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Chemically standardised extract of *Withania somnifera* (Ashwagandha) inhibits tumour growth, promotes apoptosis and cell cycle arrest

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Withania somnifera Dunal (Family: Solanaceae), also known as ashwagandha, is indigenous to Asia and Africa. It is also known as *"Indian Ginseng*." It has been used for thousands of years in ancient Indian Ayurvedic medicine to reduce stress and pain, increase energy, improve nutrition, and treat various health conditions. The present study aims to determine the chemopreventive potential of a chemically standardised extract of *W. somnifera* against lung cancer (A549) and breast cancer (MCF-7) cell lines. The anticancer activity of *W. somnifera* was determined against lung and breast cancer using A549 and MCF-7 cell lines, respectively, in different anticancer models, and the chemical constituent was determined by the GC-MS method. After being treated for 48 hours in MTT assay, *W. somnifera* successfully reduced the viability of the MCF-7 cell line with an IC_{50} value of 41.83 µg/mL in a dose-dependent manner. *W. somnifera* extract treatment reduced cyclin D1 and E2 expression in A549 cell lines. It was correlated to cell cycle arrest in the G0/G1 phase. Additionally, it decreased nuclear factor *β*-translocation, a pro-survival signal. In the human lung cancer cell line (A459), the anticancer activity was evaluated by Cas 3 gene expression investigation by RT-PCR. The existence of fatty acids in higher concentrations was revealed by GC-MS analysis of the methanolic fraction from the 70% ethanolic extract of *W. somnifera*, which produced 28 compounds. Given its apoptogenic and antiproliferative qualities, the observations point to the chemopreventive potential of *W. somnifera* extract. Additional research is required to investigate these effects in *in vivo* models.

Keywords: A549 cells, Breast cancer, GC-MS, Lung carcinoma, MCF-7 cells, MTT assay, *Withania somnifera*

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Introduction

As the worldwide burden of cancer continues to rise, cancer is becoming a serious health issue for global public health[1]. Chemotherapy is still the best option for prolonging life span and enhancing the quality of life in cancer patients[2]. Despite these advancements, medication resistance continues to be a major barrier to effective therapy, and current therapies have little effect on the 5-year overall survival of patients with advanced stage. As a powerful source of anticancer drugs, herbal products have been essential. According to estimates, up to 35-40% of global anticancer medications come from plant sources[3]. A substantial amount of interest remains in the study of medicinal plants to prevent and treat cancer.

High rates of morbidity and mortality are associated with lung cancer. Because of this, it is frequently linked to a great deal of discomfort and a general decline in quality of life. Herbal remedies are a major source of novel pharmaceuticals and are acknowledged as an attractive treatment option for lung cancer with few adverse effects[4]. Because lung cancer is sometimes not discovered until the disease has advanced significantly and the patient's quality of life has been greatly diminished, it is now the malignant tumour with the highest fatality rate worldwide[5]. Smoking, exposure to air pollution and radiation, and some occupational exposures to arsenic, nickel, and chromium are only a few of the possible causes of lung cancer. Smoking is the leading risk factor, and lung cancer incidence rates are often higher in males than in women[6]. Many herbal medicines and nutraceuticals made in India utilise *Withania somnifera* Dunal (Ashwagandha, Family: Solanaceae) in large quantities[7]. According to Singh, it is an annual herb that grows as a wild plant in dry, arid soil[8]. It is widely documented in Ayurveda, the traditional Indian system of plant medicine, for

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immunomodulation and antiaging[9,10]. *W. somnifera* also has analgesic[11], anti-inflammatory[12], anti-tumour, and radio-sensitising effects[13]. The goal of the current study was to assess the antiproliferative capability of 70% ethanol extract of *W. somnifera* leaves (analysed by GC-MS) against A549 (lung cancer) and MCF-7 (breast cancer) cancer cell lines using MTT assay, cell cycle arrest by flow cytometry and RT-PCR method.

Materials and Methods

Plant materials and chemicals

The leaves of *Withania somnifera* (WS) were collected in March 2023 from the Madurai District of Tamil Nadu, India. The plant sample was authenticated and kept in the Department of Pharmacognosy, Centre for Advanced Research in Indian System of Medicine (CARISM), SASTRA University, Thanjavur, Tamil Nadu, India (voucher specimen: 0065). Human lung cancer (A549) and human breast cancer (MCF-7) were purchased from the National Centre for Cell Science (NCCS), National Repository cell line campus branch, Pashan, Pune, India. H-reverse transcriptase, RNase, and superscript II were received from Gibco (USA). Cas 3 and B-actin forward and reverse primers were purchased from Xcelris Pvt. Ltd., India. All other chemicals and solvents were of analytical grade.

Preparation of the leaf extract of W. *somnifera*

The gathered raw material was dried for fifteen days in the shade. The extraction process employed powdered raw material. Ethanol (70%) was used for extraction, which was done using the cold percolation method. A Rota-evaporator was used to concentrate the extract, which was later kept in a refrigerator. Calculations showed that the extract yield was found to be 3.52%.

GC-MS analysis and identification of phytoconstituents of W. somnifera

About 10 mg of *W. somnifera* (WS) extract was dissolved in methanol. The methanol fraction separated from the ethanolic extract was injected for GC-MS analysis. The following experimental conditions for sample analysis by GC-MS were applied on the PerkinElmer Clarus 500 GC: Elite 5 column (5% biphenyl, 95% dimethylpolysiloxane, column size 30 mm × 0.32 mm), gas loading 1 mL/min of helium, column temperature of 50°C to 285°C, speed 10°C/min for 5 min at 285°C, the temperature of the injector and the detector was 290°C, the sample is injected in Split mode, injection volume: 0.5 µL (2 mg/100 mL methanolic solution) and the total run time was 30 min. Mass spectra were acquired using a Perkin Elmer-Turbo Mass Gold mass detector. Transmission line temperature: 230℃, source temperature: 230℃, and scanning range 40-450 amu, ionisation technology, electron ionisation technology.

The chemical component was identified by comparing the mass spectrum of the compound with the available NIST and Wiley mass spectrometry libraries. The quantitative composition was obtained by normalising the peak area[14].

MTT assay to assess cell cytotoxicity

The MTT assay was used to evaluate the possible cytotoxic and antiproliferative effects of *W. somnifera* on human MCF-7 cell lines (human breast cancer)[15]. The MTT experiment was used with a few minor modifications. According to the methodology, MCF-7 cell lines were grown in liquid Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 µg/mL streptomycin, and 100 µg/mL penicillin. These conditions were kept at 37°C in a 5% CO_2 atmosphere. Trypsinisation was used to harvest the cultivated MCF-7 cell lines, which were then gathered in a 15 mL tube. The cells were then seeded into a 96-well tissue culture plate at a density of 1×10^5 cells/mL cells/well (200 µL) in DMEM media with 10% FBS and 1% antibiotic solution for 24-48 hours at 37°C. In a serum-free DMEM medium, the wells were cleaned with sterile PBS before being treated with various doses of *W. somnifera* extract. The cells were incubated for 24 hours at 37°C in a humidified 5% CO_2 incubator, with each sample being replicated three times. The cells were then treated for a further 2-4 hours with MTT (20 µL of 5 mg/mL) until purple precipitates were visible under an inverted microscope. The medium and MTT (220 µL) were then aspirated out of the wells and rinsed with 1X PBS (200 µL). DMSO (100 µL) was added, and the plate was agitated for 5 minutes to dissolve the formazan crystals. Using a fluorescence multi-detection reader, the OD was measured at 570 nm (Thermo Fisher Scientific, USA). Cells that had not been treated served as a control.

Cell cycle analysis by flow cytometry

Using A549 cells and the propidium iodide staining technique, the *W. somnifera* sample was examined for

cell cycle analysis. In a 15 mL tube, the cultivated A549 cells were collected and seeded into a 6-well tissue culture plate at a density of 1×10^6 cells/mL in DMEM media with 10% FBS and 1% antibiotic solution for 24-48 hours at 37°C. The wells were treated with 41.83 µg/mL of the *W. somnifera* sample in a serum-free DMEM medium and incubated at 37°C in a 5% CO_2 incubator for 24 hours after being cleaned with sterile PBS. After incubation, the cells were centrifuged for five minutes at 1500 rpm to wash them in PBS. Furthermore, cold 70% ethanol was added dropwise to the cell pellet to fix the cells for 30 min at 4°C. After incubation, the cells were rinsed twice with sterile PBS, centrifuged for five minutes at 1500 rpm, and the supernatant was discarded[16]. At 4°C, until flow cytometry analysis, the cell pellet was then treated with 50µL of RNase (100 µg/mL) and 500 µL of PI (100 µg/mL) (BD Accuri C6 flow cytometer, Inc., Ann Arbor, USA)

Cas3 gene expression by quantitative RT-PCR

Cell culture and cell treatment

The A549 (Human lung cancer cells) cell line was grown in liquid medium (DMEM) with 10% Fetal Bovine Serum (FBS), 100 µg/mL penicillin, and 100 µg/mL streptomycin. It was maintained at 37°C in a 5% CO_2 environment. Using the A549 cell line, the impact of WS on the regulation of gene expression was examined. Briefly, trypsinisation was used to harvest the cultivated A549 cells, which were then collected in a 15 mL tube. The cells were then plated into a 6-well tissue culture plate at a density of 1×10^6 cells/mL cells/well (1 mL) in DMEM media containing 10% FBS and 1% antibiotic solution for 24 hours at 37°C. The wells were treated with 41.83 µg/mL of the WS sample in a serum-free DMEM medium and rinsed with sterile PBS before being incubated for 24 hours at 37°C in a humidified 5% CO_2 incubator. After incubation, the A549 cell line's total RNA was isolated using the Trizol procedures[17].

RNA isolation

According to the manufacturer's instructions, the TRIZOL technique isolated total RNA. The samples were centrifuged at 5000 rpm for 10 min in diethylpyrocarbonate-treated centrifuge tubes to obtain the cell pellet. To lyse the cells, 700 µL of TRIZOL was added to the cell pellet (1×10^7 cells). After being quickly pipetted into 1.5 mL tubes, the lysate was collected. Chloroform (300 µL) was added, and the mixture was vigorously stirred at room temperature for 5 min. Centrifugation separated the aqueous layer for 20 min at 4°C at 12000 rpm. A 1.5 mL tube was used to capture the aqueous layer. Isopropanol (700 µL) was added to precipitate the RNA[18]. Centrifugation was used to pellet the precipitated RNA. It took place at 12000 rpm for 20 min at 4°C. The pellet was then washed with 70 % ethanol. The air-dried RNA pellet was then dissolved in 30 µL of double-distilled autoclaved water and kept at -80°C until it was needed again. The quantity and quality of the isolated RNA were assessed using a 1.5% agarose gel and a Labman UV-visible spectrometer.

DNase treatment

The DNase process eliminated any DNA contamination that may have occurred during RNA production. The reaction volume, which included 1 U of DNase, was set to 20 µL. It was then added 20 µM of 2 µL EGTA and further incubated at 66°C for 10 min after being incubated at 37°C for 30-45 min. Absolute ethanol (2V) and sodium acetate (1/10 V) were added, and the mixture was then incubated at -20°C for 60 min. The pellet was then rinsed with 500 µL of 75 % ethanol following a centrifugation step at 12000 rpm for 20 min at 4°C. The sample was air-dried, dissolved in 20 µL of Milli-Q grade water, and then stored until it was needed again.

cDNA synthesis

With a reaction mixture comprising reverse transcriptase, 1.5 µg of total RNA was transformed into cDNA (MMLV). The cDNA synthesis was performed at 25°C for 10 minutes, then at 42°C for 59 minutes. Reverse transcriptase inactivation and denaturation of the cDNA and RNA hybrid were performed at 99°C for 5 sec, followed by a hold at 4°C.

Gene-level expression detection of micrometastases

The obtained cDNA was employed as a template to identify apoptosis. SYBR® Green JumpStart™ Taq Ready Mix™ in ABI StepOne Plus (Applied Biosystems, CA, USA) was the calibrator for quantitative real-time PCR (qRT-PCR). The threshold cycle was used to express the findings (Ct). The comparative Ct technique (ΔΔCt), by the manufacturer's instructions, was used to determine the relative quantity of the target transcripts. The relative changes in gene expression were examined using the $2-\Delta\Delta Ct$ technique. Control PCR assays were run without reverse transcription to ensure that the total RNA was not tainted with genomic DNA.

RT-PCR condition

The initial melting temperature was maintained at 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, annealing at 51°C for thirty seconds, and the extension temperature was maintained at 72°C for 1 minute, with final extension continuing at 10 min and being kept at 4°C. At the end of each extension stage, the real-time data was taken.

Results and Discussion

GC-MS analysis of *W. somnifera* extract

W. somnifera secondary metabolites were identified using the GC-MS method (Fig. 1), and the results are shown in Table 1. GC-MS analysis of *W. somnifera* extract revealed 28 chemical components among which (*E*)-9-octadecenoic acid ethyl ester (18.92%), butanedioic acid, 3-hydroxy-2,2-dimethyl-, diethyl ester (18.81%), palmitic acid, ethyl ester (13.34%), squalene (10.24%), 1,2-benzenediol (7.67%), α-(carboethoxy) cyclopentanone (5.16%), and Octadecanoic acid, ethyl ester (4.01%) was found as major chemical constituents. Furfural, *n*-hexadecanoic acid, *D*-mannose, *L*-glucose, *L*-glutamic acid, and palmitic acid were found as minor components in the leaves of *W. somnifera* extract. The main phytochemicals identified in WS were withanolides, withaferin A, and withanolide D[19].

Withanolides and withaferin are important phytoconstituents present in *W. somnifera* leaves and known for their anticancer, anti-inflammatory, antioxidant, antibacterial, antifungal, hypolipidemic, memory-improving, and rejuvenating properties. *W. somnifera* is a significant medicinal herb from the Indian region[20,21]. GC-MS analysis of ethanolic extract of *W. somnifera* yielded 28 compounds where (*E*)-9-octadecenoic acid ethyl ester, butanedioic acid, 3-hydroxy-2,2-dimethyl-, diethyl ester, palmitic acid, ethyl ester, squalene, 1,2-benzenediol, α-(carboethoxy) cyclopentanone, and octadecanoic acid, ethyl ester lwere found as major chemical compounds. These biomolecules are responsible for immune system enhancement, antioxidant protection, anti-inflammatory relief, antimicrobial activity, and antitumor effects[22-24]. According to numerous studies, *W. somnifera* roots and leaves are a more abundant source of withanolides and related chemicals. These withanolides have been discovered to exhibit antitumor activity, immunomodulatory properties, and neuroprotective properties[25]. Studies on the treatment of various cancers, including "breast cancer"[26,27], "prostate cancer"[28], and myeloid cells, are among the most recent therapeutic uses of *W. somnifera*[29,30].

Cell cytotoxicity

MCF-7 cells were tested using the MTT assay to determine their cytotoxicity against human breast cancer cell lines. The WS suppressed MCF-7 cells' ability to proliferate. Fig. 2 displays the MTT assay findings. Within 24 hours, WS inhibits the growth of

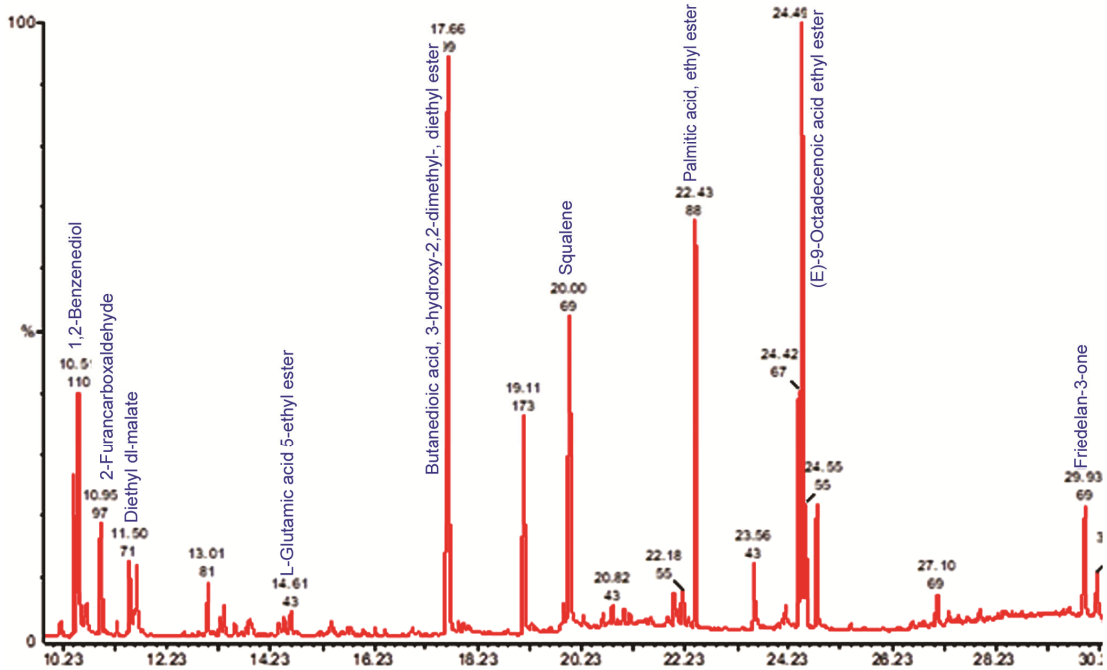

Fig. 1 — GC-MS chromatogram of *W. somnifera* extract.

Table 1 — Chemical compounds identified in *W. somnifera* extract by GC-MS

S. No	Peak Name	Retention Time	% Composition
1	3(2H)-Furanone, dihydro-2-methyl-	4.35	0.16
2	Furfural	4.74	2.87
3	2(5H)-Furanone	5.97	0.31
4	2-Furancarboxaldehyde, 5-methyl-	6.83	0.31
5	2-Hydroxypentanediamide	7.11	0.59
6	1,5-Heptadiene, 2,3,6-trimethyl-	7.45	0.49
7	Levulinic acid, ethyl ester	8.37	0.88
8	ç-Ethoxybutyrolactone	8.45	0.13
9	α-(Carboethoxy)cyclopentanone	10.43	5.16
10	1,2-Benzenediol	10.51	7.67
11	2-Furancarboxaldehyde, 5-(hydroxymethyl)	10.95	3.63
12	Benzeneacetic acid, ethyl ester	11.26	0.48
13	Diethyl dl-malate	11.50	2.25
14	α-*D*-Lyxofuranose, cyclic 2,3-(ethylboronate)	11.65	2.09
15	Ethyl α-*D*-glucopyranoside	13.54	0.36
16	*D*-Mannose	13.81	0.43
17	*L*-Glucose	14.48	0.51
18	*L*-Glutamic acid 5-ethyl ester	14.61	0.75
19	Palmitic acid, ethyl ester	15.40	0.41
20	α-Muurolene	15.72	0.24
21	Butanedioic acid, 3-hydroxy-2,2-dimethyl-, diethyl ester	17.66	18.81
22	Ethyl citrate	17.85	0.33
23	Squalene	20.00	10.24
24	*n*-Hexadecanoic acid	22.02	1.03
25	Palmitic acid, ethyl ester	22.43	13.34
26	(*E*)-9-Octadecenoic acid ethyl ester	24.49	18.92
27	Octadecanoic acid, ethyl ester	24.77	4.01
28	Friedelan-3-one	29.93	3.57

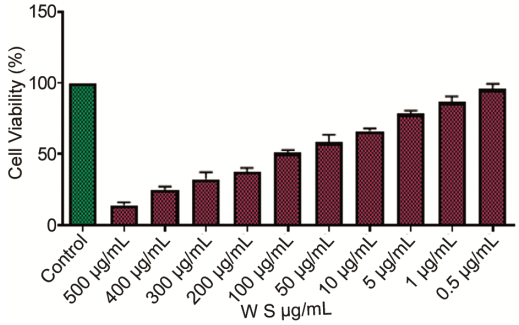

Fig. 2 — Cytotoxic effect assessed by MTT assay of *W. somnifera* extract.

MCF-7 cells at different concentrations (0.5–500 µg/mL). For MCF-7 cell lines, the *W. somnifera* extract IC_{50} value was 41.43 µg/mL at 24 hours. MTT is a water-soluble substance that the live cell can take up. For calorimetric measurement, a water-insoluble blue formazan, the reduction product of MTT, must be dissolved. As seen in Fig. 3a, even after the incubation period was extended to 24 hours, the untreated MCF-7 cells kept their original morphology and proximity to one another. MCF-7 cells, in contrast, started to lose their original form after 24 hours of WS treatment. The elongated spindle-shaped morphology of the MCF-7 cells was no longer present. Suspension cells (dead cells) were found when the therapy was prolonged to 48 hours, and more suspension cells were seen at 24 hours. (Fig. 3b–g). The current study demonstrates that *W. somnifera* extract has favourable cytostatic effects.

Gene-level expression detection of micrometastases

By employing the relative quantification (2^-ΔΔCT) method of qRT-PCR, the expression levels of the chosen genes (Table 2: Primer sequence) were evaluated. β-Actin was used as the endogenous control to normalise expression, and control cells served as the calibrator. The initial melting temperature of 94°C for three min was followed by 30 cycles of 94°C for 30 sec, annealing at 51°C for 30 sec, and extension temperatures of 72°C for 1 min with 72°C for a final extension of 10 min, and hold at 4°C (Table 3).

A549 cancer cell lines were used to examine the impact of *W. somnifera* on cell cycle analysis using the propidium iodide (PI) staining technique[31]. One of

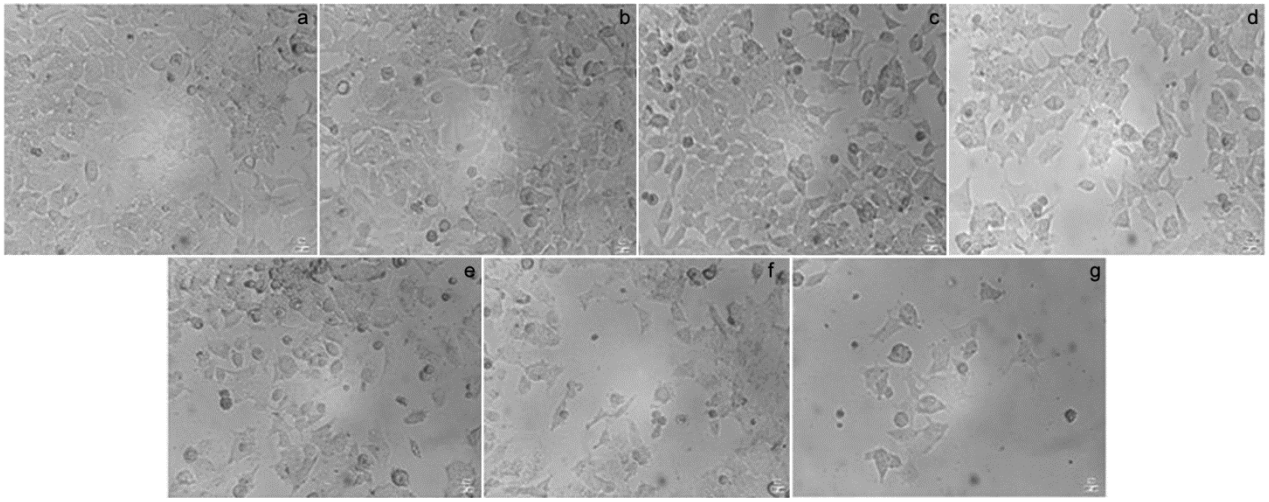

Fig. 3 — Cytotoxic effect of WS extract assessed by MTT method, where a**)** control cell, and b**-**g**)** concentration of WS extract 1 to 500 µg/mL, respectively.

Table 2 — Primers used in Cas 3 gene expression study

S. No	Primers Name	Oligo Sequence (5' to 3')
1	CAS 3 F	**5'-**AGCAAACCTCAGGGAAACATT-**3'**
2	CAS 3 R	**5'-**CTCAGAAGCACACAAACAAAACT-**3'**
3	β-actin F	**5'-**ATCGTGCGTGACATTAAGGAGAAG-**3'**
4	β-actin R	**5'-**AGGAAGGAAGGCTGGAAGAGTG-**3'**

Table 3 — The working conditions of qRT-PCR

Fragments	Initial denaturation	Number of cycles	Denaturation	Annealing	Final Extension
CAS 3	94°C -3 min	30	94°C - 30 sec	51°C - 30 sec	72°C -10 min
β-actin	94°C -3 min	30	94°C - 30 sec	51°C - 30 sec	72°C -10 min

the first uses for flow cytometry was investigating the cell cycle by quantifying DNA content. Propidium Iodide can stain the DNA of mammalian cells (PI). The PI will bind in proportion to the amount of DNA present in the cell. As a result, S-phase cells will have more DNA than G1-phase cells. Until their DNA content is doubled, they will absorb proportionately more dye and fluoresce more brightly. The G2 cells will be almost twice as luminous as the G1 cells. The cells must be fixed or permeabilised to allow the dye to enter the cells, which are otherwise actively pushing out the dye by living cells. Aldehydes or alcohols are frequently employed for fixing. Alcohol is a fixative that dehydrates and permeabilises. This will make it simple for the dye to reach the DNA and produce high-quality profiles.

Cell cycle analysis by flow cytometry

W. somnifera was displaying its pharmacological activity to ascertain the prospective impact. The effect of *W. somnifera* on cell cycle distribution in human lung cancer A549 cell lines at a dose of 41.43 g/mL resulted in an increased fraction of cells in the G0/G1 interphase with 23.20, 20.45, and 4.35%, respectively. The total apoptosis rate of the control group cells was 1.23%, while the treatment group cells had a rate of 52%. Additionally, *W. somnifera* showed a decrease in the percentage of cells in the G2/M phase. Fig. 4a depicts the untreated group, while Fig. 4b demonstrates the incorrect distribution of the cell cycle.

Understanding how the ethanolic extract of *W. somnifera* controls the cell death pathway will give us a better idea of how things might work and help us develop more powerful and selective anticancer herbs. One of the best methods for preventing tumour growth in cancer cells is thought to be the inhibition of cell cycle progression[16]. Our findings revealed that a decrease in S and G2/M phase cells accompanied the substantial increase in G0/G1 phase cells.

Cas3 gene expression by quantitative RT-PCR

The growth-inhibitory effects of *W. somnifera* on the A549 human lung cancer cell lines were examined for anticancer effects. The MTT assay's IC_{50} concentration was 41.83 µg/mL. A panel of human

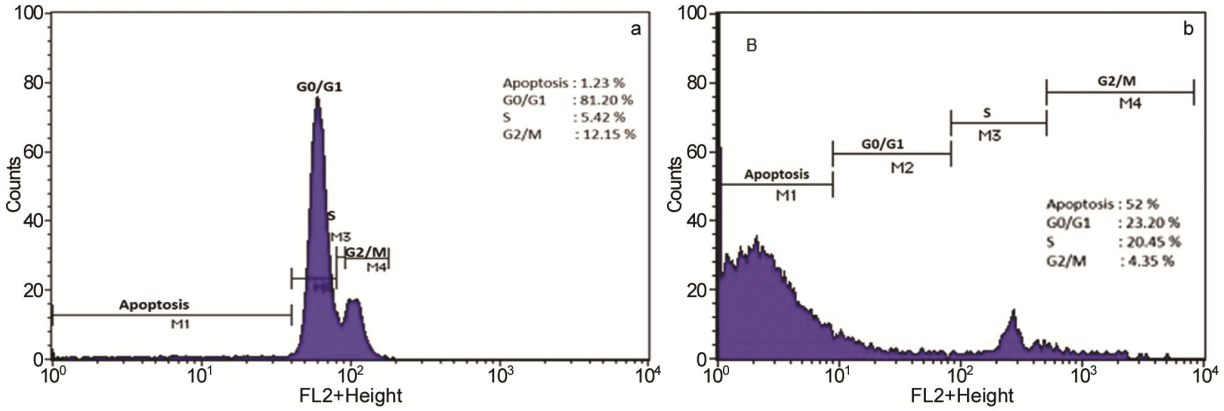

Fig. 4 — a) Cell cycle arrest for the control group, and b) Treated with *W. somnifera* extract (41.83 µg/mL).

Table 4 — Gene expression of CAS3 in A549 (Human lung cancer cells) with treatment of *W. somnifera*.

Sample	Reference gene - β-actin	Target gene- CAS 3	Δ Ct control	ΔΔCt (treatment) CAS 3	2^-ΔΔ Ct (treatment)	Log2 expression (fold variation) CAS 3
β-actin	23.90	13.80	4.73	-6.42	85.36	0.97
WS extract	28.63	12.12				

lung cancer cell lines was used to demonstrate an approximately 10-fold improvement in the antiproliferative properties of the *W. somnifera* extract. Evaluation of Cas 3 expression using the human lung cancer cell line (A549) is shown in Table 4.

Fig. 5a–c shows that (qRT)-PCR analysis showed that *W. somnifera* therapy caused a greater rise in the mRNA level of p21 and a decrease in the mRNA level of E2F1, which quantifies the rises in the expression of the two proteins. These findings imply that transcriptional regulation regulates the inhibitory effects of *W. somnifera* therapy on the expression of proteins associated with cell cycle and death. The data demonstrated that the Cas 3 protein was not expressed compared to the control group. Actin protein acted as an endogenous regulator to keep Cas 3 gene expression appropriate (Fig. 5a,b, and c). The Cas 3 gene (bcl-2) protein levels from numerous quantitative real-time RT-PCR runs are also shown in Table 4, about normalised levels of β-actin. The outcomes demonstrated that, following treatment with *W. somnifera* extract, the Cas 3 protein was upregulated with a value of 0.97. The threshold cycle was used to express the findings (Ct). The comparative Ct technique ((ΔΔCt)), by the manufacturer's instructions, was used to determine the relative quantity of the target transcripts. The relative changes in gene expression were examined using the 2−ΔΔCt technique. Control PCR assays without reverse transcription were carried out to ensure that the total RNA was free of genomic DNA contamination. Fig. 5d result showed positive expression of Cas 3 protein compared to the control group. β-actin protein was used as an endogenous control for the normalisation of expression of Cas 3 protein. Fig. 5e shows the amplifications of a plot for different cell cycles.

Overexpression of p21 inhibits cyclin-CDK4 kinase activity[32]. D-type cyclins generated by mitogenic triggers form functional complexes with CDK4 or CDK6 to phosphorylate and inactivate pRb. This, in turn, dissolves pRb complexes with transcription factors from the E2F family and related chromatin-modifying enzymes, permitting the transcription of genes essential for the S phase[33]. A variety of genes whose expression is necessary for the G1 to S transition are activated by E2F1 as cells advance from the G1 phase of the cell cycle to the S phase[34]. Alkaloids, steroidal lactones, and saponins, which comprise the majority of phytoconstituents of *W. somnifera*, have been demonstrated to reduce survival in human lung cancer cells to comparable degrees.

When overexpressed, the Cas 3 protein causes the development of cancer. It is essential for cellular activities such as cell formation, differentiation, and proliferation. Numerous studies have found a positive correlation between Cas 3 and lung cancer, and low expression or downregulation of Cas 3 is typically

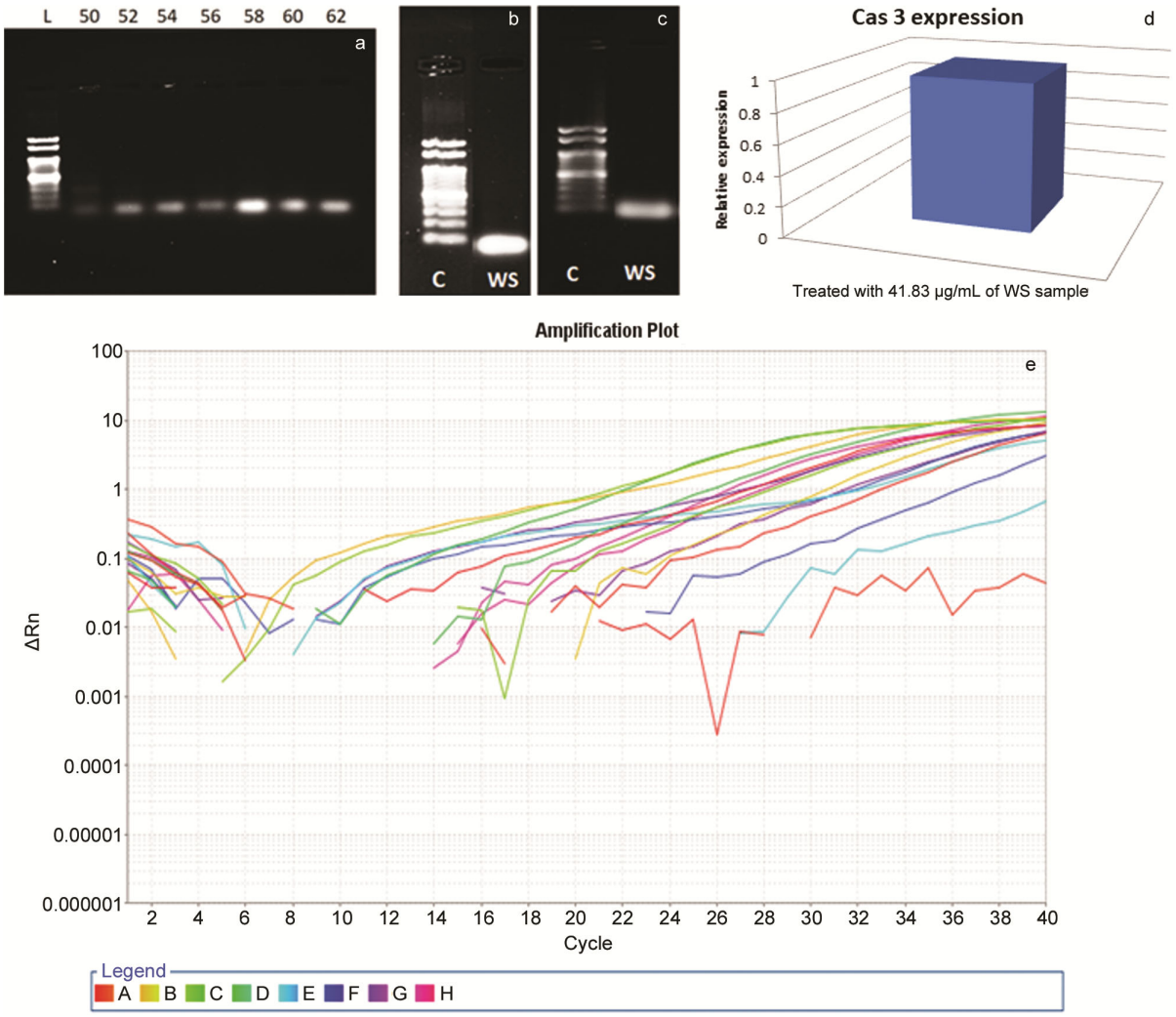

Fig. 5 — a) Gradient PCR analysis of Cas 3 gene expression at various degree temperatures, b) Real -time quantitative measurement of Cas 3 gene expression analysis in lung cancer line, c) β-actin gene expression analysis with WS extract, d) Fold variation of Cas 3 gene expression with WS extract treated A549 cells, and e) Amplification plot for each cycle.

associated with a favourable prognosis for cancer. The effect of withanolides on the development pattern of mammary cancer cells is based on the prevention of epithelial-mesenchymal transition and reduction in vimentin protein expression[35,36]. Withaferin was found to cause apoptosis in the NSCLC cell line A549 when used as a lung cancer treatment, according to an annexin V/PI assay[37].

Additionally, *W. somnifera* reduced the amount of A549 cells that proliferated because treated cells had more cells in the G0/G1 phase. Serine-threonine protein kinase pathway, the anti-apoptotic protein Bcl-2, and cleaved cas-3 were all dose-dependently decreased by *W. somnifera*. Studies show that resveratrol and silibinin increased p21 expression, caused G1 arrest, and induced apoptosis[38].

Conclusion

The ethanolic extract of leaves of *W. somnifera* was determined by the GC-MS methods, and 9-octadecenoic acid ethyl ester, butanedioic acid, 3-hydroxy-2,2-dimethyl, diethyl ester, palmitic acid, squalene, 1,2-benzenediol were found as major phytoconstituents. The *in vitro* study of MTT assay inhibition activity of the ethanolic extract of *W. somnifera* showed a concentration-dependent IC_{50} value. The results of the study also suggest that *W. somnifera* may have anticancer properties because they revealed positive Caspase 3 protein expression in the human lung cancer cell line (A549) and MTT assay in the breast cancer cell line (MCF7). *W. somnifera* slowed down the growth of cancer cells, prolonging the overall lifespan of cancer patients.

More research is required to isolate the chemical compounds in *W. somnifera* and assess their potential anticancer signaling pathway in the coming days.

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Conflict of interest

The authors declare that they have no conflict of interest.

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