

Research Article

A Study to Identify a Potent Withanolide From *Withania somnifera* Targeting Bcl-2 Family Proteins in Cervical Cancer Through *In Silico* and *In Vitro* Approach

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Abstract

Background: Natural compounds remain a vital reservoir of novel drug candidates because of their structural diversity and therapeutic potential for managing cancer and drug-resistant infectious diseases. Attention has increasingly focused on medicinal plants and related natural sources for preventing and treating various human cancers. Cervical cancer represents the fourth most frequently diagnosed malignancy among women worldwide. The therapeutic effectiveness of natural products in cervical cancer may be further enhanced by precisely targeting key molecular pathways involved in disease progression. **Methods:** This study used molecular docking and *in vitro* analyses to investigate the potential anticancer effects of *Withania somnifera* withanolides by inducing apoptosis in cervical cancer cells. Additionally, the pharmacokinetic behavior of selected compounds was predicted through Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) and drug-likeness analyses. **Results:** The results showed strong binding affinity for the B-cell lymphoma 2 (Bcl-2) family anti-apoptotic proteins, favorable ADMET properties, and selection of the more potent withanolide, withanolide A, for further analysis. *In vitro* evaluation revealed a dose- and time-dependent reduction in cell viability in withanolide A-treated CaSki cells, with significant induction of early and late apoptosis. Gene expression analysis showed dose-dependent downregulation of anti-apoptotic genes (Myeloid Cell Leukemia 1 (*MCL-1*), BCL2-like 10 (*BCL2L10*), *BCL-2* and BCL2-like 1 (*BCL2L1*)), suggesting activation of the intrinsic apoptotic pathway. **Conclusion:** The current study suggests that withanolide A may function as a promising apoptosis-inducing agent targeting Bcl-2 family proteins. Furthermore, *in vivo* validation and mechanistic studies are needed to support its development as a potential anti-cervical cancer therapeutic.

Keywords: withanolides; *Withania somnifera*; cervical cancer; apoptosis; Bcl-2 family proteins

1. Introduction

The global incidence and mortality rates of cancer are rising [1]. According to 2024 statistics, approximately 2 million new cancer cases and 611,720 cancer-related deaths were expected in the United States [2]. Cervical cancer, largely caused by persistent human papillomavirus (HPV) infections, is the fourth most frequent cancer among women, with 604,127 new cases and 341,831 deaths reported in 2020 [3]. Current treatment options include surgical intervention, radiotherapy, chemotherapy, immunotherapy, and molecularly targeted approaches, yet issues such as tumor recurrence, therapeutic resistance, and off-target toxicity remain significant concerns [4].

Recently, researchers have explored natural products from medicinal plants that modulate multiple molecular pathways, such as autophagy and apoptosis, as alternatives to chemically synthesized drugs [5,6,7]. *Withania somnifera* (L.) Dunal, commonly referred to as Ashwagandha, belongs to the Solanaceae (nightshade) family and is considered a pharmacologically important plant. *Withania somnifera* is found mainly in the Indian subcontinent and has been used in Ayurvedic and indigenous medicine for more than 1000 years [8]. The plant is particularly valued for its diverse array of bioactive compounds, the withanolides, which are naturally occurring C-28 steroidal lactones characterized by an ergostane-type structure. Researchers have identified more than 300 withanolides from



this species. Among them, withanolides A and D are considered the principal compounds responsible for many of its reported pharmacological activities. Additionally, *Withania somnifera* contains biologically active components including withanolides, sitoindosides, alkaloids, and related steroidal lactones, which contribute to its broad therapeutic potential [9,10]. Various studies have identified several molecular targets of *Withania somnifera*, including inhibition of nuclear factor kappa-B (NF- κ B) activation and induction of apoptosis in cancer cells [11,12].

Bcl-2 family proteins are frequently upregulated in various tumors, leading to resistance to apoptosis. Scientific reports have shown that both the Bcl-2 family and endonuclease G (Endo G) play crucial roles in regulating mitochondrial outer membrane permeabilization (MOMP) and subsequent caspase activation. Thus, these proteins serve as critical regulators of the intrinsic (mitochondrial) apoptotic pathway [13]. The Bcl-2 family comprises approximately 20 structurally related proteins, categorized by the presence and composition of Bcl-2 homology (BH) domains. These proteins are broadly divided into: anti-apoptotic multi-domain proteins, containing homology BH1–BH4 domains (e.g., Mcl-1, Bcl-2, Bcl-B, Bcl-w, and Bcl-xL); pro-apoptotic multi-domain effector proteins harboring BH1–BH3 domains (primarily Bax and Bak); pro-apoptotic BH3 only proteins, including Bid, Bad, Noxa, and Bik [14,15]. This classification reflects structural and functional distinctions that dictate their roles in apoptotic regulation. Upon activation, Bax and Bak undergo conformational changes and oligomerize within the mitochondrial outer membrane to form pores, thereby inducing MOMP. Conversely, anti-apoptotic proteins regulate Bax/Bak activity and prevent their homomultimerization [16]. Numerous studies have demonstrated that the dimerization of pro-apoptotic and anti-apoptotic proteins ultimately determines cellular fate [17,18]. A balance between these proteins is required for healthy cellular function. Disruption of this balance, particularly through upregulation of anti-apoptotic proteins such as Bcl-2, is a hallmark of oncogenesis. Early evidence of this mechanism was demonstrated in human B-cell lymphomas, where aberrant Bcl-2 overexpression was shown to impair programmed cell death and promote malignant cell survival [19]. This study aimed to investigate the interaction between *Withania somnifera* withanolides and Bcl-2 family proteins using *in silico* approaches and *in vitro* studies (Fig. 1). The objective was to identify potential phytochemicals that could serve as anticancer agents for treating cervical cancer by downregulating *BCL-2* family genes.

2. Materials and Methods

2.1 *In Silico* Computational Analysis

2.1.1 Identification and Selection of Target Proteins

The Bcl-2 family of anti-apoptotic proteins, including Mcl-1 (Protein Data Bank (PDB) ID: 5FDO), Bcl-B (PDB

ID: 4B4S), Bcl-2 (PDB ID: 6O0K), and Bcl-xL (PDB ID: 2YXJ), was selected for ligand docking studies. The three-dimensional (3D) structures of these proteins were obtained from the PDB in .pdb format.

2.1.2 Selection of Ligands

Owing to the numerous health benefits of withanolides from *Withania somnifera* and their frequent inclusion in various herbal and Ayurvedic remedies, this study focused on four primary withanolides: A, D, E, and F. Additionally, the standard drug 5-fluorouracil (5-FU) was included for comparison. The 3D structures of these compounds were obtained in .sdf format from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) for further investigation.

2.1.3 Drug-Likeness and Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET) Analysis of Ligands

The ADMET characteristics of selected withanolides (A, D, E, and F) from *Withania somnifera* were evaluated using the PkCSM tool (<https://biosig.lab.uq.edu.au/pkcsml/>) by entering the unique one-line canonical simplified molecular-input line-entry system (SMILES) for each phytocompound [20]. This parameter reflects the drug-like properties of withanolides, suggesting their potential as anticancer agents. Furthermore, the drug-likeness of these phytocompounds was assessed using Lipinski's Rule of Five.

2.1.4 Molecular Docking

Interactions between Bcl-2 family proteins and withanolides were analyzed using the Cavity detection-guided Blind Docking-2 (CB-Dock2) tool (<https://cadd.labshare.cn/cb-dock2/>) [21]. This web-based tool automatically identifies protein cavities and calculates the centers and dimensions of the top N cavities, with the default set to five (n = 5). For each ligand, the Vina score (kcal/mol) with the lowest value was selected. Typically, the interactions between the largest cavity and the specific ligand with the lowest Vina score are shown at the top.

2.2 *In Vitro* Cellular Experiments

2.2.1 Cell Culture

The cervical cancer CaSki cell line and the 3T3 mouse embryonic fibroblast cells were obtained from the National Center for Cell Science (NCCS), Pune, India, and were used for experiments between passages 3 and 9 (P3–P9). Both cell lines were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) (RM10434, HiMedia Laboratories Pvt. Ltd., Mumbai, India) and 1% antibiotic–antimycotic solution (A002, HiMedia Laboratories Pvt. Ltd., Mumbai, India) in a humidified atmosphere with 5% CO₂ at 37 °C. All cell lines were validated by STR profiling and tested negative for mycoplasma. Before commencing

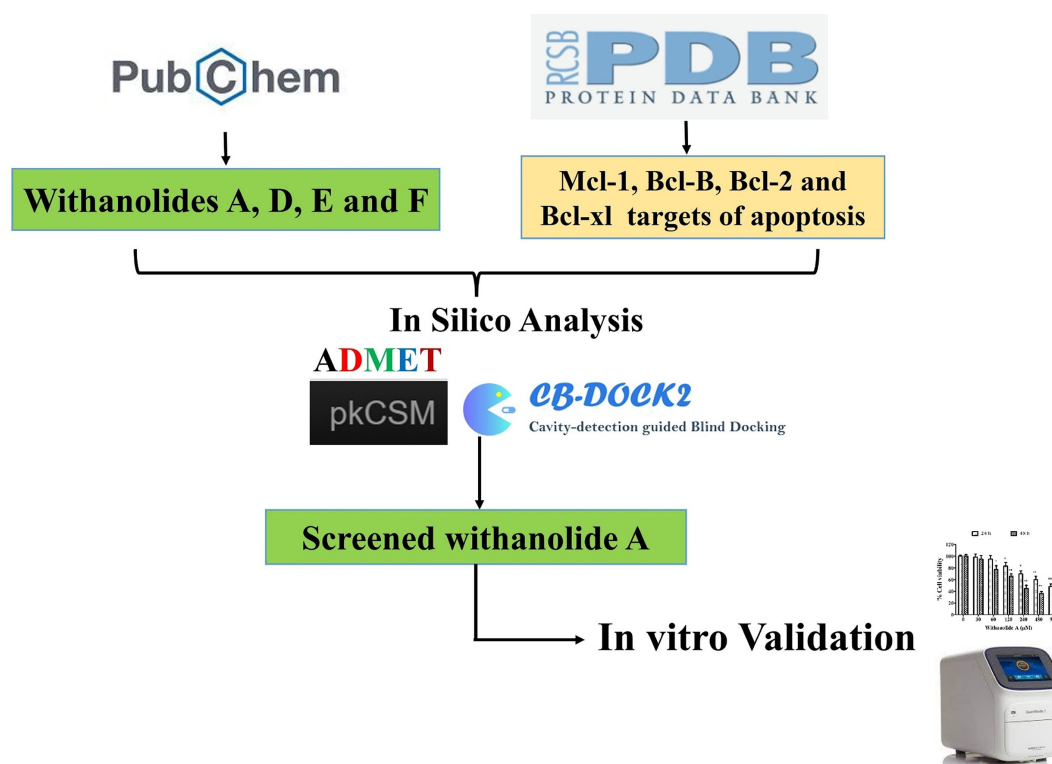


Fig. 1. Schematic presentation of the experimental workflow used to evaluate the efficacy of withanolides against anti-apoptotic Bcl-2 family proteins. Mcl-1, Myeloid Cell Leukemia 1; Bcl-2, B-cell lymphoma 2; Bcl-B, B-cell lymphoma B; Bcl-xL, B-cell lymphoma-extra large; PDB, Protein Data Bank.

the experiments, the cells were tested for mycoplasma contamination using the EZdetect™ PCR kit for mycoplasma detection (HiMedia Laboratories Pvt. Ltd., India), per the manufacturer's instructions, and were confirmed to be free of mycoplasma contamination.

2.2.2 Cell Viability Assay

The MTT assay was used to assess the viability of CaSki cervical carcinoma cells and normal cells. Both CaSki and normal 3T3 cells were seeded at 5000 cells/well in a 96-well plate and incubated for 24 h to promote cell adherence. The next day, CaSki cells were treated with varying concentrations (30–960 μM) of withanolide A (74776, ≥95.0% HPLC, Sigma-Aldrich, St. Louis, MO, USA) for 24 or 48 h, or with 5-FU for 48 h. While, 3T3 cells were treated with different concentrations (30–480 μM) of withanolide A for 48 h. Then, 10 μL MTT (M2128, 98%, powder, Sigma-Aldrich, St. Louis, MO, USA) was added, and the plate was incubated at 37 °C for 2 h. Next, each well was supplemented with 100 μL dimethyl sulfoxide (DMSO) to dissolve the formazan crystals, and the optical density at 540 nm was measured using a multifunction microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

2.2.3 Apoptosis Evaluation

To assess apoptosis, an Annexin V-FITC/propidium iodide (PI) staining kit (BD Biosciences Clontech, Moun-

tain View, CA, USA) was used. Briefly, CaSki cells were grown in 40 mm culture dishes and exposed to withanolide A at doses of 120, 240, and 480 μM, or to a control containing 0.1% DMSO, for 48 h. Cells were resuspended in 100 μL of binding buffer containing Annexin V-FITC (0.125 μg/mL) and PI (5 μg/mL), then incubated at room temperature in the dark for 15 min. Flow cytometry was performed using a BD FACSCalibur™ Flow Cytometer (BD Biosciences, San Diego, CA, USA), and the acquired events were analyzed using FlowJo software (BD Biosciences, San Diego, CA, USA). For compensation and gating controls, unstained cells, cells stained with Annexin V-FITC only, and cells stained with PI exclusively were prepared concurrently. At least 10,000 events were acquired for each sample. Following Annexin V-FITC/propidium iodide (PI) staining, the acquired events were first evaluated using a forward scatter (FSC) versus side scatter (SSC) dot plot. An initial gate was set to select the main intact cellular population and exclude events with low FSC/SSC signals corresponding to cellular debris and other non-cellular events. Doublets and aggregates were excluded by FSC-H versus FSC-A singlet gating. The resulting gated cell population was then analyzed for Annexin V-FITC and PI fluorescence using quadrant analysis. The lower-left quadrant represented viable cells (Annexin V–/PI–), the lower-right quadrant represented early apoptotic cells (Annexin V+/PI–), the upper-right quadrant represented late apop-

Table 1. Primer sequences.

Gene	Forward primer	Reverse primer	Reference
<i>MCL-1</i>	5'-GGACATCAAAAACGAAGACG-3'	5'-GCAGCTTTCTTGGTTTATGG-3'	[22]
<i>BCL2L10</i> (Bcl-B)	5'-GCCTTCATTATCTCTGGACAC-3'	5'-AAGGTGCTTCCCTCAGTTC-3'	[23]
<i>BCL-2</i>	5'-ACTGGGGGAGGATTGTGGCCTT-3'	5'-ATCTCCCGTTGACGCTCTCCA-3'	[24]
<i>BCL2L1</i> (Bcl-xL)	5'-GAATCTCTTTCTCTCCCTTCAGA-3'	5'-GCTCAACCAGTCCATTGTCC-3'	[24]
<i>β-actin</i>	5'-TGGTGGGCATGGGTCAGAAGGA-3'	5'-ATGCCGTGCTCGATGGGGTACT-3'	[24]

BCL2L10, B-cell lymphoma 2-like 10; *BCL2L1*, BCL2-like 1.

totic cells (Annexin V+/PI+), and the upper-left quadrant represented necrotic cells (Annexin V−/PI+). Quadrant boundaries were established using appropriate unstained and single-stained controls and applied consistently across samples. The percentage of cells in each quadrant was calculated relative to the total number of events within the gated cell population. The total apoptotic population was calculated as the sum of early (lower right) and late (upper right) apoptotic cells and expressed as a percentage of the total gated cell population.

2.2.4 Real-Time Quantitative PCR (RT-qPCR)

Total RNA from CaSki cells treated with withanolide A was collected and converted into cDNA using the Verso cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, MA, USA). The effect of withanolide A on the target *BCL-2* family genes *MCL-1*, *BCL2L10* (encodes Bcl-B), *BCL-2*, and *BCL2L1* (encodes Bcl-xL) was evaluated by RT-qPCR using gene-specific primers. The DyNAmoColor-Flash SYBR Green qPCR kit (Thermo Fisher Scientific, Waltham, MA, USA) was used to quantify mRNA expression levels of the target genes on an Applied Biosystems 7500 real-time PCR system. RT-qPCR was performed in optical-grade PCR tubes in a final reaction volume of 20 µL. The reaction mixture contained 10 µL SYBR Green Master Mix, 0.5 µM each primer, and 100 ng cDNA. PCR cycling conditions included an initial denaturation at 95 °C for 7 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C. All data were analyzed in triplicate and standardized against the reference gene, *β-actin*. The comparative Ct method was used to analyze the data, and the $2^{-\Delta\Delta C_t}$ method was used to assess changes in gene expression. RT-qPCR was performed using the following primer sequences for the specific targets (Table 1, Ref. [22,23,24]):

2.3 Statistical Analysis

All experimental results are presented as the mean ± standard error of the mean (SEM) from three independent experiments performed in triplicate (n = 3). Results were analyzed using one-way analysis of variance (ANOVA) with Dunnett's multiple-comparison post hoc test in Graph-Pad Prism (GraphPad Software, Boston, MA, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1 ADMET and Drug-Likeness Assessment

This study selected four anti-apoptotic Bcl-2 family proteins, specifically Mcl-1, Bcl-B, Bcl-2, and Bcl-xL, from the Protein Data Bank. Additionally, four anticancer withanolides (A, D, E, and F) from *Withania somnifera* were identified in PubChem (<https://pubchem.ncbi.nlm.nih.gov/>) for further investigation (Table 2).

Table 2. Selected target protein ligands and their corresponding identifiers.

Target proteins	PDB ID	Ligand/compound	PubChem CID
Mcl-1	5FDO	Withanolides A	11294368
Bcl-B	4B4S	Withanolides D	161671
Bcl-2	6O0K	Withanolides E	301751
Bcl-xL	2YXJ	Withanolides F	135887

The selected withanolides (A, D, E, and F) were analyzed using Lipinski's Rule of Five and ADMET characteristics. First, the PkCSM tool was used to determine whether the selected withanolides A, D, E, and F adhere to Lipinski's Rule of Five, a preliminary criterion for considering a compound a potential drug candidate. All selected compounds adhered to Lipinski's Rule of Five, with no criteria breached (Table 3). Subsequently, the compounds were assessed by calculating their ADMET properties and using the PkCSM tool to provide insights into their pharmacokinetic attributes. Detailed ADMET property information for the compounds is presented in Tables 4,5,6,7,8. The analysis indicated that the selected withanolides met the criteria and possessed promising drug-likeness properties.

3.2 Molecular Docking Analysis of Anti-Apoptotic Proteins and Withanolides

A blind docking experiment was conducted between Bcl-2 family proteins and selected *Withania somnifera* withanolides using the online docking platform CB-Dock2, which is based on the AutoDock Vina algorithm. Additionally, the Bcl-2 family proteins and 5-FU were docked simultaneously. The 3D structures of the target proteins were processed separately and docked with each withanolide. CB-Dock2 results were derived from potential receptor–ligand pose configurations, with the optimal pose selected

Table 3. Lipinski's Rule of Five analysis of withanolide compounds.

S. no.	Ligand	Molecular weight (≤ 500 Da)	cLogP (≤ 5)	H-bond donor (≤ 5)	H-bond acceptor (≤ 10)	Rotatable bonds (≤ 10)
1.	A	Yes	Yes	Yes	Yes	Yes
2.	D	Yes	Yes	Yes	Yes	Yes
3.	E	Yes	Yes	Yes	Yes	Yes
4.	F	Yes	Yes	Yes	Yes	Yes

cLogP, calculated logarithm of the partition coefficient.

Table 4. Absorption properties of selected withanolide compounds.

S. no.	Withanolides	Water solubility (log mol/L)	Caco2 permeability (log Papp in 10 ⁻⁶ cm/s)	Intestinal absorption (human) (%) absorbed)	Skin permeability (log Kp)	P-glycoprotein substrate	P-glycoprotein-I inhibitor	P-glycoprotein-II inhibitor
1.	A	-5.096	0.846	100	-3.285	Yes	Yes	Yes
2.	D	-5.127	0.831	99.2	-3.267	Yes	Yes	Yes
3.	E	-5.252	0.742	100	-2.968	Yes	Yes	No
4.	F	-5.209	0.668	87.631	-3.285	Yes	No	No

Table 5. Distribution properties of selected withanolide compounds.

S. No.	Ligand	VDss (human) (log L/kg)	Fraction unbound (human)	BBB permeability (log BB)	CNS permeability (log PS)
1.	A	-0.097	0.096	-0.255	-2.681
2.	D	-0.048	0.093	-0.315	-2.696
3.	E	0.093	0.286	-0.956	-3.098
4.	F	-0.075	0.301	-0.9	-3.108

VDss, Volume of distribution at steady state; BBB, Blood-brain barrier; BB, Blood-brain distribution; PS, Permeability-surface area product.

Table 6. Metabolic properties of selected withanolide compounds.

S. No.	Ligand	CYP2D6 substrate	CYP3A4 substrate	CYP1A2 inhibitor	CYP2C19 inhibitor	CYP2C9 inhibitor	CYP2D6 inhibitor	CYP3A4 inhibitor
1.	A	No	Yes	No	No	No	No	No
2.	D	No	Yes	No	No	No	No	No
3.	E	No	Yes	No	No	No	No	No
4.	F	No	Yes	No	No	No	No	No

CYP, Cytochrome P450.

Table 7. Excretion properties of selected withanolide compounds.

S. No.	Ligand	Total clearance (log mL/min/kg)	Renal OCT2 substrate
1.	A	0.337	Yes
2.	D	0.347	No
3.	E	0.378	No
4.	F	0.489	No

OCT2, Organic Cation Transporter 2.

based on the Vina score, cavity dimensions, grid map, and other criteria. Interaction models were generated by predicting the extent to which anti-apoptotic Bcl-2 proteins and various withanolides would bind to each other using a curvature-dependent surface area model. After calculating the binding energy values (Vina scores), the interac-

tion with the lowest binding energy was selected (Table 9). When binding energies were equal, the contact in the larger cavity was selected throughout the docking process. The lowest predicted Vina score for the Mcl-1 protein was observed for withanolide A (-11.1 kcal/mol). In the subsequent analysis, withanolide A again showed a lower Vina score (-8.2 kcal/mol) against the Bcl-B protein. In contrast, withanolide D showed lower Vina scores (-9.1 and -9.4 kcal/mol) against the Bcl-2 and Bcl-xL proteins (Figs. 2,3,4,5). Despite being extensively researched, the control drug 5-FU showed weaker binding affinity (-5.1, -4.4, -4.8, and -5.7 kcal/mol) than the selected withanolides (Fig. 6). Conversely, the withanolides A, D, E, and F have superior binding free energy compared with the standard drug 5-FU, with estimated values ranging from -11.1 to -7.4. Based on various criteria, including the Vina score, drug-likeness, and molecular interactions with target proteins,

Table 8. Toxicity prediction for selected withanolides.

S. no.	Ligand	Ames toxicity	Max. tolerated dose (human) log mg/kg/day	hERG-I inhibitor	hERG-II inhibitor	Oral rat acute toxicity (LD ₅₀) (mol/kg)	Oral rat chronic toxicity (log mg/kg_bw/day)	Hepatotoxicity	Skin sensitization	<i>T. pyriformis</i> toxicity (log µg/L)	Minnow toxicity (log mM)
1.	A	No	−0.967	No	No	2.913	1.739	No	No	0.298	1.22
2.	D	No	−0.867	No	No	2.831	1.776	No	No	0.305	1.178
3.	E	No	−0.768	No	No	3.503	2.154	No	No	0.286	2.578
4.	F	No	−0.613	No	No	2.494	2.122	No	No	0.286	2.725

hERG, Human Ether-à-go-go-Related Gene.

Table 9. Vina scores for withanolides A, D, E, and F, and for 5-fluorouracil (5-FU) with Bcl-2 family proteins, assessed using the CB-Dock2 tool.

Compound/ligand	Vina score (kcal/mol)			
	Mcl-1 (PDB ID: 5FDO)	Bcl-B (PDB ID: 4B4S)	Bcl-2 (PDB ID: 6O0K)	Bcl-xL (PDB ID: 2YXJ)
Withanolide A	−11.1	−8.2	−8.8	−9.3
Withanolide D	−10.5	−7.7	−9.1	−9.4
Withanolide E	−9.3	−7.4	−8.4	−7.9
Withanolide F	−9.8	−7.8	−8.1	−8.1
5-FU	−5.1	−4.4	−4.8	−5.7

withanolide A was selected for additional *in vitro* experimental studies (Table 10).

3.3 Withanolide A Inhibited Tumor Cell Proliferation

The MTT assay results showed that withanolide A (30–960 µM) reduced CaSki cell viability in a dose- and time-dependent manner (Fig. 7A). The half-maximal inhibitory concentration (IC₅₀) values for CaSki cells at 24 h and 48 h were 349.6 ± 2.82 µM and 150.4 ± 3.14 µM, respectively (Fig. 7B,C). Based on these IC₅₀ values, appropriate concentrations of withanolide A were selected for further experiments. Treatment with the standard drug 5-FU also significantly decreased CaSki cell viability in a dose-dependent manner (Fig. 7D). In contrast, 3T3 cells were less sensitive to withanolide A and exhibited minimal cytotoxicity, observed only at higher concentrations (Fig. 7E).

3.4 Withanolide A Induced Apoptosis in CaSki Cells

To elucidate the molecular mechanism of withanolide A-induced cytotoxicity in CaSki cells, its pro-apoptotic effects were evaluated using the Annexin V/PI double-staining assay. The findings showed that after 48 h of exposure to withanolide A at varying doses (120, 240, and 480 µM), the proportion of apoptotic cells, calculated as the sum of early and late apoptotic populations, increased significantly to 9.33 ± 1.87% (**p* < 0.05), 23.67 ± 3.98% (***p* < 0.001), and 41.50 ± 4.63% (***p* < 0.001) (Fig. 8A,B).

3.5 Withanolide A Modulated Target Gene Expression

The mRNA expression levels of the important apoptotic markers *MCL-1*, *BCL2L10* (encoding Bcl-B), *BCL-2*,

and *BCL2L1* (encoding Bcl-xL) were examined in CaSki cells treated with withanolide A. In CaSki cells, withanolide A treatment downregulated the expression levels of *MCL-1*, *BCL2L10* (encoding Bcl-B), *BCL-2*, and *BCL2L1* (encoding Bcl-xL) in a concentration-dependent manner (Fig. 9A,B). At 120 µM, only *BCL-2* showed a significant reduction, whereas changes in *MCL-1*, *BCL2L10*, and *BCL2L1* were not statistically significant. At 240 and 480 µM, we observed significant reductions in all four genes. These findings suggest that withanolide A modulates the expression of survival-associated *BCL-2* family members, with more pronounced effects at higher concentrations.

4. Discussion

Natural compounds and their analogs from medicinal plants exhibit significant biological properties, including antioxidant, antimicrobial, antidiabetic, and antitumor activities [25,26,27]. *Withania somnifera* is a highly regarded herb in the Indian medicinal tradition owing to its pharmacologically active constituents. The roots and leaves contain a variety of phytochemicals, including phenols, flavonoids, steroids, and quinones. These phytochemicals are distributed across different plant tissues in varying amounts. These chemicals support diverse applications, including pharmaceutical formulations, cosmetics, food ingredients, and traditional medicine [28,29,30,31]. The bioactive constituents of *Withania somnifera* include withanolides (0.001%–1.5% of dry weight), alkaloids (0.13%–0.31%), and flavonoids (43.51 mg RE/g), which confer medicinal effects [32]. Among these, withanolides are used in pharmaceutical formulations owing to their medicinal properties. However, there remains uncertainty regarding

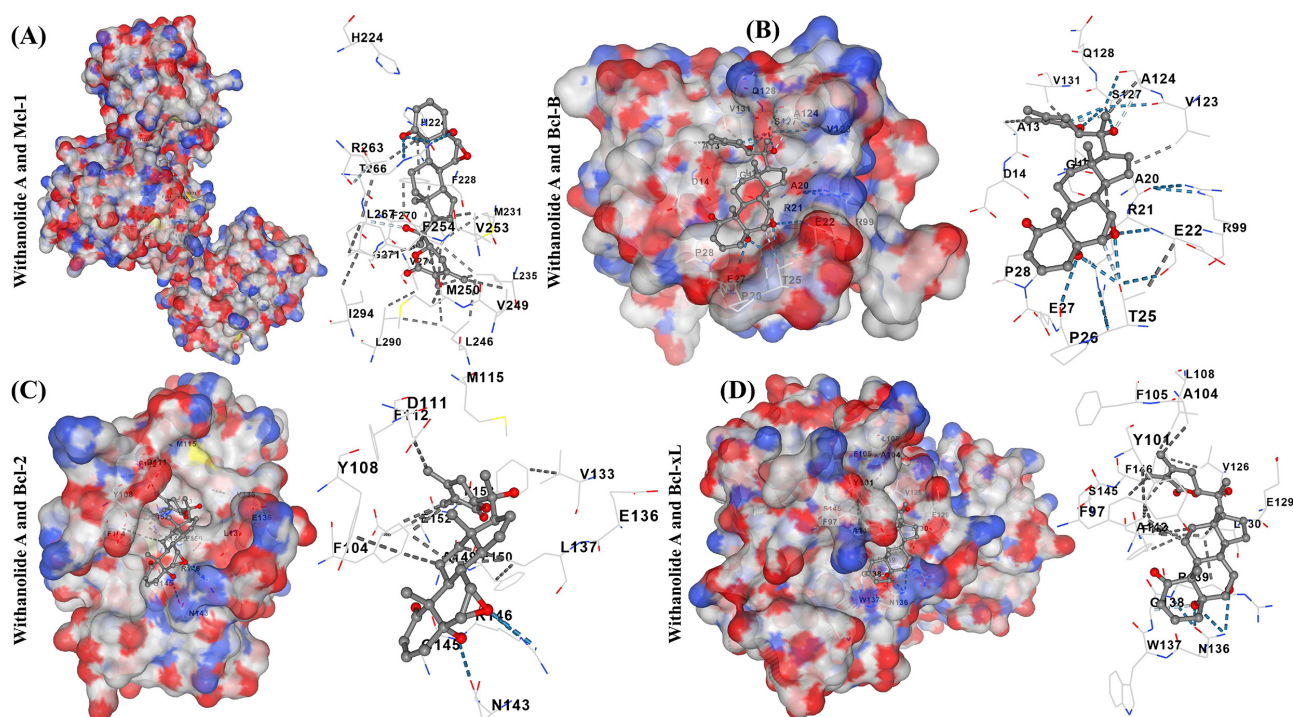


Fig. 2. Interaction of withanolide A with different target proteins of the Bcl-2 family, as determined by CB-Dock2 molecular docking analysis: (A) Mcl-1, (B) Bcl-B, (C) Bcl-2, and (D) Bcl-xL.

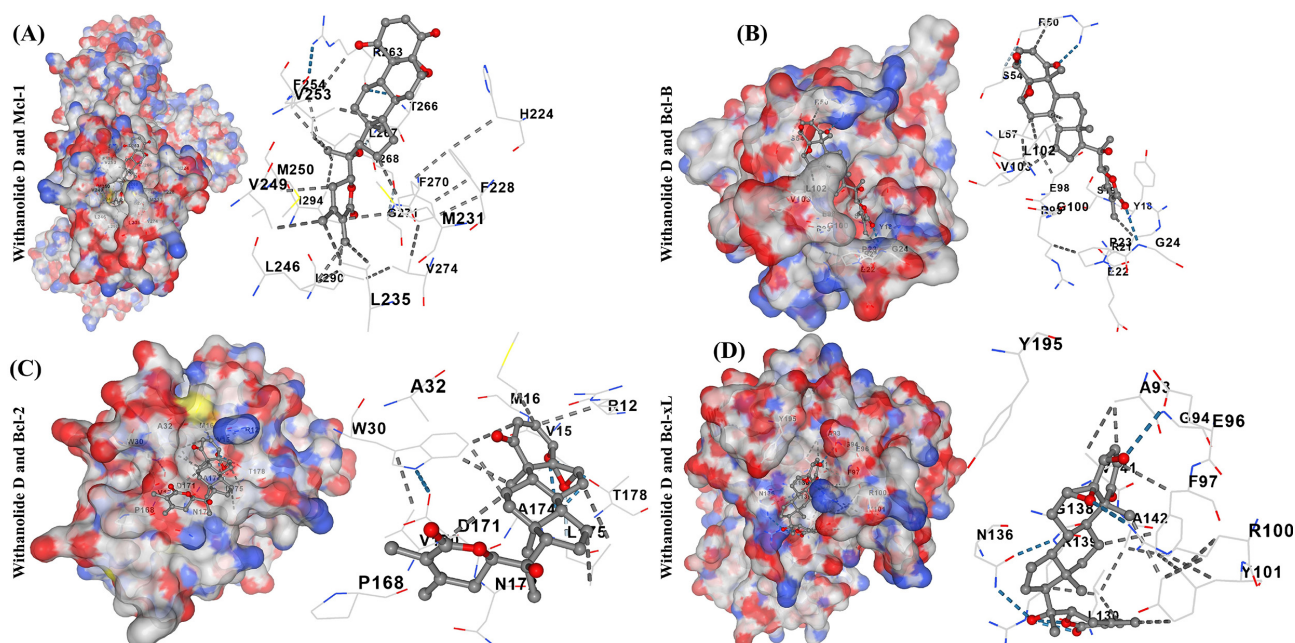


Fig. 3. Interaction of withanolide D with different target proteins of the Bcl-2 family, as determined by CB-Dock2 molecular docking analysis: (A) Mcl-1, (B) Bcl-B, (C) Bcl-2, and (D) Bcl-xL.

the scientific validation of their anticancer effects, specifically with regard to cervical cancer. Therefore, this study aimed to address this gap by investigating the anticancer potential of selected withanolides targeting Bcl-2 family proteins using an integrated *in silico* and *in vitro* approach. To our knowledge, limited studies have investigated this mech-

anism in cervical cancer, and our findings provide additional evidence supporting the potential of withanolides in cervical cancer research.

ADMET analysis indicates that the withanolides generally satisfy Lipinski's Rule of Five and exhibit favorable absorption, distribution, metabolism, excretion, and toxic-

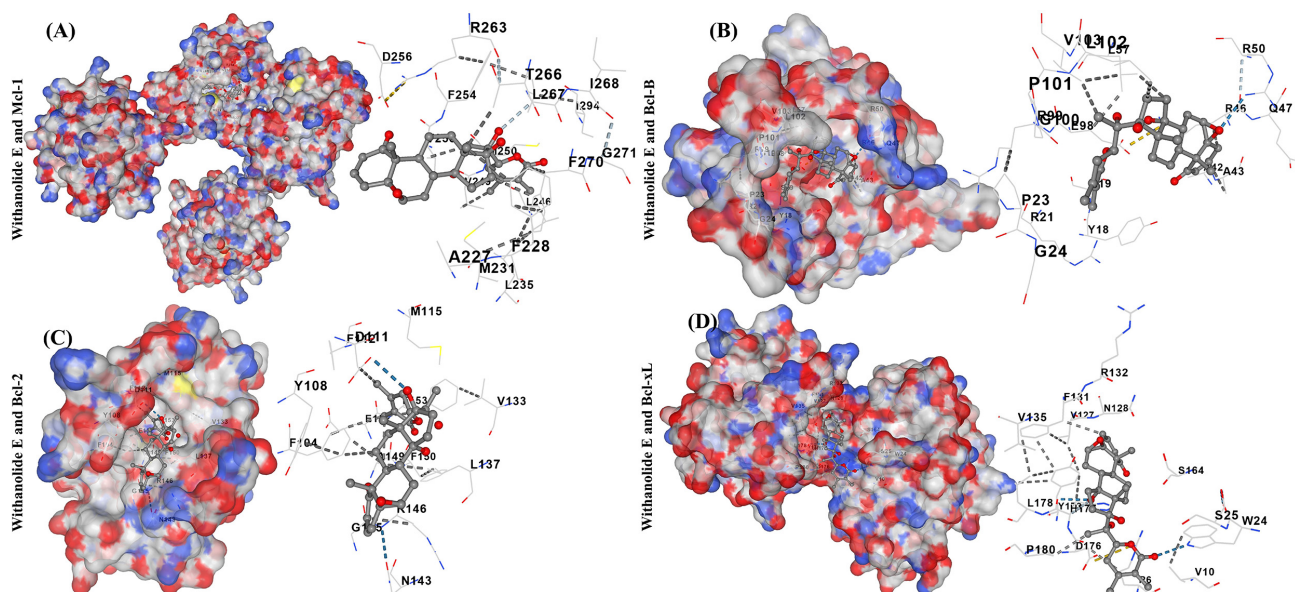


Fig. 4. Interaction of withanolide E with different target proteins of the Bcl-2 family, as determined by CB-Dock2 molecular docking analysis: (A) Mcl-1, (B) Bcl-B, (C) Bcl-2, and (D) Bcl-xL.

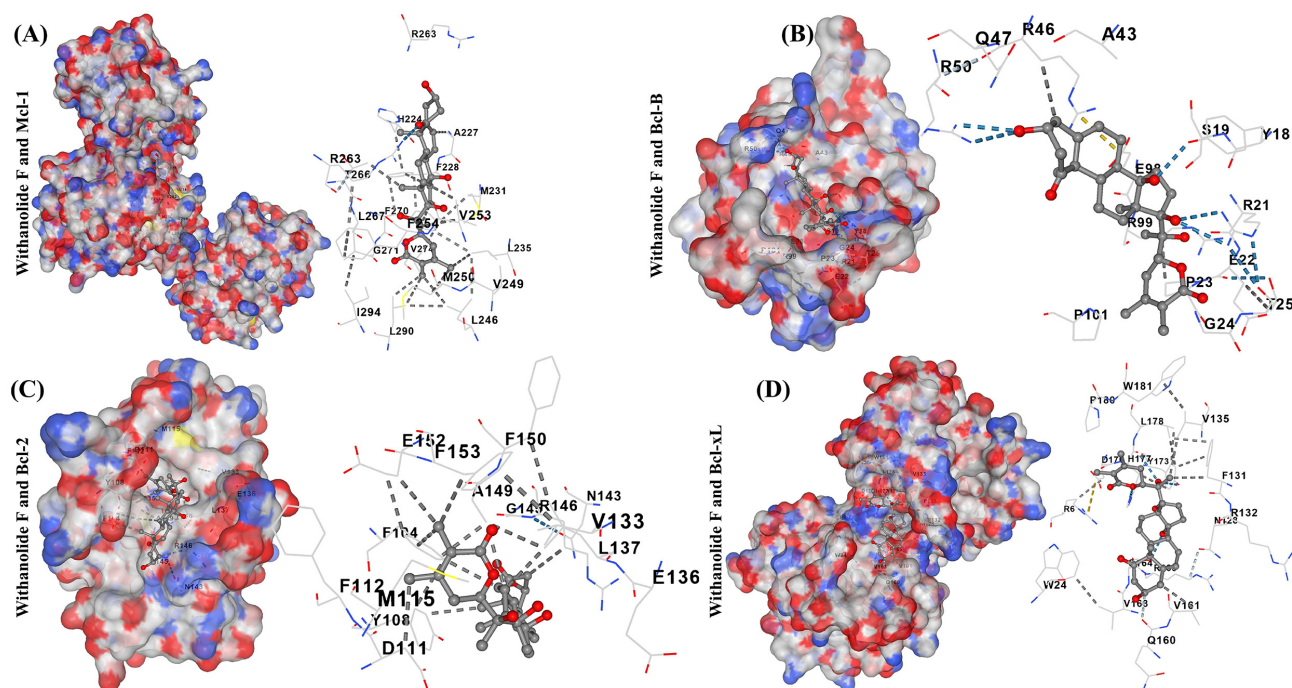


Fig. 5. Interaction of withanolide F with different target proteins of the Bcl-2 family, as determined by CB-Dock2 molecular docking analysis: (A) Mcl-1, (B) Bcl-B, (C) Bcl-2, and (D) Bcl-xL.

ity profiles. Low predicted toxicity, acceptable GI absorption, and no predicted cytochrome P450 inhibition suggest a reasonable safety margin and oral bioavailability. These computational pharmacokinetic predictions strengthen the translational relevance of the selected withanolides and support their progression to further analysis. The findings of the present study are consistent with earlier reports showing that computational screening of natural compounds revealed diverse anticancer activities [33,34]. In addition, re-

cent advances in bioinformatics, integrating machine learning and artificial intelligence techniques, have further facilitated the identification and prioritization of potential drug candidates [35,36].

CB dock analysis indicates that withanolides A, D, E, and F bind effectively within the functional pocket of specific Bcl-2 family proteins, including Mcl-1, Bcl-B, Bcl-2, and Bcl-xL. Among the selected withanolides, A and D show superior binding affinities across all target Bcl-

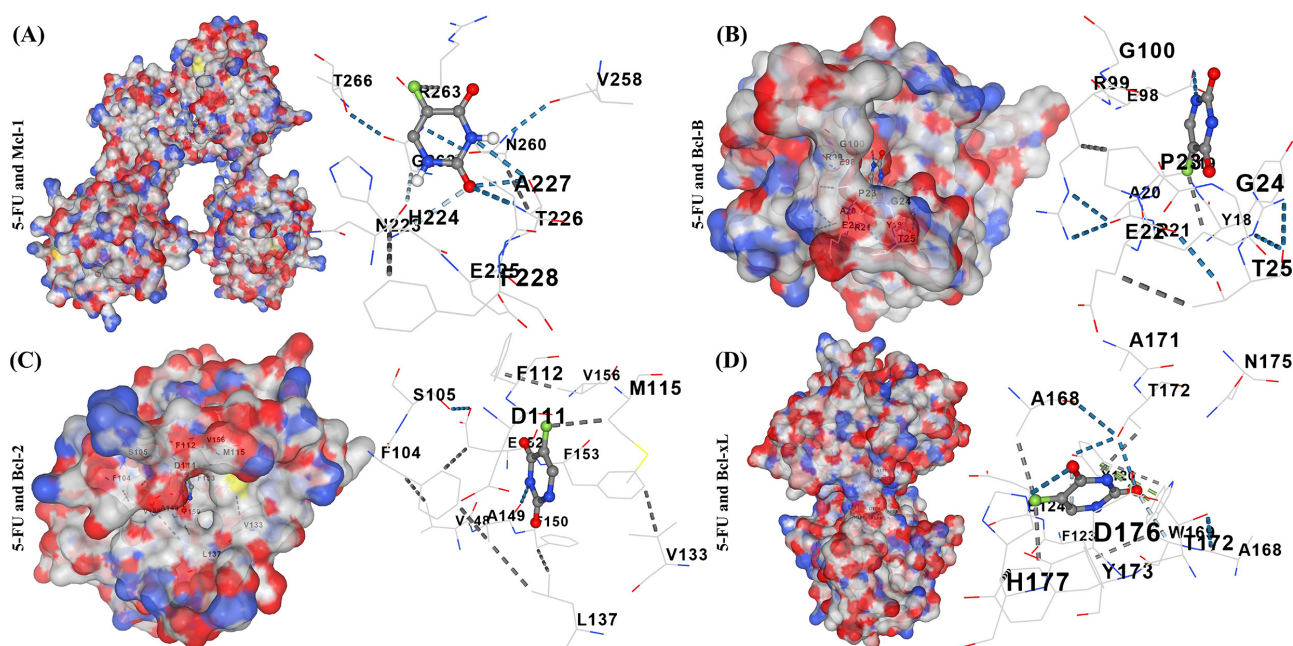


Fig. 6. Interaction of 5-fluorouracil with different target proteins of the Bcl-2 family, as determined by CB-Dock2 molecular docking analysis: (A) Mcl-1, (B) Bcl-B, (C) Bcl-2, and (D) Bcl-xL.

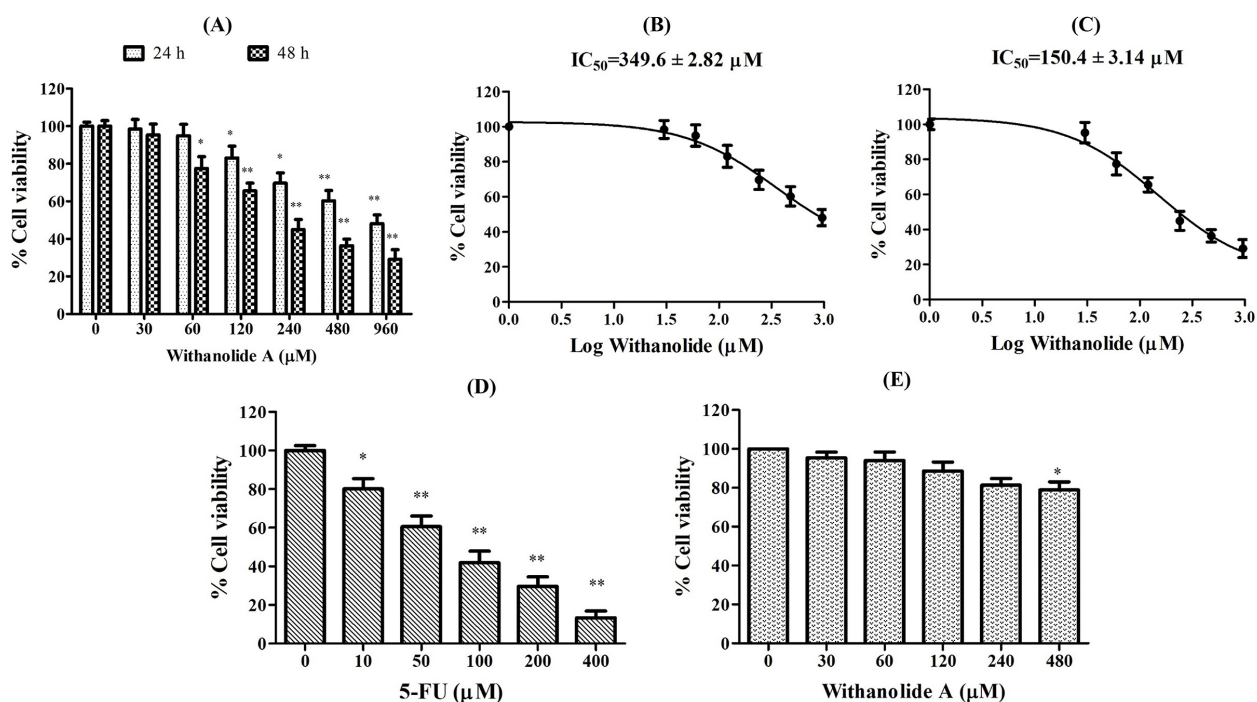


Fig. 7. Effects of withanolide A on the viability of cervical cancer CaSki cells. (A) Inhibitory effects of withanolide A (30–960 μM) on CaSki cell viability. (B) IC₅₀ curve of withanolide A as a function of dose at 24 h and (C) 48 h. (D) Effects of varying doses of the standard drug 5-FU (10–400 μM) on CaSki cell viability. (E) Evaluation of the effects of withanolide A on 3T3 cell viability at 48 h. Data are shown as the mean ± standard error of the mean (SEM) from three separate experiments. (*p < 0.05, **p < 0.001 compared with the control group). IC₅₀, half-maximal inhibitory concentration.

2 family proteins. Low binding energies and numerous non-covalent interactions with key amino acid residues of the target proteins facilitate stable protein–ligand interac-

tions. These interactions suggest that these phytochemicals may induce apoptosis by disrupting anti-apoptotic signaling and promoting mitochondrial membrane perme-

Table 10. Amino acid residues of Bcl-2 family proteins interacting with withanolides A, D, E, and F and 5-FU, assessed using the CB-Dock2 tool.

Compound/ligand	Interacting amino acid residues			
	Mcl-1 (PDB ID: 5FDO)	Bcl-B (PDB ID: 4B4S)	Bcl-2 (PDB ID: 6O0K)	Bcl-xL (PDB ID: 2YXJ)
Withanolide A	Chain A: HIS224	Chain A: ALA13 ASP14	Chain A: PHE104 TYR108	Chain B: PHE97 TYR101
	Chain B: HIS224 PHE228	LEU16 GLY17 ALA20	ASP111 PHE112 MET115	ALA104 PHE105 LEU108
	MET231 LEU235 LEU246	ARG21 GLU22 THR25	VAL133 GLU136 LEU137	VAL126 GLU129 LEU130
	VAL249 MET250 VAL253	PRO26 GLU27 PRO28	ASN143 GLY145 ARG146	ASN136 TRP137 GLY138
	PHE254 ARG263 THR266	ARG99 VAL123 ALA124	ALA149 PHE150 GLU152	ARG139 ALA142 PHE143
	LEU267 PHE270 GLY271	SER127 GLN128 VAL131	PHE153	SER145 PHE146
	VAL274 LEU290 ILE294			
Withanolide D	HIS224 PHE228 MET231	Chain A: TYR18 SER19	Chain A: PHE104 TYR108	Chain B: ALA93 GLY94
	LEU235 LEU246 VAL249	ARG21 GLU22 PRO23	ASP111 PHE112 MET115	GLU96 PHE97 ARG100
	MET250 VAL253 PHE254	GLY24 ARG50 SER54	VAL133 GLU136 LEU137	TYR101 LEU130 ASN136
	ARG263 THR266 LEU267	LEU57 GLU98 ARG99	ASN143 GLY145 ARG146	GLY138 ARG139 VAL141
	ILE268 PHE270 GLY271	GLY100 LEU102 VAL103	ALA149 PHE150 GLU152	ALA142 TYR195
	VAL274 LEU290 ILE294		PHE153	
Withanolide E	Chain B: ALA227 PHE228	Chain A: TYR18 SER19	Chain A: PHE104 TYR108	Chain B: VAL127 ASN128
	MET231 LEU235 LEU246	ARG21 PRO23 GLY24	ASP111 PHE112 MET115	PHE131 ARG132 VAL135
	VAL249 MET250 VAL253	ALA42 ALA43 ARG46	VAL133 LEU137 ASN143	TYR173 ASP176 HIS177
	PHE254 ASP256 ARG263	GLN47 ARG50 LEU57	GLY145 ARG146	LEU178 PRO180
	THR266 LEU267 ILE268	GLU98 ARG99 GLY100	ALA149 PHE150 GLU152	
	PHE270 GLY271 ILE294	PRO101 LEU102 VAL103	PHE153	
Withanolide F	Chain A: ARG263	TYR18 SER19 ARG21	Chain A: PHE104 TYR108	Chain A: ARG6 TRP24
	Chain B: HIS224 ALA227	GLU22 PRO23 GLY24	ASP111 PHE112 MET115	GLN160 VAL161 VAL163
	PHE228 MET231 LEU235	THR25 ALA43 ARG46	VAL133 GLU136 LEU137	SER164 ARG165
	LEU246 VAL249 MET250	GLN47 ARG50 GLU98	ASN143 GLY145 ARG146	Chain B: ASN128 PHE131
	VAL253 PHE254 ARG263	ARG99 PRO101	ALA149 PHE150 GLU152	ARG132 VAL135 TYR173
	THR266 LEU267 PHE270		PHE153	ASP176 HIS177 LEU178
	GLY271 VAL274 LEU290			PRO180 TRP181
5-FU	ILE294			
	Chain A: VAL258 ASN260	Chain A: TYR18 SER19	Chain A: PHE104 SER105	Chain A: ALA168
	GLY262 ARG263 THR266	ALA20 ARG21 GLU22	ASP111 PHE112 MET115	ALA171 THR172 ASN175
	Chain B: ASN223 HIS224	PRO23 GLY24 THR25	VAL133 LEU137 VAL148	Chain B: TYR120 PHE123
	GLU225 THR226	GLU98 ARG99 GLY100	ALA149 PHE150 GLU152	GLU124 ALA168 TRP169
	ALA227 PHE228		PHE153 VAL156	THR172 TYR173 ASP176
				HIS177

abilization. Although these *in silico* methods demonstrate promising molecular compatibility, these methods are insufficient to ensure biological efficacy. Therefore, additional *in vitro* experimental validation is necessary. Similar studies have also emphasized the role of other natural substances as Bcl-2 family protein inhibitors [37,38,39]. Based on the *in silico* parameters, withanolide A was selected for further *in vitro* validation.

MTT-based cell viability assays show a concentration- and time-dependent reduction in proliferation in treated CaSki cells compared with untreated controls. Withanolide A exhibits a pronounced effect at 48 h compared with 24 h. The determined IC₅₀ indicates meaningful antiproliferative activity at 48 h compared with 24 h. Thus, all subsequent experiments used 48 h as the treatment period. Additionally, minimal cytotoxicity is observed in the normal 3T3

cell line. Flow cytometric analysis using FITC-Annexin V/PI double staining provides clear evidence that decreased cell viability results primarily from apoptosis rather than non-specific necrosis. Withanolide A treatment for 48 h significantly increases both early and late apoptotic cell populations. This observation functionally confirms activation of programmed cell death pathways and supports involvement of the mitochondrial pathway of apoptosis following inhibition of anti-apoptotic Bcl-2 proteins [40].

RT-qPCR analysis strengthened the mechanistic interpretation by revealing reduced transcription of anti-apoptotic genes, including *MCL-1*, *BCL2L10* (encoding Bcl-B), and *BCL2L1* (encoding Bcl-xL), after 48 h of withanolide A treatment. These coordinated molecular alterations are characteristic of mitochondrial apoptotic activation and demonstrate that withanolide A regulates cell death

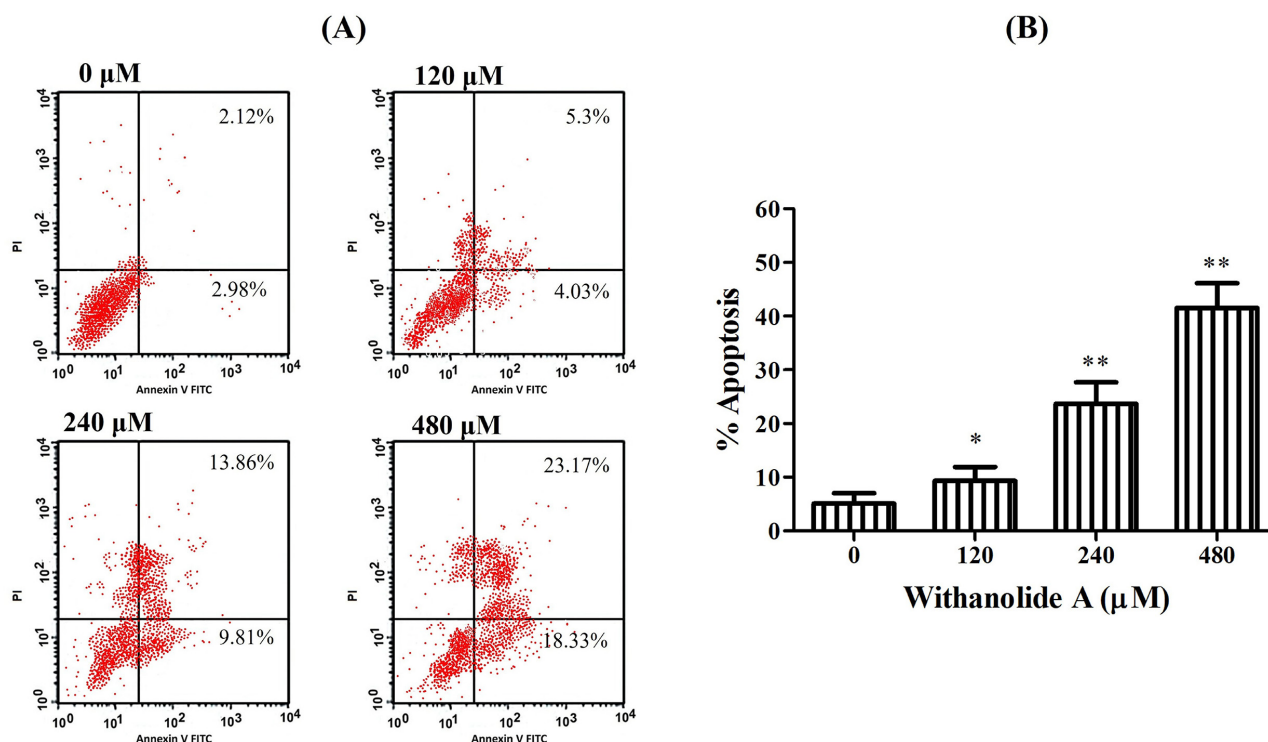


Fig. 8. Apoptosis inducing effects of Withanolide A in cervical cancer cells. (A,B) Withanolide A induced apoptosis in CaSki cells. Exposure to withanolide A promoted apoptosis in CaSki cells, as assessed by Annexin V-FITC/PI staining. The data are shown as the mean $\pm$ SEM of three separate experiments (* p < 0.05 and ** p < 0.001 were considered significant compared with control). PI, propidium iodide.

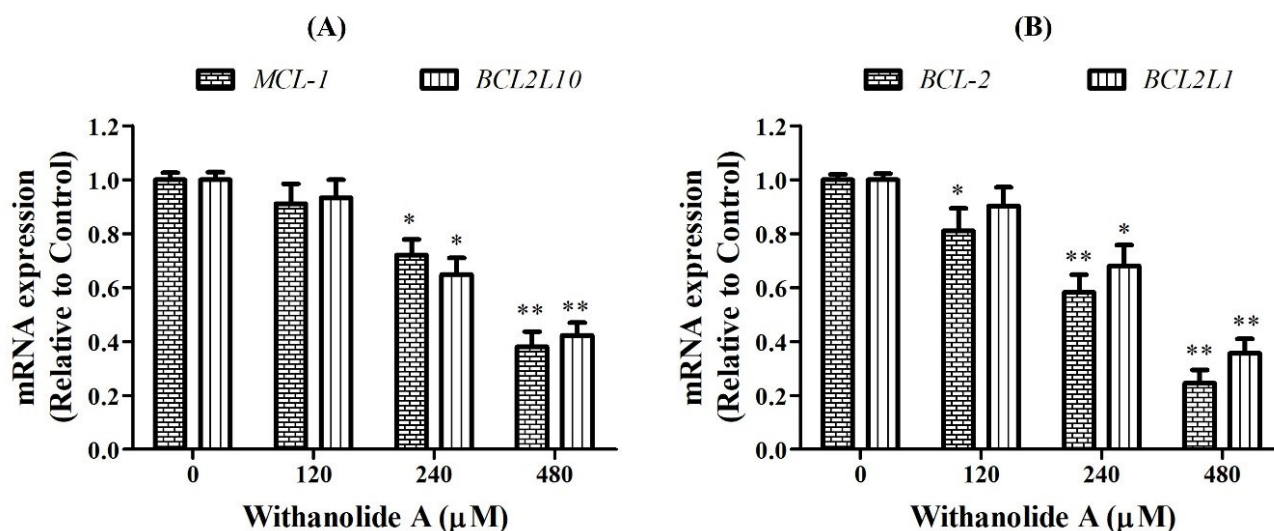


Fig. 9. Withanolide A-mediated downregulation of mRNA expression of (A) *MCL-1*, *BCL2L10* (encodes Bcl-B), and (B) *BCL-2*, *BCL2L1* (encodes Bcl-xL), as analyzed by real-time quantitative PCR (RT-qPCR). Data are shown as mean $\pm$ SEM from three separate experiments. (* p < 0.05 and ** p < 0.001 were considered significant compared with the control).

pathways at the gene expression level. Our results are consistent with previous reports of apoptotic induction by natural compounds from *Withania somnifera* plants in different cancer cell lines [41,42,43]. Altogether, these findings indicate that the investigated withanolide, withanolide A,

has a strong ability to promote apoptosis by regulating Bcl-2 family anti-apoptotic proteins (Fig. 10). Withanolide A could be a promising preliminary candidate for anticancer drug development against cervical cancer.

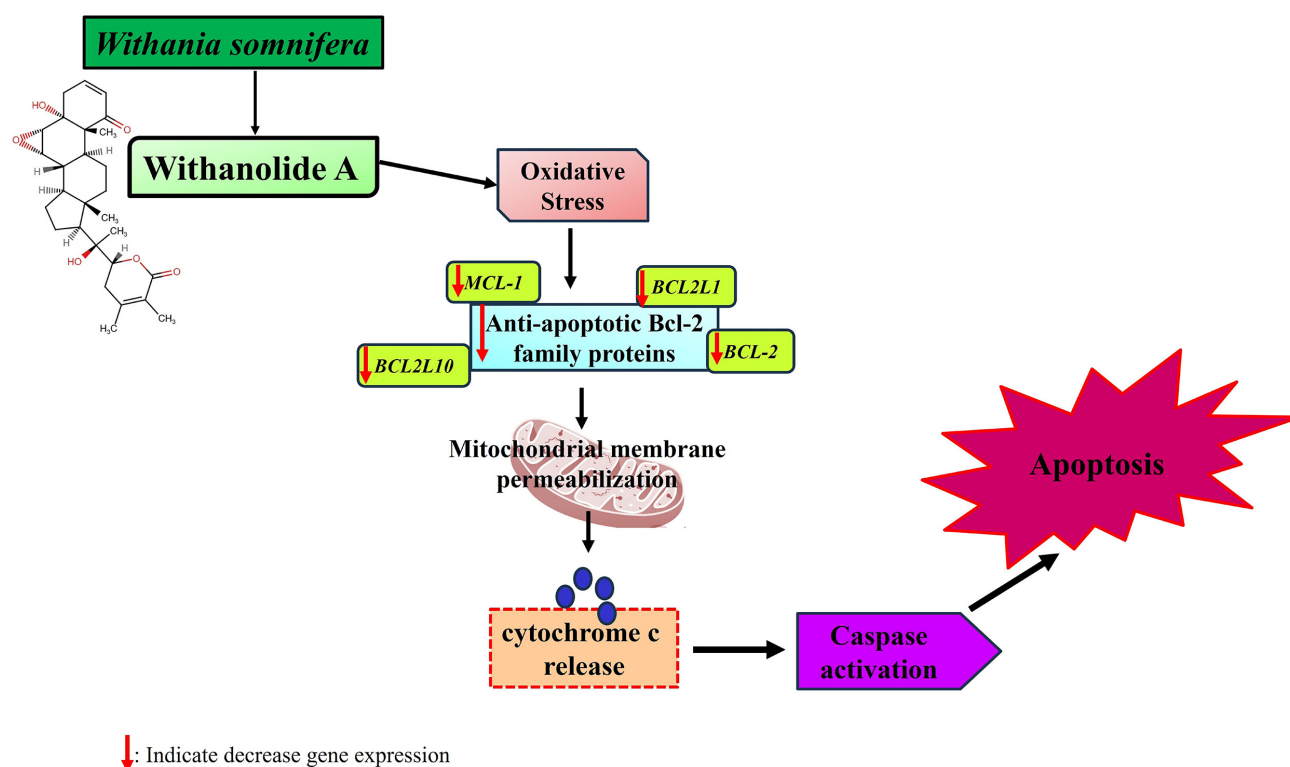


Fig. 10. Proposed mechanism through which *Withania somnifera*-derived withanolide A induces apoptosis in cervical cancer CaSki cells through downregulation of BCL-2 family gene expression (red arrows indicate downregulated gene expression).

5. Limitations

Despite encouraging outcomes, several limitations remain. First, the apoptotic-inducing potential of withanolides was investigated using only *in silico* and *in vitro* approaches, which may not fully reflect their pharmacological effects in the *in vivo* tumor environment. Second, the effect of withanolide A on selected apoptotic targets was evaluated primarily at the gene expression level; thus, further expression analysis at the protein level is highly recommended to substantiate the mechanism. Third, the present study employed only a limited set of experiments to explore the pro-apoptotic mechanism of withanolides in cervical cancer. Additional mechanistic and confirmatory examinations are therefore required to comprehensively explore the mode of action of these withanolides in cervical cancer management.

6. Conclusion

The combined *in silico* and *in vitro* approach employed in this study demonstrates that withanolide A can effectively target anti-apoptotic Bcl-2 family proteins and trigger mitochondrial-mediated apoptosis in cervical cancer CaSki cells. The convergence of molecular docking, pharmacokinetic prediction, cytotoxic evaluation, and apoptotic confirmation provides compelling preliminary evidence supporting withanolide A as a potential lead for anti-cervical cancer therapy. Investigating combination therapy

and improved drug delivery approaches may further enhance the translational potential of withanolides in cervical cancer.

Availability of Data and Materials

The datasets used and analyzed during the current study are available from the corresponding authors on reasonable request.

Author Contributions

AB, DAA and MS performed the *in silico* experiments, critically revised the manuscript. PP, MV, and SS performed the *in vitro* experiments, contributed to data analysis, revised the manuscript. AMA, RAA, and FK performed experiments (*in silico/in vitro*), wrote the original draft. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflicts of Interest

The authors declare no conflicts of interest.

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