

Viwithan, a Standardized *Withania somnifera* Root Extract Induces Apoptosis in Murine Melanoma Cells

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ABSTRACT

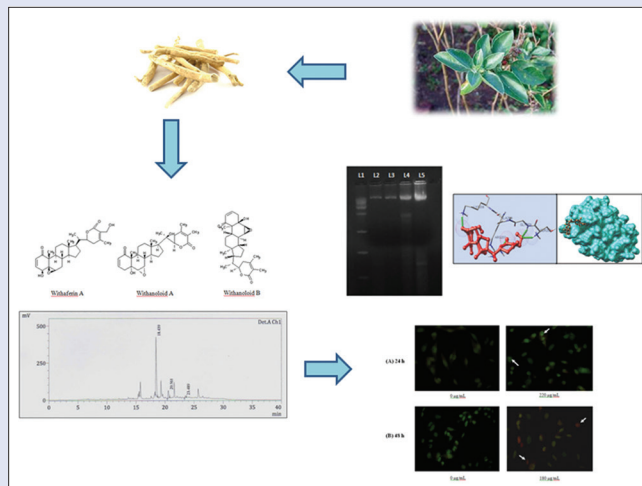
Background: *Withania somnifera* is an Indian medicinal herb known for the multipotential ability to cure various therapeutic ailments as described in the ayurvedic system of medicine. **Objective:** In the present study, we have evaluated the antiproliferative activity of a standardized *W. somnifera* root extract (Viwithan) against different human and murine cancer cell lines. **Materials and Methods:** The cytotoxicity of Viwithan was determined using thiazolyl blue tetrazolium blue assay and crystal violet staining. The apoptotic changes in B16F1 cells following treatment with Viwithan were observed by acridine orange/ethidium bromide (AO/EB) staining and DNA fragmentation assay. The binding affinity of withanolides in Viwithan with antiapoptotic proteins B-cell lymphoma 2, B-cell lymphoma-extra large, and myeloid cell leukemia 1 (MCL-1) were studied using *in silico* approach. **Results:** The half maximal inhibitory concentration (IC₅₀) values of Viwithan against liver hepatocellular carcinoma, Henrietta Lacks cervical carcinoma cells, human colorectal carcinoma cell line, and Ehrlich ascites carcinoma cells were 1830, 968, 2715, and 633 µg/ml, respectively. Interestingly, Viwithan was highly effective against B16F1 cells with an IC₅₀ value of 220 µg/ml after 24 h treatment. The morphological alterations of apoptotic cell death were clearly observed in the AO/EB-stained cells after treatment with Viwithan. Viwithan induced late apoptotic changes in treated B16F1 cells as evident by the ladder formation of fragmented DNA in a time-dependent manner. The findings of molecular docking showed that withanolides present in Viwithan have a more binding affinity with the antiapoptotic proteins, particularly MCL-1. **Conclusion:** We have reported for the first time that Viwithan with 5% withanolides has a potent cytotoxic effect, particularly against B16F1 murine melanoma cells among the different cancer cell lines tested.

Key words: Apoptosis, B16F1 cells, cytotoxicity, *Withania somnifera*

SUMMARY

- The present study reports for the first time that Viwithan, a standardized 5% *Withania somnifera* root extract, has potent cytotoxicity against B16F1 murine melanoma cells
- We have investigated the *in vitro* cytotoxicity of Viwithan in different human and murine cancer cells. Interestingly, we found that Viwithan was particularly very effective against B16F1 melanoma cells with a half maximal inhibitory concentration value of 220 µg/ml
- The microscopic observations following acridine orange/ethidium bromide staining and DNA fragmentation assays clearly indicated that Viwithan might initiate late apoptosis in B16F1 cells
- The binding affinity of withanolides in Viwithan with antiapoptotic proteins of B-cell lymphoma 2 family was predicted using AutoDock tool. The results from *in silico* studies indicated a plausible synergistic effect of withanolides

attributing to the Viwithan-induced apoptosis through suppression of intrinsic pathway for carcinogenesis.



Abbreviations used: MTT: Thiazolyl blue tetrazolium blue; DMSO: Dimethyl sulfoxide; BSA: Bovine serum albumin; DMEM: Dulbecco's minimum essential medium; NCCS: National Centre for Cell Science; PBS: Phosphate-Buffered Saline; HepG2: Liver hepatocellular carcinoma; HeLa: Henrietta Lacks cervical carcinoma cells; HCT-116: Human colorectal carcinoma cell line; EAC: Ehrlich ascites carcinoma cells; IC₅₀: Half maximal inhibitory concentration; AO/EB: Acridine orange/Ethidium bromide; BCL-2: B-cell lymphoma 2; BCL-XL: B-cell lymphoma-extra large; MCL-1: Myeloid cell leukemia 1; PDB: Protein Data Bank; ANOVA: Analysis of variance.

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INTRODUCTION

Withania somnifera (family Solanaceae) is commonly known as Ashwagandha or Indian Ginseng. It is considered as the King of Herbs due to wide ranging health benefits as documented in the ayurvedic system of medicine in India. Various forms of medication prepared from *W. somnifera* roots are implicated in a vast range of diseases. The root of the plant is extensively used as aphrodisiac, diuretic, astringent, and anthelmintic. The root is prescribed in Ayurveda for

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snake bite as well as in scorpion sting.^[1] *W. somnifera* improves the cell-mediated immunity helping in body's defense against disease. Scientific studies have shown it to be effective in enhancing the physical endurance and alleviating the stress-induced gastric ulcer and hepatotoxicity.^[2] Efficacy of *W. somnifera* in the treatment of several stress-induced diseases like diabetes, hypertension, premature aging, and malignancy have been scientifically evaluated^[3] and support the clinical use of the plant in health-care management. Research findings lend support to the neuroprotective effect of *W. somnifera* as documented in Ayurveda.^[4-6] *W. somnifera* is effective against several types of cancer. The detailed mechanism of therapeutic action needs to be addressed. *In vitro* cell culture system is a suitable model to study the molecular targets of antiproliferative compounds.

Viwithan is a standardized hydroalcoholic extract of *W. somnifera* root. Chemically, it contains a high proportion of Withaferin A and moderate amounts of Withanolides A and B together constituting 5% withanolides. In this study, we investigated the effects of Viwithan supplied by the Department of Phytochemistry, Vidya Herbs Pvt. Ltd., against several cancer cell lines such as liver hepatocellular carcinoma (HepG2), Henrietta Lacks cervical carcinoma cells (HeLa), human colorectal carcinoma cell line (HCT-116), Ehrlich-Lette ascites carcinoma (EAC), and B16F1 cells. Among the cells analyzed, B16F1 murine melanoma cells showed a particularly increased susceptibility to Viwithan. To better understand the mechanisms that mediate Viwithan action, we have evaluated the antiproliferative effect in B16F1 melanoma cells in detail.

MATERIALS AND METHODS

High-performance liquid chromatography analysis

The high-performance liquid chromatography (HPLC) analysis was performed on a C18 column (4.6 mm × 250 mm, Phenomenex Kinetex) at a UV detection of 227 nm (HPLC-LC 2010HT). The isocratic mobile phase of acetonitrile/0.05% phosphoric acid was flowed at 1.5 ml/min through the column.

General

Thiazolyl blue tetrazolium blue (MTT), crystal violet staining solution, Dimethyl sulfoxide (DMSO), RNAase A, Proteinase K, Agarose, and bovine serum albumin were purchased from Sigma-Aldrich. All other chemicals used in this study were obtained from Sigma.

Cell culture

The cancer cell lines used in the study such as HepG2, HeLa, HCT-116, Ehrlich-Lette ascites carcinoma (EAC), and B16F1 cells mouse melanoma cell lines were procured from National Centre for Cell Science, Pune. Cells were maintained in Dulbecco's minimum essential medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco). The cells were cultured at 37°C in 5% CO₂ humid atmosphere.

Thiazolyl blue tetrazolium blue assay

5 × 10³ cells were seeded in 100 µl of growth medium in the presence or absence of increasing concentrations of Viwithan in 96-well plates. The cells were cultured at 37°C in 5% CO₂ for 24, 48, and 72 h. After the stipulated time, MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (0.5 mg/ml) was added to each well and incubated for 3 h. The purple formazan crystals were dissolved in 100 µl of DMSO (HiMedia) by proper mixing.

Crystal violet assay

Cells were seeded in 96-well plates at a density of 2 × 10³ cells/well for 48 h in the presence or absence of different concentrations of Viwithan. After incubation, medium was removed, washed with phosphate-buffer saline (PBS) twice, and then stained with 0.5% crystal violet for 20 min, followed by three rinses in water. Air-dried plates were photographed. For the detection of optical density value, 100 µl of methanol was applied per well to decolorize. Absorbance was read at 570 nm with a microplate reader (Multiskan EX, Thermo scientific). The experiments were repeated three times, each time in triplicate.

Morphological study

B16F1 cells were treated with different Half maximal inhibitory concentration (IC50) (220, 180 and 100 µg/ml) of Viwithan for 24, 48, and 72 h. Following incubation, the cells were observed under phase contrast microscope. The images were photographed at ×20 magnification using TC capture software (Lawrence and Mayo India (P) Ltd.).

Apoptosis assay with acridine orange/ethidium bromide staining method

B16F1 cells were seeded on to 60 mm dishes at a density of 1.5 × 10⁵ cells and incubated for 24 h. The medium was removed and replaced with medium containing respective IC50 concentrations of Viwithan for 24 and 48 h. After incubation, the medium was removed and cells washed with PBS. A volume of 400 µl of acridine orange/ethidium bromide (AO/EB) dye mix (AO, 100 µg/ml; EB, 100 µg/ml) was added to the dish and cells were observed under fluorescent microscope.

DNA fragmentation assay

B16F1 cells were cultured in 6-well plates at a density of 2.5 × 10⁵ cells/well and treated for 24, 48, and 72 h with their respective IC50 concentrations (220, 180, and 100 µg/ml) of Viwithan. After incubation, cells were washed with PBS twice and trypsinized. The cell was lysed in digestion buffer containing 100 mM NaCl, 10 mM Tris, 25 mM EDTA, 0.5% SDS, and 0.1 mg/ml proteinase K. DNA was extracted by phenol/chloroform extraction and was precipitated by ethanol. The precipitated DNA was dissolved in Tris EDTA and treated with RNAase A.

Molecular docking

AutoDock tools were utilized to generate grids, calculate dock score, and evaluate the conformers of inhibitors bound in the active site of antiapoptotic proteins as targets for antiproliferative activity. AutoDock 4.2 was employed to get docking and binding scores, which is implemented by Lamarckian genetic algorithm method. The ligand molecules, withanolides were designed, and the structure was analyzed using ACD/Chemsketch. The PRODRG server was used to minimize the energy of drug compounds and three-dimensional coordinates were prepared. The protein structure files for B-cell lymphoma 2 (BCL-2), B-cell lymphoma-extra large (BCL-XL), and myeloid cell leukemia 1 (MCL-1) (protein data bank [PDB] ID: 1GJH, 2YXJ, and 3MK8) were taken from PDB and edited by removing the hetero atoms using Python molecule viewer. The grid map was centered at particular residues of the protein and was generated with AutoGrid. The Lamarckian genetic algorithm and the pseudo-Solis and Wets methods were applied for minimization, using default parameters.^[7]

Statistical analysis

The data were expressed as a mean ± standard error of the mean and analyzed by one-way analysis of variance followed by Dunnett's *t*-test using GraphPad Prism version 5 (GraphPad Software, Inc., San Diego, California, USA). The data were considered statistically significant at *P* < 0.05.

RESULTS AND DISCUSSION

High-performance liquid chromatography analysis of Viwithan

In the present study, Viwithan was characterized for the presence of withanolides using HPLC analysis. The HPLC chromatogram showed the presence of 5% of withaferin A and withanolides A and B [Figure 1].

Viwithan treatment showed reduction in viability of B16F1 cells

MTT assay was performed to evaluate the cytotoxic effect of Viwithan on different human and murine cancer cell lines. The IC₅₀ values recorded were 1830, 968, 2715, and 633 µg/ml, respectively, against HepG2, HeLa, HCT-116, and EAC cells [Table 1 and Figure 2]. Interestingly, we found that Viwithan was more effective against B16F1 mouse melanoma cells with an IC₅₀ value of 220 µg/ml. Hence, we chose B16F1 cells to further validate the cytotoxic potentials of Viwithan.

B16F1 cells were treated with different concentrations of Viwithan for 24, 48, and 72 h. A considerable reduction in the cell viability was observed in a dose- and time-dependent manner when compared with the control treated cells. The calculated IC₅₀ values were 220, 180, and 100 µg/ml for 24, 48, and 72 h, respectively [Figure 3]. We further confirmed the cytotoxic effect of Viwithan through crystal violet staining at 48 h time point [Figure 4]. B16F1 cells were treated with different concentrations of Viwithan; following the incubation time, the cells were stained and the absorbance recorded. IC₅₀ value was recorded to be 180 µg/ml.

Morphological observations of B16F1 cells after treatment with Viwithan

B16F1 cells were treated with respective IC₅₀ concentrations (220, 180, and 100 µg/ml) of Viwithan for 24, 48, and 72 h, observed under a phase contrast microscope. Morphological alterations were observed in treated cells as compared to control treated. The cells turned round and detached from the surface following treatment with IC₅₀ concentrations of Viwithan. The morphological changes were more significant with an increase in duration of treatment [Figure 5]. Further, Viwithan treated cells were observed for the morphological features of apoptosis following a treatment of 24 and 48 h. As shown in Figure 6, the control cells were stained with uniformly green fluorescence and no apoptotic features observed. Viwithan-treated cells were stained as green yellow after 24 h indicating signs of apoptosis. The cells were densely stained as orange after 48 h treatment of Viwithan demonstrating the late apoptosis.

Viwithan treatment induced DNA fragmentation in B16F1 cells

DNA fragmentation is one of the hallmark signs of apoptosis. DNA fragmentation assay was carried out in B16F1 cells after 24, 48, and 72 h of treatment with Viwithan. DNA fragmentation was not seen in the control cells and the 24 h treated cells. However, results of the study demonstrated distinct ladder formation in 48 and 72 h of Viwithan treatment as compared to untreated cells [Figure 7].

Viwithan showed inhibitor specificity against antiapoptotic proteins

BCL-2 family of proteins plays a major role in the regulation of apoptosis through interaction with proapoptotic proteins. In the present study, binding affinity of withanolides in Viwithan was studied against antiapoptotic proteins through bioinformatics approach using AutoDock tool. The docking scores revealed that withanolides had favorable

Table 1: The antiproliferation effect of Viwithan on different cancer cell lines

Cell lines	IC ₅₀ (µg/ml)
HepG2 (hepatocarcinoma cells)	1830
HeLa cells (cervical cancer cells)	968
HCT-116 (human colon cancer cells)	2715.48
EAC (Ehrlich-Lette ascites carcinoma cells)	633.4
B16F1 (murine melanoma cells)	220

IC₅₀: Half maximal inhibitory concentration

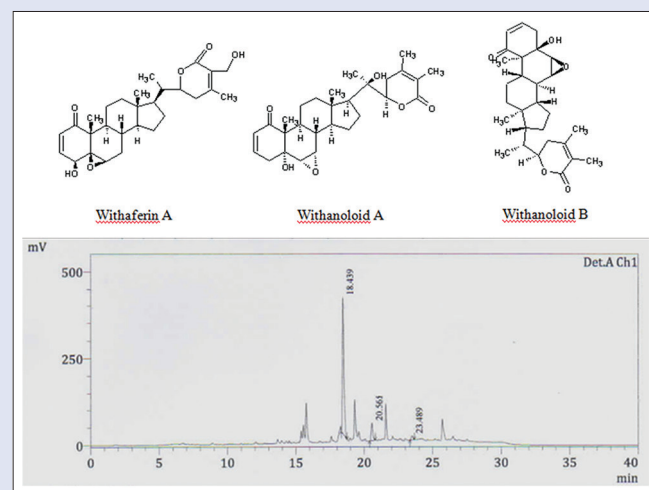


Figure 1: High-performance liquid chromatography spectra of Viwithan from *Withania somnifera*

interactions with BCL-2, BCL-XL, and MCL-1 proteins [Table 2]. However, the molecules had a more binding affinity with MCL-1. Comparatively, Withaferin A was predicted to have a profound interact with a binding energy of -6.41 kJ/mol in the MCL-1 active site and had four hydrogen bonds with Gly203, Ser202, Arg207, and Lys208 residues of MCL-1 [Figure 8] whereas Withanolide A and B exhibited a slightly lesser binding affinity (-6.19 and -5.78 kJ/mol) with the active site residues. Withaferin A had a strong covalent interaction with MCL-1 as indicated by the bond distance (1.8 – 2.25 Å) [Figure 8].

Cancer is a complex disease involving malignant tumors causing morbidity and mortality. Progression of cancer is a multistep process leading to the transformation of normal cells into highly malignant derivatives. There are several approaches available in chemotherapy to treat cancer. Chemotherapeutic agents, however, are associated with a lot of side effects. Hence, researchers are searching for safe and nontoxic alternative medicine from natural sources.^[8,9]

W. somnifera is one of the important medicinal herbs valued for its vast therapeutic potentials in the ayurvedic system of medicine. Commonly known as “Indian ginseng,” *W. somnifera* has been used in formulations to treat conditions such as rheumatoid arthritis, inflammation, constipation, sexual dysfunction, anxiety, and nervous breakdown. *Withania* extracts have been reported to be effective against several types of cancers. Studies revealed that the constituents of genus *Withania* play a significant role in antiproliferative activity against cancer cells such as MCF-7, colon cancer cells, pancreatic, prostate, and fibrosarcoma cells.^[10-12] The present study is focused on validating the antiproliferative effects of Viwithan, a standardized *W. somnifera* extract against B16F1 murine melanoma cells.

The cytotoxic effect of Viwithan on different human and murine cancer cell lines was evaluated using MTT assay. We found that Viwithan treatment was particularly effective against B16F1 melanoma

Table 2: Molecular docking score of withanolides with antiapoptotic proteins

Ligand	BCL-2			BCL-XL			MCL-1		
	Binding energy (kJ/mol)	H-bonds	Interactions	Binding energy (kJ/mol)	H-bonds	Interactions	Binding energy (kJ/mol)	H-bonds	Interactions
Withaferin A	-6.89	3	His20, Glu42, Thr41	-7.02	2	Val135, Trp181	-6.41	4	Gly203, Ser202, Arg207, Lys208
Withanolide A	-6.77	1	Gly33	-7.29	2	His177	-6.19	4	Gly203, Ser202, Arg214
Withanolide B	-8.47	2	Asn39, Glu42	-7.21	2	Val192, Glu193	-5.78	4	Gly203, Ser202, Arg207

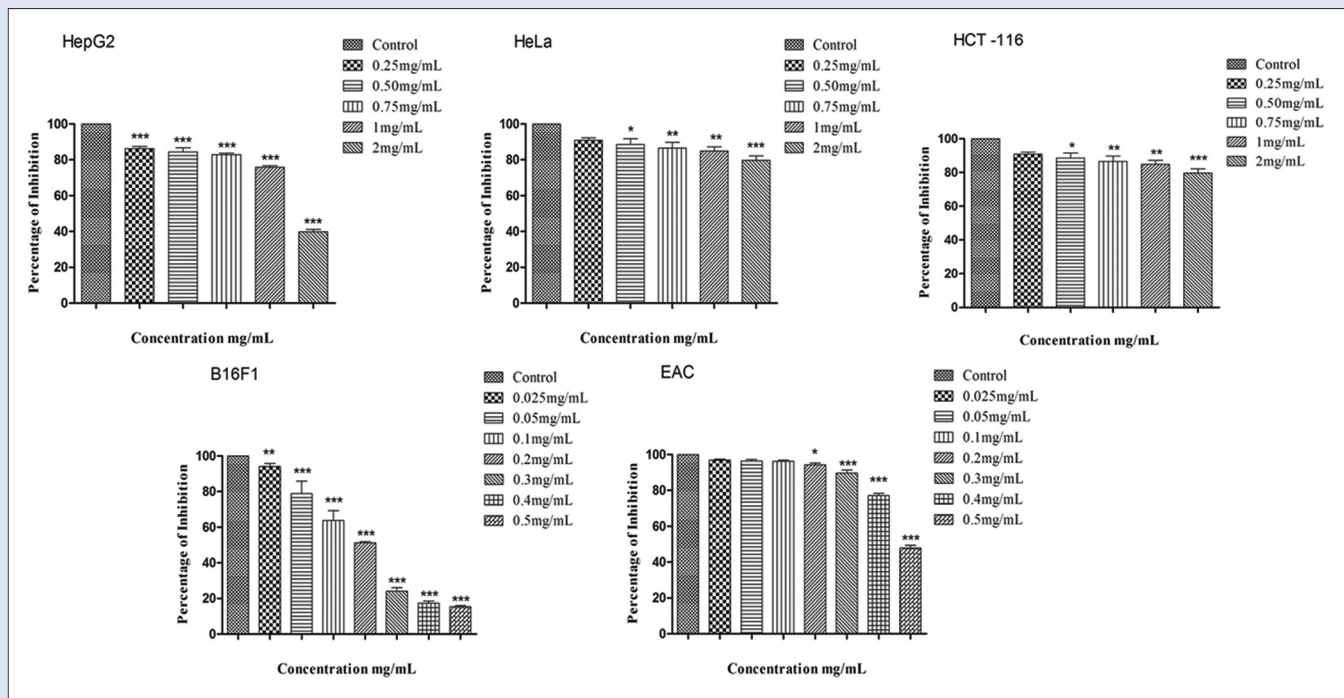


Figure 2: Determination of cytotoxicity of Viwithan in different cancer cell lines. Cells were treated with different concentrations of Viwithan for 24 h. Data are expressed as mean \pm standard error of the mean of three independent experimental observations ($*P < 0.05$, $**P < 0.01$, $P < 0.001$ versus control)

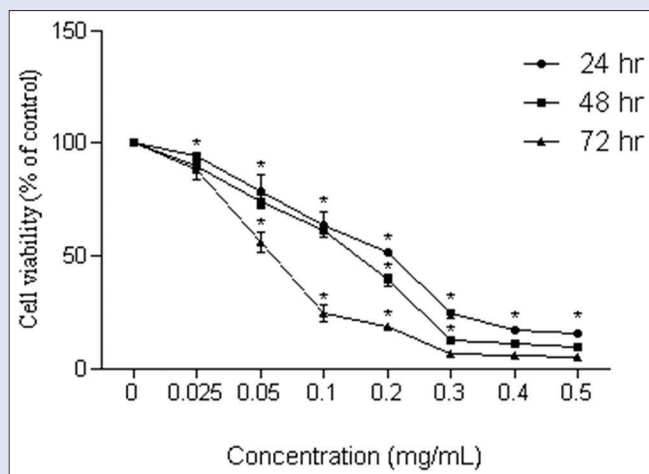


Figure 3: Treatment with different concentrations of Viwithan showed cytotoxic effect on B16F1 cell line at different time points of incubation. Cells were treated with different concentrations of Viwithan for 24, 48, and 72 h. All data are expressed as mean of three independent experimental observations

cells (IC₅₀: 220 μ g/ml) compared to other cell lines tested. We further determined the cytotoxicity of Viwithan against B16F1 cells at varying lengths of time (24, 48, and 72 h). As compared to control cells, there was a significant reduction in cell viability in Viwithan-treated cells. Effect of Viwithan on cell viability was confirmed by crystal violet staining. From these data, we concluded that the cytotoxicity of Viwithan was dose and time dependent in B16F1 cells. The toxic effects of Viwithan can be correlated to the morphological changes in treated cells, as evident from the observations in phase contrast microscopy.

Cancer development and progression in most cases is mediated by the suppression of apoptosis.^[13] AO/EB detects cellular apoptosis as a consequence of differential nuclear staining. The normal and early apoptotic cells are characterized by intact membranes; hence, we considered that AO penetrated these cells fluorescing green when bound to DNA. EB binds to the apoptotic bodies or DNA fragments emitting orange-red fluorescence in the late apoptotic cells.^[14] In our study, followed by treatment with Viwithan for 24 h, the early stage apoptotic cells were marked by yellow-green nuclear staining. Further, AO/EB-stained cells showed asymmetrically localized orange staining after 48 h treatment with Viwithan indicating the signs of late apoptosis. Cleavage of chromosomal DNA into oligonucleosomal fragments is a hallmark of apoptosis.^[15] We extended the study to check if Viwithan

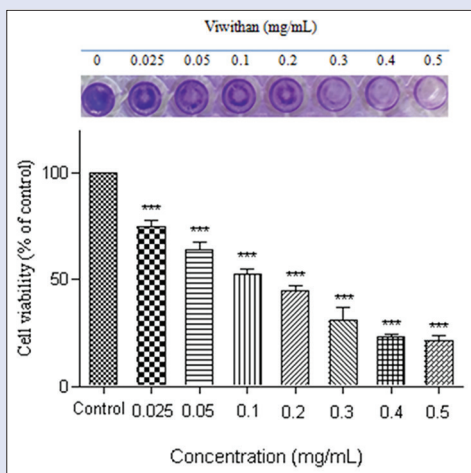


Figure 4: Treatment with different concentrations of Viwithan showed cytotoxic effect on B16F1 cell line after 48 h incubation. Cells were treated with different concentrations of Viwithan for 48 h. All data are expressed as mean of three independent experimental observations

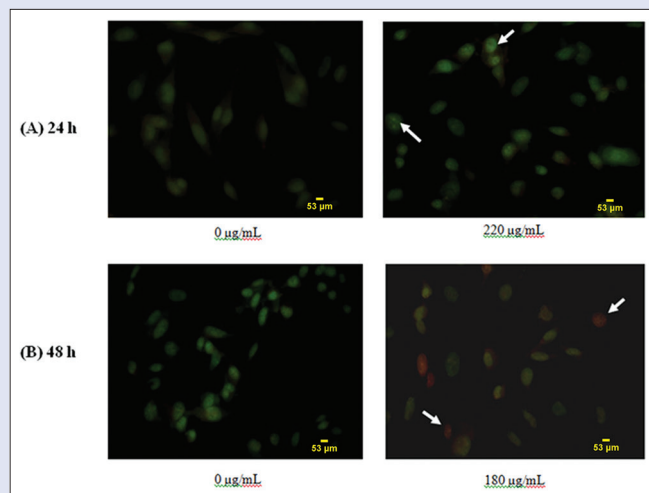


Figure 6: Apoptotic changes observed in Viwithan-treated B16F1 cells stained with acridine orange and ethidium bromide. Cells were treated with 220 and 180 µg/ml of Viwithan for (A) 24 and (B) 48 h, respectively

treatment could induce DNA fragmentation in B16F1 cells. Cells were treated with different IC50 concentrations of Viwithan for respective time points (24, 48, and 72 h). The DNA in control and 24 h treated cells remained intact. Interestingly, there was a clear ladder formation in DNA samples from 48 and 72 h treated cells. It can be speculated that the DNA fragmentation seen at 48 and 72 h incubation could be due to a late apoptotic change. Together, microscopic observations and DNA fragmentation assays clearly indicated that Viwithan might initiate late apoptosis in B16F1 cells.

In silico docking is a bioinformatics approach used to predict the drug targets and an indispensable tool in the drug discovery. Antiapoptotic proteins are key regulators of mitochondria-dependent apoptosis and major targets for cancer chemotherapy. Virtual screening of potent inhibitors for BCL-2 family proteins enables to search for candidate drugs against cancer. Many researchers have determined the structure-activity relation profile of small molecules as inhibitors of antiapoptotic members from BCL-2 family.^[16,17] In the present study, molecular docking was

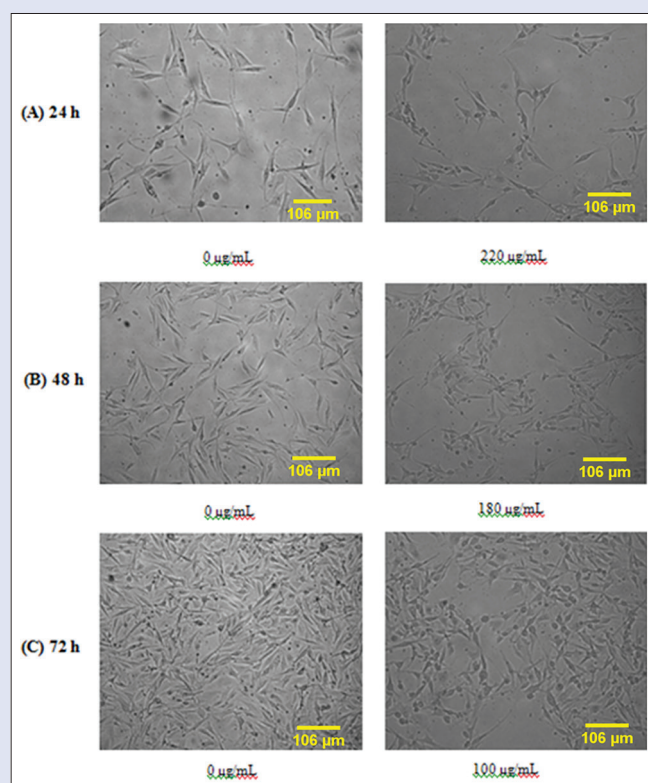


Figure 5: Morphological changes observed in Viwithan treated B16F1 cells. Cells were treated with 220, 180, and 100 µg/ml of Viwithan for (A) 24, (B) 48, and (C) 72 h, respectively

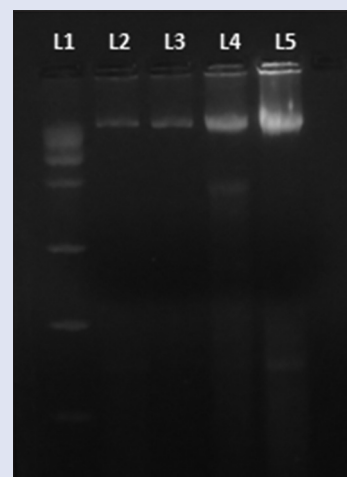


Figure 7: Viwithan treatment induces DNA fragmentations. L denotes lane in the gel, L1 = 1 kb DNA ladder, L2 = control (0 µg/ml), L3 = 24 h treatment with 220 µg/ml, L4 = 48 h treatment with 180 µg/ml, L5 = 72 h treatment with 100 µg/ml

performed to predict the interaction of withanolides present in Viwithan with the antiapoptotic proteins such as BCL-2, BCL-XL, and MCL-1. In our study, the docking score revealed the favorable interaction between the target proteins and the withanolides. Withaferin A exhibited more binding affinity with all the apoptotic proteins screened. The molecule interacted strongly with the active site residues of MCL-1 having four hydrogen bonds with covalent bonding [Figure 8]. The results from

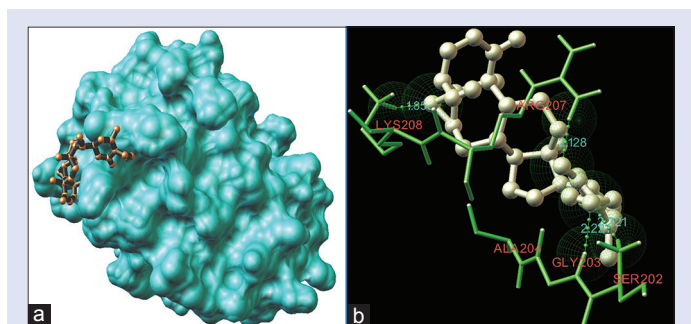


Figure 8: Molecular docking of Withaferin A and myeloid cell leukemia 1. (a) Molecular surface representation of docked withaferin A with active site of myeloid cell leukemia 1. (b) Cartoon representation of myeloid cell leukemia 1 residues (green) interacting with withaferin A (white). The H-bond interactions are shown as green spheres and the donor-acceptor distance displayed in blue color

in silico studies indicate a plausible synergistic effect of withanolides attributing to the Viwithan-induced apoptosis through suppression of intrinsic pathway for carcinogenesis.

Withanolides are the major phytoconstituents present in root and leaves of *W. somnifera*. These secondary metabolites possess immunomodulatory and neuroprotective effects.^[18-20] Extracts from different parts of *W. somnifera* with withanolides have been evaluated previously for cancer cell cytotoxicity.^[21,22] In the present study, cytotoxic potentials of Viwithan containing 5% of withanolides were investigated on several cancer cell lines. To conclude, this is the first study reporting the antiproliferative effect of Viwithan against B16F1 melanoma cancer cells. Our data indicate that Viwithan-mediated death of B16F1 cells might be due to the induction of intrinsic pathway of apoptosis. However, to determine the potency of Viwithan, further studies addressing the molecular mechanism of action are to be performed.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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