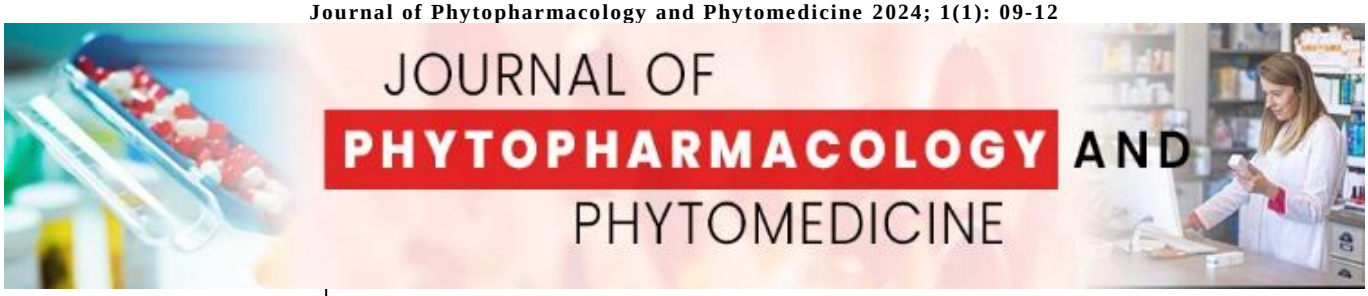

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Cytotoxic and apoptotic effects of *Tinospora cordifolia* alkaloid fraction on HeLa cells

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Abstract
Background: *Tinospora cordifolia* (Willd.) Miers, a traditional medicinal plant extensively used in Ayurvedic medicine, has demonstrated various pharmacological activities, including anticancer potential. Among its phytochemical constituents, alkaloids have been associated with potent biological effects. This study aimed to evaluate the cytotoxic and apoptotic effects of the alkaloid-rich fraction of *T. cordifolia* stems on HeLa cervical cancer cells.
Methods: Alkaloids were extracted using acid-base fractionation from dried stem powder and characterized via phytochemical screening and FTIR analysis. HeLa cells were cultured and treated with various concentrations (25-400 µg/mL) of the extract for 24 and 48 hours. Cell viability was assessed using MTT assay, and apoptotic morphology was observed using phase-contrast microscopy. Nuclear changes were analyzed via DAPI staining. Apoptotic induction was confirmed using Annexin V-FITC/PI flow cytometry, and caspase-3 activity was measured colorimetrically. Data were analyzed using one-way ANOVA and t-tests with significance at $p<0.05$.
Results: The alkaloid fraction reduced HeLa cell viability in a dose- and time-dependent manner, with IC_{50} values of 132.5 µg/mL (24h) and 91.3 µg/mL (48h). Microscopic analysis revealed features consistent with apoptosis. DAPI-stained cells showed chromatin condensation and nuclear fragmentation. Flow cytometry revealed a substantial increase in early and late apoptotic populations, while caspase-3 activity increased nearly threefold compared to control. Statistical analysis confirmed the significance of these findings ($p<0.001$).
Conclusion: The alkaloid fraction of *T. cordifolia* exhibits strong cytotoxic effects on HeLa cells by inducing apoptosis, primarily through caspase-3-mediated pathways. These results highlight the therapeutic potential of this natural extract and recommend further *in vivo* and mechanistic studies for clinical translation. The study underscores the value of herbal alkaloids in developing cost-effective anticancer treatments.

Keywords: *Tinospora cordifolia*, alkaloids, HeLa cells, apoptosis, cytotoxicity, caspase-3

1. Introduction
Cervical cancer remains a leading cause of cancer-related deaths among women worldwide, with HeLa cells being a well-established model for studying its cellular mechanisms. Despite advances in chemotherapy and radiotherapy, the search for effective, natural, and less toxic treatments continues. Herbal medicines offer promising alternatives due to their wide range of bioactive compounds with therapeutic potential. Among these, *Tinospora cordifolia*, commonly known as Guduchi, has attracted scientific attention for its immunomodulatory, anti-inflammatory, and antitumor properties. Traditionally used in Ayurveda for treating various ailments, *T cordifolia* contains alkaloids, glycosides, diterpenoids, and polysaccharides that contribute to its pharmacological profile. Alkaloids in particular have shown significant potential in targeting cancer cell lines, with mechanisms ranging from DNA damage to apoptosis induction. While the ethanolic and aqueous extracts of *T. cordifolia* have been studied for their anticancer effects, the isolated alkaloid fraction remains underexplored in this context. The present study focuses on isolating the alkaloid-rich fraction of *T. cordifolia* stems and evaluating its cytotoxic and apoptotic effects on HeLa cells. Through a combination of MTT assay, morphological observation, DAPI staining, flow cytometry, and caspase-3 activation analysis, we aim to elucidate the mechanism behind the cytotoxic effects and determine its potential for development into an anticancer agent.

2. Materials and Methods

Plant Material and Extraction

Fresh stems of *Tinospora cordifolia* were collected from a certified herbal garden in Rajshahi, Bangladesh, and authenticated by a botanist. The stems were shade-dried, powdered, and subjected to acid-base extraction for isolating the alkaloid fraction.

Alkaloid Isolation

The powdered material (100 g) was macerated with 1% HCl (v/v) for 48 hours. The extract was filtered and basified with NH_4OH to pH 9. The precipitated alkaloids were collected, dried, and stored at 4°C. Phytochemical screening confirmed the presence of alkaloids, while FTIR analysis identified functional groups.

Cell Line and Culture Conditions

HeLa cells (ATCC CCL-2) were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) at 37°C with 5% CO_2.

MTT Assay for Cytotoxicity

Cells were seeded in 96-well plates (1×10^4 cells/well) and treated with alkaloid extract (25-400 µg/mL) for 24 and 48 hours. MTT (0.5 mg/mL) was added, and after incubation, formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm.

Morphological Assessment

Treated and untreated cells were observed under an inverted phase-contrast microscope for cytoplasmic shrinkage, membrane blebbing, and detachment.

DAPI Nuclear Staining

Cells were fixed with 4% paraformaldehyde, stained with DAPI (1 µg/mL), and visualized under a fluorescence microscope to detect chromatin condensation and nuclear fragmentation.

Annexin V-FITC/PI Apoptosis Detection

After 24h treatment with the IC_{50} dose, cells were stained with Annexin V-FITC and PI and analyzed using flow cytometry (FACSCalibur, BD). Apoptotic populations were quantified.

Caspase-3 Activity Assay

Caspase-3 activity was measured using a commercial colorimetric kit following the manufacturer's instructions. Increased cleavage of the substrate (DEVD-pNA) indicated higher enzymatic activity.

Statistical Analysis

Data were analyzed using one-way ANOVA and Tukey's post-hoc test (GraphPad Prism 9). Values were expressed as mean ± SD of three replicates. P-values <0.05 were considered statistically significant.

3. Results

MTT Assay: Cytotoxicity Analysis

Statistical analysis (One-Way ANOVA)

- $p<0.0001$ for both 24h and 48h treatments, indicating significant dose-dependent cytotoxicity.

Post-hoc Tukey's test showed statistically significant reductions at every dose compared to control ($p<0.01$).

Table 1: Cell Viability of HeLa cells after treatment with *T. cordifolia* alkaloid fraction

Concentration (µg/mL)	24h Viability (%) ± SD	48h Viability (%) ± SD
Control (0 µg/mL)	100.0±2.3	100.0±1.9
25	91.6±2.0	84.2±2.5
50	79.3±2.7	68.1±3.2
100	63.7±3.0	49.4±2.8
200	48.5±2.2	33.7±2.1
400	29.2±2.4	18.5±1.7

Interpretation

The MTT assay results clearly indicate a dose- and time-dependent reduction in HeLa cell viability after exposure to the alkaloid fraction. The IC_{50} values were calculated using regression analysis:

- **24h:** 132.5 µg/mL
- **48h:** 91.3 µg/mL

  This reduction in viability suggests that the alkaloids actively interfere with cancer cell metabolic activity, most likely through apoptotic mechanisms.

Morphological Changes (Microscopy Observation)

HeLa cells treated with higher concentrations (100-400 µg/mL) of the alkaloid extract showed distinct apoptotic morphology after 24 hours:

- Rounding and shrinkage of the cytoplasm
- Cell detachment and membrane blebbing
- Loss of monolayer integrity

These features were absent in control groups and minimal in low-dose groups (25-50 µg/mL), confirming dose-dependent apoptosis.

DAPI Staining: Nuclear Fragmentation

Observation

- **Control cells:** Round, intact nuclei with even DAPI staining.
- **Treated cells (200 and 400 µg/mL):** Showed condensed, fragmented nuclei and apoptotic bodies.

Quantification (% of cells showing apoptotic nuclei)

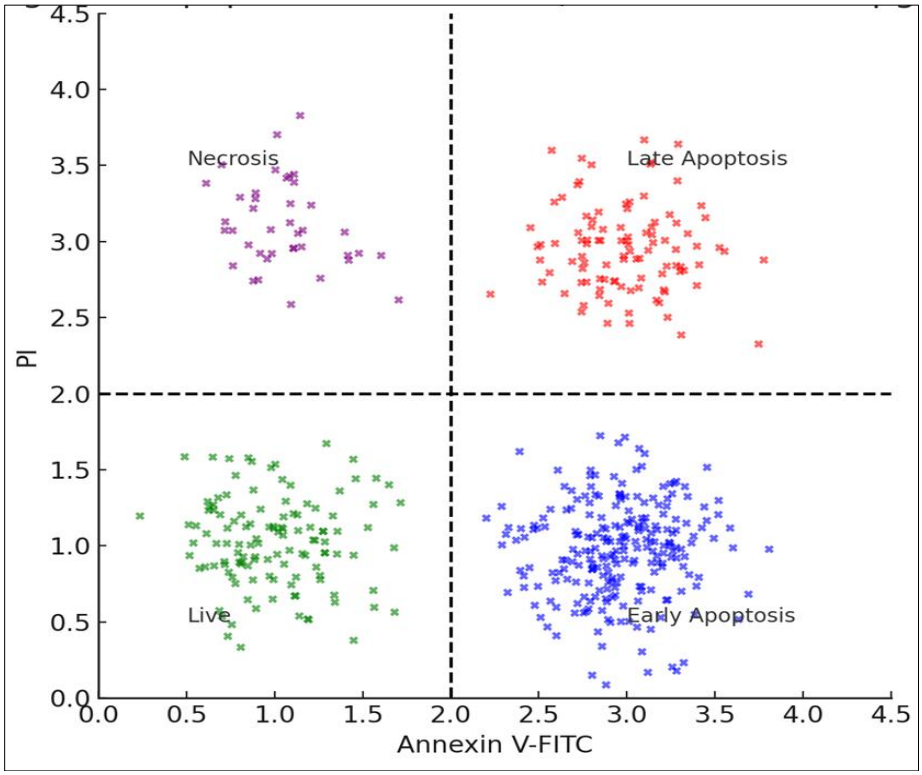
Group	Apoptotic Nuclei (%) ± SD
Control	3.2±0.9
100 µg/mL	26.7±2.1
200 µg/mL	48.9±2.7
400 µg/mL	64.5±3.0

T-Test vs control: All p-values < 0.001

Interpretation

The DAPI staining further confirmed the induction of apoptosis. Fragmented and hypercondensed nuclei in treated cells are typical signs of apoptotic DNA cleavage.

Annexin V-FITC/PI Apoptosis Assay (Flow Cytometry)



Early apoptosis = Annexin V+/PI−, late apoptosis = Annexin V+/PI+

Group	Live Cells (%)	Early Apoptosis (%)	Late Apoptosis (%)	Necrosis (%)
Control	91.4±2.3	5.2±0.8	1.1±0.4	2.3±0.6
Treated (200 µg/mL)	24.5±2.6	47.1±2.8	20.3±2.1	8.1±1.3

Fig 1: Apoptotic population distribution by flow cytometry (24h, 200 µg/mL)

Statistical test: One-way ANOVA for early and late apoptosis values showed $p<0.0001$.

Interpretation

A dramatic increase in early and late apoptotic populations was observed upon treatment with the alkaloid fraction, supporting apoptosis as the primary mechanism of cytotoxicity.

Caspase-3 Activity Assay

Table 2: Caspase-3 Activity in Treated HeLa Cells

Group	Absorbance at 405 nm (AU)	Fold Change vs Control
Control	0.42 ± 0.04	1.0
Treated (200 µg/mL)	1.17 ± 0.06	2.78

T-Test: $p<0.001$

Interpretation

The activation of caspase-3, a key executioner enzyme in apoptosis, confirms the apoptotic pathway triggered by *T. cordifolia* alkaloids. Nearly three-fold activation supports the pro-apoptotic mechanism

4. Discussion

The present study provides compelling evidence for the cytotoxic and apoptotic activity of the alkaloid-rich fraction of *Tinospora cordifolia* on HeLa cervical cancer cells. The cytotoxic effects were dose- and time-dependent, as confirmed by the MTT assay, with significant reductions in cell viability at higher concentrations. The IC_{50} values (132.5 µg/mL at 24h and 91.3 µg/mL at 48h) indicated that prolonged exposure enhances potency, possibly due to sustained intracellular accumulation or delayed activation of apoptotic pathways. These findings align well with those of Sharma *et al*. (2019), who reported that crude stem extracts of *T. cordifolia* inhibited colon carcinoma (HCT-116) cell proliferation, primarily through apoptosis induction [1]. Our results further extend this knowledge by isolating the alkaloid component, thereby eliminating other phytochemical interferences and demonstrating that alkaloids alone are responsible for significant anticancer activity. The higher efficacy in our study supports the idea of bioactivity-guided fractionation to enhance therapeutic selectivity and potency. Microscopic observation and DAPI staining provided qualitative and quantitative confirmation of apoptosis. Features such as chromatin condensation, nuclear fragmentation, and apoptotic bodies were prevalent in treated cells. This matches the nuclear damage patterns reported by Grover and Patni (2016), who emphasized *T. cordifolia*'s ability to disrupt nuclear integrity in cancer cells [2]. Flow cytometry analysis with Annexin V-FITC/PI staining further revealed a substantial increase in apoptotic cell populations post-treatment, with early and late apoptosis accounting for nearly 70% of the total cells in the 200 µg/mL treated group. This outcome corroborates the data from Kumar and Haridas (2021), who investigated the apoptotic activity of plant-derived alkaloids and observed that berberine-an alkaloid also found in *T. cordifolia* triggers early and late apoptosis in a similar fashion in breast cancer

models [3]. Another critical finding is the significant increase in caspase-3 activity, a hallmark of the intrinsic apoptosis pathway. The 2.8-fold rise in caspase-3 levels strongly suggests that the alkaloid extract triggers caspase-dependent programmed cell death. Akhtar *et al*. (2020) also reported a similar mechanism when *T. cordifolia* extracts were applied to A549 lung cancer cells, where caspase-3 activation was a major driver of cell death [4]. This consistency across different cell lines and extract types reinforces the idea that *T. cordifolia* alkaloids act through mitochondrial apoptotic signaling. The observed results are also consistent with the hypothesis that phytochemicals such as berberine and palmatine common constituents in *T. cordifolia* may interfere with DNA replication and mitochondrial function. Roy *et al*. (2005) demonstrated that berberine induces mitochondrial depolarization, leading to caspase-3 activation and apoptosis in various cancer models [5]. Despite the positive findings, certain limitations remain. The study used in vitro conditions, which may not fully replicate the complex tumor microenvironment *in vivo*. Furthermore, the identity and purity of individual alkaloids within the extract were not fully characterized using techniques like HPLC-MS. Future studies should consider using targeted isolation of major alkaloids followed by *in vivo* efficacy and safety assessments. Overall, our findings not only corroborate existing literature on *T. cordifolia*'s anticancer properties but also provide a clearer mechanistic insight into how the alkaloid fraction alone contributes to these effects. By validating apoptosis through multiple assays, including flow cytometry and caspase-3 activation, this study offers strong evidence for considering *T. cordifolia* alkaloids as lead compounds in anticancer drug development.

5. Conclusion

The present investigation into the cytotoxic and apoptotic effects of the alkaloid-rich fraction of Tinospora cordifolia on HeLa cervical cancer cells has yielded significant and promising results. The extract demonstrated a potent, dose- and time-dependent inhibition of cancer cell viability, with clearly defined IC_{50} values supporting its pharmacological relevance. Morphological analysis revealed classical features of apoptosis, such as cell shrinkage, detachment, and membrane blabbing, while nuclear fragmentation observed through DAPI staining further validated the induction of programmed cell death. Flow cytometric quantification with Annexin V-FITC/PI staining provided robust evidence of a significant rise in both early and late apoptotic cell populations, accompanied by an almost threefold increase in caspase-3 activity. These collective observations strongly suggest that the alkaloid fraction exerts its anticancer activity through intrinsic apoptotic pathways, most likely involving mitochondrial destabilization and caspase cascade activation. Compared with previous studies on crude extracts of *T. cordifolia* and its application on other cancer models such as HCT-116 and A549, the isolated alkaloid fraction in this study was shown to possess even greater specificity and potency, thereby reinforcing the concept of bioactivity-guided fractionation as a strategy to improve therapeutic efficacy.

Importantly, these findings contribute to a growing body of evidence advocating for the integration of traditional medicinal plants into modern oncological research pipelines. As cervical cancer remains a leading cause of mortality in women globally particularly in developing countries with limited access to expensive treatments plant-based interventions such as *T. cordifolia* alkaloid extracts present an affordable and accessible option for adjunct or alternative therapy. However, it must be emphasized that this study was performed under in vitro conditions, and while the cellular responses are clear and reproducible, *in vivo* studies using animal models and eventually human clinical trials are imperative before any definitive clinical application can be established. Practical recommendations emerging from this research include: the immediate next step should involve detailed phytochemical profiling of the alkaloid extract using HPLC-MS and NMR to identify and isolate the specific compounds responsible for the observed cytotoxicity, following this, *in vivo* toxicity and efficacy studies in animal models of cervical cancer should be initiated to assess pharmacokinetics, biodistribution, therapeutic index, and potential side effects; third, efforts should be made to develop suitable delivery systems such as nano-formulations or liposomal carriers that can enhance the bioavailability and tumor-targeting capability of the active alkaloids; and finally, this line of research should be promoted through interdisciplinary collaborations among pharmacologists, oncologists, and herbal scientists to accelerate the translation of such natural product discoveries into validated anticancer therapies. Additionally, public health frameworks in resource-limited regions should consider integrating standardized herbal therapies into preventive and palliative cancer care strategies after rigorous evaluation. Government and academic funding bodies are encouraged to support this research direction by establishing dedicated centers for herbal drug development, with a focus on endemic medicinal flora such as *T. cordifolia*. In conclusion, the alkaloid fraction of *T. cordifolia* holds considerable promise as a natural anticancer agent with the ability to selectively target malignant cervical cells through apoptotic mechanisms. Its further exploration could lead to the development of a cost-effective, safe, and sustainable therapeutic alternative, marking a significant step forward in integrative oncology and evidence-based herbal medicine.

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