± 1.1% late apoptosis, accompanied by minimal necrosis (6.2 ± 0.4%). Cell cycle analysis revealed significant Sub-G1 accumulation (31.3% in HL-60) and G0/G1 phase arrest across all cell lines.

**Conclusions**
The hydro-alcoholic leaf extract of Withania somnifera (L.) demonstrated significant antileukemic activity through apoptosis induction and cell cycle arrest, with HL-60 cells being the most sensitive. These findings support its potential as an adjunct therapeutic agent in leukemia management and warrant further in vivo investigation.

**Keywords:** Withania somnifera (L.), Apoptosis, Cytotoxicity, Leukemia, Flow cytometry.
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## 1. Introduction

Leukemia, a heterogeneous group of hematological malignancies originating from the clonal proliferation of abnormal white blood cells, represents a major global health burden. According to the GLOBOCAN 2022 estimates, leukemia accounted for approximately 487,000 new cases and 305,000 deaths worldwide, making it one of the leading causes of cancer-related mortality [1,2]. The disease encompasses several subtypes, including chronic myeloid leukemia (CML), acute promyelocytic leukemia (APL), and acute monocytic leukemia, each characterized by distinct pathogenic mechanisms, clinical presentations, and treatment responses [3].
Conventional chemotherapeutic agents, while effective in inducing remission, are associated with significant dose-limiting toxicities, development of drug resistance, and adverse effects on normal tissues, particularly the bone marrow, gastrointestinal epithelium, and immune system [4,5]. These limitations have driven the search for alternative or complementary therapeutic strategies that are effective, safer, and capable of overcoming chemoresistance. In this context, phytochemicals derived from traditional medicinal plants have

emerged as promising candidates for anticancer drug discovery [6,7].

*Withania somnifera* (L.) Dunal, commonly known as Ashwagandha or Indian Ginseng, is one of the most important medicinal plants in the traditional Indian system of medicine, Ayurveda. Classified as a Rasayana (rejuvenator) and Balya (tonic), it has been employed for centuries in the management of a wide range of ailments including stress, fatigue, inflammation, and immune dysfunction [8,9]. The plant belongs to the family Solanaceae and is widely distributed across the Indian subcontinent, the Mediterranean region, and parts of Africa [10].

The pharmacological versatility of *Withania somnifera* (L.), is primarily attributed to its rich phytochemical profile, which includes withanolides (steroidal lactones), alkaloids, saponins, flavonoids, and glycosides [11,12]. Among these, withanolides-particularly Withaferin A, Withanolide D, and Withanolide E-have garnered significant research attention for their anticancer properties. These compounds have been demonstrated to induce apoptosis, inhibit cell proliferation, suppress angiogenesis, and modulate key oncogenic signaling pathways including NF-κB, PI3K/Akt, and MAPK in various cancer models [13-16].

While the root of *Withania somnifera* (L.), has been the most extensively studied plant part in traditional formulations, recent pharmacological evidence suggests that the leaves contain a higher concentration of certain bioactive withanolides, particularly Withaferin A, compared to the roots [17,18]. This observation renders the leaf a potentially superior source of anticancer phytoconstituents and justifies its selection for the present investigation.

Despite the growing body of evidence supporting the anticancer potential of *Withania somnifera* (L.), comprehensive studies specifically evaluating the cytotoxic, apoptosis-inducing, and cell cycle-modulatory effects of its leaf extract on human leukemia cell lines remain limited. Most existing studies have focused on solid tumors such as breast, colon, and lung cancers, and comparatively fewer investigations have addressed hematological malignancies [19,20].

Therefore, the present study was designed to evaluate the *in-vitro* antileukemic potential of hydro-alcoholic leaf extract of *Withania somnifera* (L.), against three human leukemia cell lines representing distinct subtypes of hematological malignancies: K562 (chronic myeloid leukemia), HL-60 (acute promyelocytic leukemia), and THP-1 (acute monocytic leukemia). The study employed the MTT assay for cytotoxicity evaluation, Annexin V-FITC/PI dual staining for apoptosis detection, and PI-based flow cytometric cell cycle analysis to elucidate the mechanisms underlying the observed antiproliferative effects. Doxorubicin, a clinically established chemotherapeutic agent, was used as the positive control for comparative evaluation.

## 2. Materials and Methods

### *2.1 Preparation of Plant Extract*

Fresh, healthy leaves of *Withania somnifera* (L.) were collected, authenticated, and shade-dried at room temperature for 7–10 days to preserve thermolabile constituents. The dried leaves were pulverized using a laboratory-grade electric grinder, sieved to ensure uniform particle size, and stored in airtight glass containers protected from light and moisture. Extraction was performed using the hydro-alcoholic method with a 70:30 ethanol-to-distilled water ratio by cold maceration over 72 hours with intermittent stirring. The filtrate was concentrated under reduced pressure using a rotary evaporator at 40–45°C to yield a semi-solid residue, which was dried in a vacuum desiccator and stored at 4°C in airtight amber-colored vials [21,22]. For cell culture experiments, stock solutions were prepared in dimethyl sulfoxide (DMSO) at a final concentration of $\leq$0.1% (v/v) to preclude solvent-induced cytotoxicity [23].

### *2.2 Cell Lines and Culture Conditions*

Three human leukemia cell lines were selected to evaluate the broad-spectrum cytotoxic potential of the extract: (a) K562, a chronic myeloid leukemia (CML) cell line derived from a female patient in blast crisis, known for its resistance to apoptosis [24]; (b) HL-60, an acute promyelocytic leukemia (APL) cell line established from a 36-year-old female patient, highly responsive to agents that induce differentiation and apoptosis [25]; and (c) THP-1, a monocytic leukemia cell line derived from an acute monocytic leukemia patient, frequently employed in studies on monocyte-macrophage differentiation and immune responses [26]. All cell lines were obtained from a certified cell repository and verified for authenticity and absence of mycoplasma contamination.

Cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 IU/mL penicillin and 100 µg/mL streptomycin). Cultures were maintained in T-25 flasks at 37°C in a humidified incubator with 5% $CO_2$ and 95% air. Sub-culturing was performed every 48–72 hours. Prior to experiments, cell viability was confirmed at $\geq$95% using the trypan blue exclusion test [27,28].

### *2.3 MTT Assay (Cytotoxicity Evaluation)*

Cytotoxicity was evaluated using the MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which measures the reduction of MTT by mitochondrial succinate dehydrogenase in viable cells to insoluble purple formazan crystals [29–31]. Cells were seeded in 96-well plates at a density of 5,000–10,000 cells per well in 100 µL of growth medium and incubated at 37°C with 5% $CO_2$ for 24 hours. Following attachment, cells were treated with serial dilutions of the extract and Doxorubicin (positive control) at concentrations of 10, 25, 50, and

100 µg/mL for 24 hours. Untreated cells served as the negative control. After treatment, 10 µL of MTT solution (5 mg/mL in PBS) was added to each well, and plates were incubated for 3–4 hours at 37°C in the dark. The medium was carefully removed, and 100 µL of DMSO was added to solubilize the formazan crystals. Absorbance was measured at 570 nm (reference wavelength: 630–690 nm) using a microplate reader. Cell viability was calculated as: Cell Viability (%) = (Absorbance of treated cells / Absorbance of control cells) × 100. All experiments were performed in triplicate [32,33].

***2.4 Apoptosis Detection (Annexin V-FITC/PI Assay)***

To investigate whether the cytotoxicity was associated with apoptosis, Annexin V-FITC/Propidium Iodide (PI) dual staining was performed [34,35]. Cells (1–2 × $10^5$ per well) were seeded in 96-well plates, incubated overnight at 37°C in 5% $CO_2$, and treated with the extract and Doxorubicin at equivalent concentrations. Untreated cells served as controls. After incubation, both floating and adherent cells were harvested, washed twice with cold PBS (centrifugation at 1200–1500 rpm for 5 min), and resuspended in 100 µL of 1× Annexin V binding buffer. Cells were stained with 5 µL Annexin V-FITC and 5 µL PI, gently vortexed, and incubated for 15 minutes at room temperature in the dark. Following addition of 400 µL binding buffer, samples were analyzed immediately on a flow cytometer using FL1 (FITC) and FL2/FL3 (PI) channels, collecting at least 10,000 events per sample. Annexin V binds to phosphatidylserine exposed on the outer membrane leaflet during early apoptosis, while PI stains DNA in cells with compromised membrane integrity, characteristic of late apoptosis or necrosis [36,37].

***2.5 Cell Cycle Analysis***

Cell cycle distribution was analyzed by PI staining followed by flow cytometry to detect extract-induced alterations in cell cycle progression [38,39]. Cells (2–3 × $10^5$ per well) were seeded, treated with selected concentrations of the extract for 24–48 hours, and harvested. Cells were washed twice with cold PBS, resuspended gently in 500 µL cold PBS, and fixed by dropwise addition of 4.5 mL ice-cold 70% ethanol while vortexing gently to prevent clumping. Fixation was carried out overnight at −20°C. After removal of ethanol by centrifugation and PBS wash, cells were resuspended in 500 µL PI staining solution containing 50 µg/mL PI, 100 µg/mL RNase A, and 0.1% Triton X-100, and incubated for 30 minutes at room temperature in the dark. Stained cells were analyzed using a flow cytometer (FL2/FL3 channel), collecting at least 10,000 events per sample. Data were interpreted using flow cytometry software to determine the percentage distribution of cells in Sub-G1, G0/G1, S, and G2/M phases [40,41].

***2.6 Statistical Analysis***

All experiments were performed in triplicate (n = 3). Data are expressed as mean ± standard error (SE). Statistical significance was assessed by comparison of treated groups with untreated controls.

## 3. Results

***3.1 Cytotoxicity (MTT Assay)***

The cytotoxic potential of *Withania somnifera* (L.), leaf extract was evaluated against K562, HL-60, and THP-1 cell lines after 24-hour treatment at concentrations of 10–100 µg/mL. The results, presented in Table 1, demonstrated a dose-dependent decrease in cell viability across all three cell lines for both the test extract and Doxorubicin (positive control).

At the lowest concentration (10 µg/mL), cell viability remained relatively high across all lines (85.4 ± 0.35% for K562, 81.2 ± 0.12% for HL-60, and 88.7 ± 0.12% for THP-1), indicating modest cytotoxicity at lower doses. However, at 50 µg/mL, cell viability declined substantially, particularly in HL-60 (44.3 ± 0.12%) and K562 (47.9 ± 0.23%) cells, indicating that approximately half of the cell population was eliminated at this concentration.

At the highest concentration tested (100 µg/mL), the extract reduced cell viability to 26.7 ± 0.12% in HL-60, 28.6 ± 0.10% in K562, and 34.2 ± 0.06% in THP-1 cells, confirming marked cytotoxic activity. Among the three cell lines, HL-60 demonstrated the greatest sensitivity to the extract, followed by K562, with THP-1 showing relatively lower sensitivity. Doxorubicin exhibited stronger cytotoxicity at equivalent concentrations, reducing viability to 10.1 ± 0.05% (HL-60), 12.5 ± 0.08% (K562), and 15.3 ± 0.10% (THP-1) at 100 µg/mL.

**Table 1:** Percentage cell viability after 24 hours treatment with *Withania somnifera* (L.), leaf extract and Doxorubicin

| Conc. (µg/mL) | K562 (% ± SE) | Doxorubicin K562 (% ± SE) | HL-60 (% ± SE) | Doxorubicin HL-60 (% ± SE) | HP-1 (% ± SE) | Doxorubicin THP-1 (% ± SE) |
|---|---|---|---|---|---|---|
| 0 | 100.0 ± 0.00 | 100.0 ± 0.00 | 100.0 ± 0.00 | 100.0 ± 0.00 | 100.0 ± 0.00 | 100.0 ± 0.00 |
| 10 | 85.4 ± 0.35 | 77.3 ± 0.25 | 81.2 ± 0.12 | 75.5 ± 0.30 | 88.7 ± 0.12 | 80.4 ± 0.21 |
| 25 | 68.3 ± | 52.7 ± 0.18 | 64.5 ± | 48.9 ± 0.22 | 72.1 ± | 56.3 ± 0.16 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | 0.20 | | 0.12 | | 0.17 | |
| 50 | 47.9 ± 0.23 | 28.9 ± 0.15 | 44.3 ± 0.12 | 25.4 ± 0.13 | 51.6 ± 0.17 | 31.2 ± 0.20 |
| 100 | 28.6 ± 0.10 | 12.5 ± 0.08 | 26.7 ± 0.12 | 10.1 ± 0.05 | 34.2 ± 0.06 | 15.3 ± 0.10 |

*Values are expressed as mean ± standard error (n = 3).*

### *3.2 Apoptosis Profile (Annexin V-FITC/PI Assay)*

Annexin V-FITC/PI dual staining was performed to characterize the mode of cell death induced by the extract. The results, summarized in Table 2, revealed that the extract predominantly induced apoptotic cell death across all three leukemia cell lines, with minimal necrosis.

HL-60 cells exhibited the highest susceptibility to apoptosis, with 34.7 ± 1.5% early apoptosis and 29.3 ± 1.1% late apoptosis (total apoptosis: 64.0%), leaving only 29.8 ± 1.8% viable cells. In K562 cells, early apoptosis was 28.1 ± 1.2% and late apoptosis was 25.4 ± 0.9% (total: 53.5%), with 38.0 ± 1.6% live cells remaining. THP-1 cells showed comparatively lower apoptosis (21.6 ± 0.8% early, 19.9 ± 1.0% late; total: 41.5%), with 50.7 ± 2.1% viable cells, indicating the lowest sensitivity among the three cell lines.

Importantly, necrosis was minimal in all extract-treated groups (6.2–8.5%), confirming that the predominant mechanism of cell death was controlled apoptosis rather than unregulated necrosis. In comparison, Doxorubicin induced stronger apoptotic responses, with HL-60 showing 49.5 ± 1.8% early and 43.1 ± 1.6% late apoptosis (total: 92.6%), and correspondingly higher necrosis (10.8–14.5%), likely reflecting the more aggressive cytotoxic action of the conventional chemotherapeutic agent.

**Table 2:** Apoptosis profile post-treatment with *Withania somnifera* (L.), leaf extract and Doxorubicin

| Cell Line | Early Apoptosis (%) WS | Early Apoptosis (%) Dox | Late Apoptosis (%) WS | Necrosis (%) WS | Live (%) WS | Late Apoptosis (%) Dox | Necrosis (%) Dox | Live (%) Dox |
|---|---|---|---|---|---|---|---|---|
| K562 | 28.1 ±1.2 | 42.3 ±1.5 | 25.4 ±0.9 | 8.5 ±0.5 | 38.0± 1.6 | 38.4 ±1.2 | 12.1± 0.6 | 7.2 ±0.3 |
| HL-60 | 34.7 ±1.5 | 49.5 ±1.8 | 29.3 ±1.1 | 6.2 ±0.4 | 29.8± 1.8 | 43.1 ±1.6 | 14.5± 0.7 | 3.0 ±0.1 |
| THP-1 | 21.6 ±0.8 | 33.2 ±1.0 | 19.9 ±1.0 | 7.8 ±0.6 | 50.7± 2.1 | 31.4 ±1.4 | 10.8± 0.9 | 24.6± 1.3 |

*WS = Withania somnifera* (L.) *leaf extract; Dox = Doxorubicin. Values are mean ± SE (n = 3).*

### *3.3 Cell Cycle Distribution*

Cell cycle analysis by PI staining and flow cytometry revealed that the extract induced significant alterations in cell cycle distribution across all three leukemia cell lines (Table 3).

A notable increase in the Sub-G1 population, indicative of DNA fragmentation and apoptosis, was observed in all treated cell lines. HL-60 cells showed the most prominent Sub-G1 accumulation (31.3 ± 1.4%), followed by K562 (26.5 ± 1.1%) and THP-1 (19.7 ± 1.0%). Doxorubicin induced higher Sub-G1 accumulation in all cell lines (48.5% in HL-60, 42.3% in K562 and 28.1% in THP-1).

G0/G1 phase arrest was observed across all cell lines, with THP-1 showing the highest arrest (40.6 ± 1.8%), suggesting a cytostatic effect that prevents cells from progressing into S-phase. The S-phase population showed a moderate decrease, particularly in HL-60 (16.2 ± 0.7%) and K562 (18.4 ± 0.8%), indicating impairment of DNA replication. The G2/M phase population was maintained at 19.6–22.0% across the extract-treated groups, while Doxorubicin caused a marked reduction in G2/M (8.0–9.7%), reflecting its potent mitotic inhibition.

**Table 3:** Cell cycle distribution after treatment with *Withania somnifera* (L.), leaf extract and Doxorubicin

| Cell Line | Sub-G1 (%) WS | Sub-G1 (%) Dox | G0/G1 (%) WS | G0/G1 (%) Dox | S (%) WS | S (%) Dox | G2/M (%) WS | G2/M (%) Dox |
|---|---|---|---|---|---|---|---|---|
| K562 | 26.5±1.1 | 42.3±1.5 | 34.2±1.5 | 35.1±1.4 | 18.4±0.8 | 14.6±0.7 | 20.9±1.0 | 8.0 ±0.3 |
| HL-60 | 31.3±1.4 | 48.5±1.8 | 30.5±1.2 | 27.9±1.3 | 16.2±0.7 | 15.2±0.6 | 22.0±0.9 | 8.4 ±0.5 |
| THP-1 | 19.7±1.0 | 28.1±0.8 | 40.6±1.8 | 43.5±1.6 | 20.1±0.9 | 18.7±0.5 | 19.6±1.1 | 9.7 ±0.4 |

*WS = Withania somnifera* (L.) *leaf extract; Dox = Doxorubicin. Values are mean ± SE (n = 3).*

## 4. Discussion

The present study provides comprehensive *in-vitro* evidence for the antileukemic potential of hydro-alcoholic leaf extract of *Withania somnifera* (L.), against three human leukemia cell lines representing distinct hematological malignancy subtypes. The

findings demonstrate that the extract exerts dose-dependent cytotoxicity, induces apoptosis as the predominant mechanism of cell death, and causes cell cycle arrest, collectively supporting its candidacy as a potential complementary therapeutic agent in leukemia management.

***4.1 Dose-Dependent Cytotoxicity***

The MTT assay results revealed a clear concentration-dependent reduction in cell viability across K562, HL-60, and THP-1 cell lines following 24-hour treatment with the extract. Among the three cell lines, HL-60 cells demonstrated the highest sensitivity, with cell viability declining to 26.7% at 100 µg/mL, followed closely by K562 (28.6%) and THP-1 (34.2%). This differential sensitivity pattern is consistent with the established biological characteristics of these cell lines. HL-60 cells, being an acute promyelocytic leukemia model, are inherently more responsive to agents that induce differentiation and apoptosis compared to K562 cells, which originate from CML blast crisis and are characterized by constitutive BCR-ABL kinase activity that confers resistance to apoptosis [42,43].

The relatively lower sensitivity of THP-1 cells may be attributed to their monocytic lineage and the associated activation of survival pathways, including constitutive NF-κB signaling, which has been implicated in resistance to cytotoxic agents [44]. These observations align with earlier reports by Widodo et al. [45] and Malik et al. [46], who demonstrated that withanolide-rich extracts of *Withania somnifera* (L.), exhibit selective cytotoxicity against cancer cells while sparing normal cells, a property attributed to the preferential targeting of dysregulated oncogenic pathways.

While Doxorubicin demonstrated superior cytotoxicity at equivalent concentrations, the extract achieved clinically meaningful reductions in cell viability, particularly at 50 and 100 µg/mL, suggesting that it may serve as a complementary agent to reduce the dose and associated toxicity of conventional chemotherapeutic drugs [47].

***4.2 Apoptosis-Mediated Cell Death***

The Annexin V-FITC/PI dual staining results confirmed that apoptosis was the predominant mechanism of cell death induced by the extract. HL-60 cells exhibited the highest total apoptosis (64.0%), comprising 34.7% early and 29.3% late apoptosis, with only 6.2% necrosis. Similarly, K562 cells showed 53.5% total apoptosis (28.1% early, 25.4% late), while THP-1 displayed 41.5% total apoptosis. The consistently low necrosis rates (6.2–8.5%) across all extract-treated groups are therapeutically significant, as apoptosis represents a controlled, non-inflammatory form of cell death that avoids collateral damage to surrounding healthy tissues, unlike necrosis which triggers inflammatory cascades [48,49].

The apoptotic activity of *Withania somnifera* (L.), leaf extract can be attributed to its major bioactive constituents, particularly Withaferin A. This steroidal lactone has been demonstrated to induce apoptosis through multiple mechanisms, including inhibition of the NF-κB signaling pathway, activation of caspase cascades, downregulation of anti-apoptotic proteins Bcl-2 and Bcl-xL, upregulation of pro-apoptotic proteins Bax and Bak, generation of reactive oxygen species (ROS), and disruption of mitochondrial membrane potential [50–53]. Studies by Hahm et al. [54] and Lee et al. [55] have further demonstrated that Withaferin A can overcome chemoresistance in leukemia cells by targeting proteasomal and Hsp90 chaperone pathways.

In comparison, Doxorubicin induced substantially higher apoptosis (92.6% in HL-60) but also higher necrosis (10.8–14.5%), reflecting the more aggressive and less selective cytotoxic mechanism of conventional anthracyclines. The extract's ability to achieve significant apoptosis with markedly lower necrosis suggests a more favorable therapeutic profile and supports the rationale for its use as an adjunct agent [56].

***4.3 Cell Cycle Arrest***

Cell cycle analysis revealed that the extract induced significant Sub-G1 accumulation and G0/G1 phase arrest in all three cell lines. The Sub-G1 population, representing cells with fragmented DNA, was highest in HL-60 (31.3%), consistent with the apoptosis data and confirming DNA fragmentation as a key feature of the extract-induced cell death. The G0/G1 arrest, particularly prominent in THP-1 (40.6%) and K562 (34.2%), indicates that the extract impedes cell cycle progression at the G1/S checkpoint, preventing DNA replication and subsequent mitosis [57,58].

This pattern of G0/G1 arrest is consistent with the known mechanism of action of withanolides, which have been shown to modulate cell cycle regulatory proteins, including downregulation of cyclin D1, cyclin E, CDK2, and CDK4, and upregulation of the CDK inhibitors p21 and p27 [59,60]. Additionally, withanolides have been reported to inhibit the phosphorylation of retinoblastoma protein (pRb), thereby preventing E2F-mediated transcription of genes required for S-phase entry [61]. The relatively preserved G2/M population in extract-treated groups (19.6–22.0%) compared to the marked G2/M depletion by Doxorubicin (8.0–9.7%) suggests that the extract primarily acts at the G1/S checkpoint rather than the G2/M checkpoint, indicating a distinct mechanism of action [62].

***4.4 Therapeutic Implications***

The collective findings of this study-dose-dependent cytotoxicity, apoptosis-mediated cell death with minimal necrosis, and G0/G1 cell cycle arrest-position the hydro-alcoholic leaf extract of *Withania somnifera* (L.), as a promising candidate for further development as a complementary or adjunct agent in leukemia therapy. The differential sensitivity pattern, with HL-60 being the most responsive, suggests that the extract

may be particularly effective in acute promyelocytic leukemia and potentially other APL-related conditions.

From an integrative medicine perspective, these findings corroborate the traditional Ayurvedic use of Ashwagandha as a Rasayana with broad therapeutic potential. The concept of Rasayana in Ayurveda encompasses tissue rejuvenation, immune modulation, and enhancement of the body's innate healing mechanisms-properties that align with the observed cytotoxic selectivity and apoptosis-inducing capacity of the extract [63,64]. The present study therefore provides modern pharmacological validation for the traditional use of *Withania somnifera* (L.) and supports the growing body of evidence advocating for the integrative use of Ayurvedic medicinal plants in contemporary cancer management.

### *4.5 Limitations and Future Directions*

The present study has certain limitations that should be acknowledged. The experiments were conducted *in-vitro*, and the translation of these findings to *in-vivo* models and clinical settings requires further investigation. $IC_{50}$ values were not computed and dose-response curve modeling would strengthen the quantitative assessment of cytotoxicity. Additionally, the study employed a crude hydro-alcoholic extract and bioactivity-guided fractionation would be necessary to identify and isolate the specific compounds responsible for the observed antileukemic effects. Future studies should include *in-vivo* tumor models, combination studies with standard chemotherapeutic agents, evaluation of selectivity indices using normal cell lines, and mechanistic studies employing Western blotting, RT-PCR, and caspase activity assays to elucidate the precise molecular pathways involved.

## 5. Conclusion

The present study demonstrates that the hydro-alcoholic leaf extract of *Withania somnifera* (L.), possesses significant *in-vitro* antileukemic activity against three human leukemia cell lines (K562, HL-60, and THP-1). The extract exhibited dose-dependent cytotoxicity, with HL-60 cells being the most sensitive. Apoptosis was confirmed as the predominant mechanism of cell death through Annexin V-FITC/PI staining, characterized by high early and late apoptosis with minimal necrosis. Cell cycle analysis revealed significant Sub-G1 accumulation and G0/G1 phase arrest, supporting the antiproliferative action of the extract. These findings collectively validate the anticancer potential of *Withania somnifera* (L.), leaf extract and support its further evaluation as a complementary therapeutic agent in leukemia management. *In-vivo* studies and clinical trials are warranted to translate these promising *in-vitro* findings into therapeutic applications.

## Conflict of Interest

The authors declare no conflict of interest.

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