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RESEARCH ARTICLE

Evaluating the Anti-proliferative and Apoptotic Effects of *Sesamin* on SKOV3 Ovarian Cancer Cells: An Integrated *In Vitro* and *In Silico* Approach

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Abstract

Background: Ovarian cancer is among the most lethal gynaecological tumours because it is resistant to apoptosis and chemotherapy. **Aim:** The aim of the current study was to evaluate the cytotoxic and apoptotic effects of *Sesamin* on SKOV3 ovarian cancer cells and identify its molecular mechanisms. **Methods:** SKOV3 cells were exposed to *Sesamin* (75 µM for 48 hours) and subjected to MTT assay, immunofluorescence microscopy, flow cytometry, western blotting, gene expression analysis, and molecular docking. **Results:** *Sesamin* treatment downregulated cell viability dose dependently and caused typical apoptotic morphological alterations such as chromatin condensation and nuclear fragmentation. Flow cytometry analysis indicated cell cycle arrest in the sub-G1 and G2/M phases, whereas Annexin V-FITC staining showed an enhanced apoptotic cell population. Western blotting and gene expression analyses proved upregulation of pro-apoptotic proteins (Bax, p53, and Caspase-9) and downregulation of anti-apoptotic proteins (Bcl-2 and Bcl-xL), showing activation of the intrinsic mitochondrial apoptotic pathway. Molecular docking study further attested to *Sesamin*'s high binding affinity with crucial apoptotic regulators, further supporting *Sesamin*'s apoptosis-inducing role. **Conclusion:** *Sesamin* has potent anti-tumour activity against SKOV3 ovarian cancer cells through the induction of apoptosis, suppression of cell proliferation, and interference with major survival pathways. The induction of pro-apoptotic proteins and suppression of anti-apoptotic regulators indicate that *Sesamin* triggers the intrinsic mitochondrial apoptotic pathway, increasing susceptibility of cancer cells to apoptosis. Furthermore, molecular docking analysis confirms its robust interaction with central apoptotic regulators, further establishing its mechanism of action. Our findings offer useful information regarding the molecular impact of *Sesamin* and highlight its potential as a viable therapeutic agent for the treatment of ovarian cancer.

Major Findings: *Sesamin* showed significant cytotoxicity and pro-apoptotic activity towards SKOV3 ovarian cancer cells by causing cell cycle arrest and stimulating the intrinsic mitochondrial apoptotic pathway. Molecular docking validated strong binding affinity towards important apoptotic modulators against ovarian cancer.

Keywords: Apoptosis, Cell Arrest, Ovarian Cancer, *Sesamin*, SKOV3, Therapeutic Agent

Abbreviation: Akt - Protein Kinase B, Annexin V-FITC staining -Annexin V conjugated with Fluorescein Isothiocyanate staining, AO/EtBr - Acridine Orange/Ethidium Bromide, Bax - Bcl-2-associated X protein, Bcl-2 - B-cell lymphoma 2, Bcl-xL

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- B-cell lymphoma-extra-large, BRCA1 and BRCA2 - Breast Cancer 1 and Breast Cancer 2 genes, DAPI - 4',6-diamidino-2-phenylindole, JNK/MAPK - c-Jun N-terminal Kinase / Mitogen-Activated Protein Kinase, Mcl-1 - Myeloid Cell Leukemia-1, p53 - Tumor Protein p53, PARP - Poly (ADP-ribose) Polymerase, V-FITC - Fluorescein Isothiocyanate-labeled Annexin V

1. Introduction

Cancer is a complex and multifaceted disease involving the uncontrolled growth of abnormal cells within the body. Worldwide health issues, cancer ranking among the foremost causes of morbidity and mortality globally[1]. Cancer can occur in nearly any organ or tissue in the body, and some of the most prevalent forms are breast cancer, lung cancer, ovarian cancer, prostate cancer, and colorectal cancer. Of the different cancers, ovarian cancer ranks among the most lethal and virulent cancers in women[2]. Ovarian cancer is among the most lethal gynaecologic cancers; in many cases they are also called the "silent killer" because of its lack of symptoms in the initial stages and late diagnosis. Ovarian cancer arises in the ovaries, which are female organs in the body where they grow eggs as well as female hormones estrogen and progesterone. Ovarian cancer has numerous histological subtypes, with the most prevalent being epithelial ovarian cancer, which arises in approximately 90% of cases[3]. The precise etiology of ovarian cancer is unknown, yet there are a number of recognized risk factors, such as genetic susceptibility, age, reproductive history, hormone replacement therapy, and environmental factors[4]. Current studies emphasize enhancing early detection methods and creating personalized regimens of treatment increased patient survival rates. Since ovarian cancer continues to pose a serious health risk to women globally, ongoing research, awareness, and early detection are important in mitigating the occurrence of this disease[5]. In recent years, natural compounds have become of particular interest in cancer studies because of their promising therapeutic value with fewer side effects. Unlike traditional chemotherapy and radiation, which are frequently associated with high toxicity and resistance, plant, herb, and dietary-derived bioactive compounds provide a safer and more environmentally friendly option[6]. These natural compounds possess anti-cancer activities by modulating significant molecular mechanisms, triggering apoptosis, preventing angiogenesis, and suppressing tumour growth[7]. The main treatment for this type of cancer is often surgery, including cytoreductive or debulking procedures to remove as much of the tumour as possible. The standard first-line treatment is chemotherapy with a carboplatin and paclitaxel-based regimen[8]. Despite significant progress in treatment, challenges such as drug resistance, recurrence, and treatment-related toxicities persist, underscoring the need for innovative therapeutic strategies[9].

Sesamin, a lignan bioactive compound found in sesame seeds (*Sesamum indicum*), has generated significant interest as a result of its therapeutic property against various diseases, including cancer. As an anti-inflammatory and antioxidant compound in nature, *Sesamin* possesses highly effective anti-cancer activity against cell proliferation, apoptosis, and metastasis *via* the modulation of multiple pathways involved[10]. Studies have proven that *Sesamin* causes cell cycle arrest, encourages programmed cell death (apoptosis), and suppresses angiogenesis, hence inhibiting the growth and spread of tumours. Moreover, *Sesamin* has been found to improve the efficacy of chemotherapeutic agents without adding to their toxicity, thus holding potential for use in combination therapy[11]. Previous research has established the anti-proliferative action of *Sesamin* on numerous cancer cell lines. For example, in human colorectal cancer cells, *Sesamin* produced cell cycle arrest and apoptosis through the induction of p38 and JNK/MAPK pathways[12]. Likewise, in breast cancer MCF-7 cells, *Sesamin* brought about G1 phase arrest through cyclin D1 protein expression suppression *via* proteasome degradation[13,14]. In this research, we seek to fill this gap by rigorously exploring the cytotoxic action of *Sesamin* on SKOV3 human ovarian cancer cells. Our strategy involves measuring cell viability, determining morphological changes that reflect apoptosis, analyzing cell cycle distribution and apoptosis using flow cytometry, conducting molecular docking to determine possible interactions with major apoptotic regulators, and determining the expression levels of crucial genes and proteins that are part of apoptotic pathways. With this thorough analysis, we aim to clarify the molecular mechanisms of the anti-cancer effects of *Sesamin* in ovarian cancer cells in order to add to the evolution of

new treatment approaches for this aggressive disease. This multidisciplinary perspective yields a better understanding of the therapeutic usefulness of *Sesamin* in the management of ovarian cancer.

2. Materials and Methods

2.1 Molecular Docking

The process for docking molecules with Auto Dock Tools involves several steps; the AutoGrid method creates three-dimensional grid boxes in order to determine the binding energies to the coordinates of macromolecules. AutoGrid can create grid maps that represent the whole ligand at the target site of the actual docking. The entire ligand is placed into the binding pocket, which is then fully enclosed in cubic grids. The graphical user interface of Auto Dock (version 4.2.6), supplied by MGL Tools, is used to establish the AutoDock atom types. Within Auto Dock, the Lamarckian genetic algorithm was used it is one of the most successful free docking procedures available. Auto Dock then calculates the binding free energy and the best fit of a ligand conformation that binds in a macromolecular structure. BIOVIA Discovery Studio was used to profile the binding pocket[15]. It can be valuable for understanding the essence of binding and determining more efficacious drug options.

2.2 SKOV3 Cell Culture and Compound

Human Ovarian cancer cells (SKOV3) was obtained from NCCS, Pune, India, and was cultured in a T-75 culture flask using Ham's F-12 medium containing 10% FBS. The cells were maintained at 37° C in a humidified atmosphere of 5% CO_2 and 95% air. The cells were trypsinised and sub-cultured for additional studies when they reached 80% confluence. Sesamin (≥98% purity, CAS number: 607-80-7), purchased from Simson pharma limited, was utilized for the following experiments.

2.3 MTT Assay

Cytotoxic activity of *Sesamin* on SKOV3 cells, which are human ovarian cells, by using the MTT assay method of Mosmann, with minor modification[16]. We began by plating SKOV3 cells into 96-well plates at a cell density of 1×104 cells per well and left them to incubate overnight at 37° C with 5% CO_2 in a humidified atmosphere. After that, we treated the cells with different concentrations of *Sesamin*, ranging from 10 to 100 µM, and let them sit for 24 and 48 hours. Once the treatment was done, we added 20 µL of MTT solution (5 mg/mL in PBS) to each well and let it incubate for another 4 hours. The resultant formazan crystals were then dissolved in 100 µL of DMSO, and we performed the absorbance at 570 nm using a microplate reader. Then computed the percentage of viable cells versus the untreated control group.

$$Viable\ cells(\%) = \frac{OD\ of\ Sesa\min\ treated\ cells}{OD\ of\ control\ sample} \times 100$$

2.4 Nuclear and Cellular Staining

2.4.1 DAPI Staining (Nuclear Morphology Assessment)

SKOV3 cells (2×10^5 cells/well) were seeded into 6-well plates and exposed to *Sesamin* at its IC_{50} concentration of 48 µM for 48 hours. After treatment, the cells were fixed with 4% paraformaldehyde for 15 minutes and then washed in PBS. The fixed cells were treated with DAPI (1 µg/mL) for 10 minutes under room temperature in darkness. Following washing, we observed the nuclear morphology under a fluorescence microscope with an excitation of 358 nm and emission of 461 nm. Apoptotic cells had condensed and fragmented nuclei, whereas the control cells had a uniform nuclear staining pattern[17].

2.4.2 DCFH-DA Staining (ROS Detection)

To assess the production of intracellular Reactive Oxygen Species (ROS), SKOV3 cells were incubated in 10 µM DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) for 30 minutes at 37° C following exposure to *Sesamin*. After completing the staining, the cells were washed with PBS and viewed under a fluorescence microscope (excitation wavelength of 488 nm and emission at 525 nm). The observation of intensified green fluorescence indicated an increase in ROS production by treated cells compared to the control group[18].

2.4.3 AO/EtBr Dual Staining (Apoptosis Detection)

We stained SKOV3 cells using a blend of Acridine Orange (AO) and Ethidium Bromide (EtBr) After 48 hours of treatment with *Sesamin*, at a concentration 100 µg/mL for 5 minutes in the dark. We then examined

the cells under the fluorescence microscope. The live cells emitted green fluorescence, meaning that they were AO permeable, while the early apoptotic cells also emitted green but in addition to condensed chromatin. However, the late apoptotic or necrotic cells acquired orange to red colour due to uptake of EtBr[19].

2.5 Cell Cycle Analysis

Fluorescence-Activated Cell Sorting (FACS) was utilized to quantify the DNA content of PI-stained cells in accordance with published protocols to assess the effect of *Sesamin* on the cell cycle[20]. SKOV3 cells were grown in 25 cm^2 flasks and treated with 43 µM/L of *Sesamin* for 48 hours. After treatment, the cells were spun at 4° C for 5 minutes at 2,000 rpm, washed twice with cold phosphate-buffered saline, and fixed for one hour at -20° C in 70% ethanol. After fixation, they went through a short NP-40 incubation, PBS wash, and a 20-minute ice staining with PI. Cell cycle distribution across phases was ascertained through FACS callibur using Beckman coulter, US.

2.6 Cell Death Analysis by Flow Cytometry

SKOV3 ovarian cancer cells were cultured in MEM medium supplemented with 10% FBS, 1X NEAA, and antibiotics, at 37° C and 5% CO_2. Cells (0.1×10^6/mL) were seeded in 24-well plates, and exposed to *Sesamin* for 48h. After treatment, cells were harvested and washed using PBS, and stained using the FITC Annexin V/Dead Cell Apoptosis Kit (Invitrogen), according to the manufacturer's guidelines. Briefly, cells were harvested, resuspended in 1X annexin-binding buffer, and incubated with FITC Annexin V and PI mixture. Following a 15-minute room temperature incubation, samples were subjected to flow cytometric analysis (FL1 for FITC, FL3 for PI)[21]. Results are presented from 3 independent experiments compared by one-way ANOVA (*$P < 0.05$ was 32 significant) Invitrogen, Thermo Fisher Scientific, USA.

2.7 Gene Expression

Gene expression analysis usually includes the quantitation of RNA transcripts (mRNA) and frequently the verification of protein expression. The protocol starts with RNA extraction (with or without phenol-chloroform or column-based kits) and then reverse transcription to cDNA. Transcript levels are measured using quantitative PCR (qPCR) or Reverse Transcription PCR (RT-PCR) normalized against housekeeping genes like β-actin or GAPDH. Primer design, annealing temperature optimization, and reaction efficiency verification are part of method optimization. Protein-level verification through Western blotting augments transcript analysis, enabling correlation between transcriptional and translational modifications[22].

2.8 Protein Expression by Western Blot

In brief, 5 × 106 SKOV3 cells were seeded in a 100 mm culture plate. After treatment with *Sesamin*e for 48 h, the cells were trypsinised and proteins were harvested in RIPA buffer (#Cat no. R0278, Sigma). The total content of protein was measured by Lowry's method. The proteins were then transferred onto a PVDF membrane following separation by SDS-PAGE. Following membrane blocking with 2% blocking solution for an hour, the primary antibodies (anti-ABCE1 antibody diluted at 1:1000) were added and incubated overnight. Following washing of the membrane in TBS-T and TBS, the membrane was probed with Horseradish Peroxidase (HRP)-coupled secondary antibody (diluted at 1:5000) for 1 h. After another round of washing, Enhanced Chemiluminescence Substrate (ECL) was added. Protein band intensity was measured by the Quantity One image analysis system (Bio-Rad, USA), with β-actin used as an invariant control in this work. RNA was isolated using the Takara RNA isolation kit after an overnight incubation by the methods described. The amount of extracted RNA was measured in units of micrograms (µg) by spectrometric measurement. An activation mixture was carefully generated using Takara SyBr green master mix and custom-designed forward and reverse primers specific for the gene ABCE1 to examine mRNA expression *via* RT-PCR. The thermal cycling scheme included a pre-activation step of 5 minutes at 95° C, followed by 40 rounds of a two-step cycle process. This included denaturation at 95° C for 5 seconds and combined annealing extension for 10s at the appropriate primer temperature ranging from 55-62.5° C. For Real-Time PCR, a reaction mixture was set using Takara SyBr green master mix, in addition to specifically designed forward and reverse primers aimed at these genes.

2.8 Statistical Analysis

For the extensive analysis of data in this study, the statistical programming language R-known to be highly capable of statistical computing was used. The log-rank test was utilized with utmost caution using GraphPad prism 8 to obtain suitable p-values for determining the significance of mRNA and protein expressions. All cell experiments on drug treatment used at least three independent biological replicates. To analyze overall survival patterns, Kaplan-Meier plots were also carefully drawn. *p < 0.05, the various significance levels were employed to determine the statistical significance between the groups.

3. Results

3.1 MTT Assay

The present study investigated the cytotoxic effects of *Sesamin* on SKOV3 human ovarian cancer cells using MTT assay and morphological analysis. The MTT assay results demonstrated a significant dose- and time-dependent reduction in cell viability upon *Sesamin* treatment for 24 and 48 hours (Figure 1A). Notably, at the IC50 concentration (75 µM) for 48 hours, cell viability was substantially decreased (Figure 1B), suggesting potent anti-proliferative activity. This was further corroborated by morphological analysis, where *Sesamin*-treated cells exhibited significant alterations, including reduced cell density, rounding, and detachment, indicative of cellular stress and apoptosis. The observed cytotoxicity of *Sesamin* could be attributed to its ability to interfere with key survival pathways, potentially inducing apoptosis and inhibiting proliferation in SKOV3 cells. Statistical analysis confirmed that the reduction in cell viability was significant ($p < 0.05$), highlighting the reproducibility of the results. These findings highlight the potential of *Sesamin* as an anti-cancer agent against SKOV3 cells.

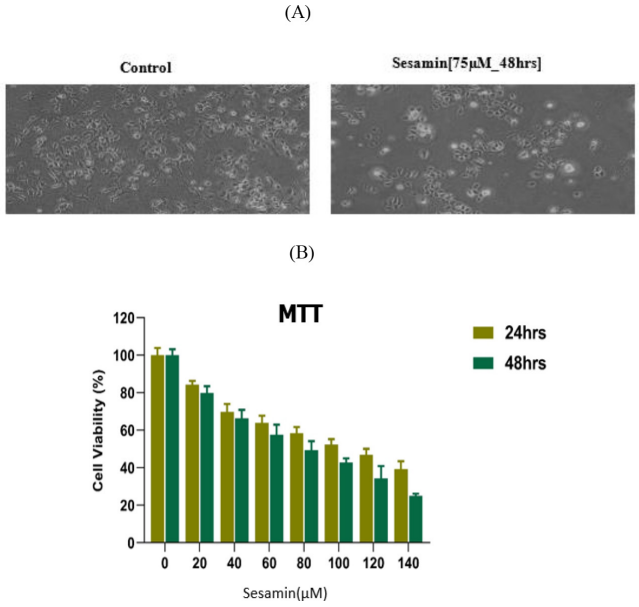


Figure 1 **(A).** Evaluating the impact of *Sesamin* on the viability of SKOV3 cells Human ovarian cancer cells: SKOV3 cells cultured and supplemented with 10% FBS with indicated different concentration of *Sesamin* for 48hrs. **(B).** Cell viability assay, the cells were treated with 75µM concentration of *Sesamin* for 48 hrs. *Sesamin* inhibited growth (as determined by MTT assay) of human AGS cells. Cell morphology for IC50 Concentration (75 µM) of *Sesamin* representative figures have been indicated. Every bar represents the mean ± SEM of six 3 independent observations. Statistical significance of control versus *Sesamin* treatment groups at p <0.05 level by Student's–Newman–Keul's test.

3.2 Immunofluorescence Assay

The fluorescence microscopy images illustrate the apoptotic effects of *Sesamin* on SKOV3 ovarian cancer

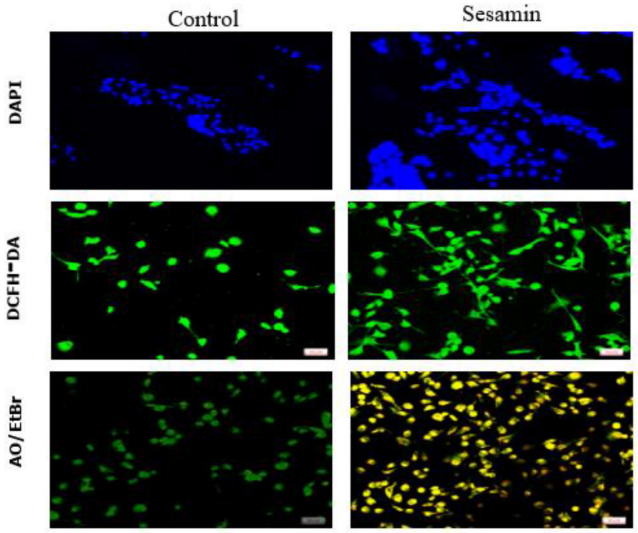


Figure 2. Effect of Sesamin on apoptosis in SKOV3 cells. The SKOV3 cells were treated with Sesamin for 48 hours and observed the morphological changes using immunofluorescence staining. DAPI for nuclear staining, DCFH-DA for intracellular ROS activity, and AO/EtBr staining for early and late apoptosis in AGS cells at 20X magnification. Images were observed by Olympus with zenoptik camera and measured using ImageJ software.

cells. Nuclear staining (DAPI) reveals chromatin condensation and fragmentation, characteristic of apoptosis. AO/EB dual staining further confirms apoptotic progression, with early apoptotic cells displaying green fluorescence and late apoptotic/ necrotic cells emitting orange/red fluorescence (Figure 2). Compared to the control, *Sesamin*-treated cells show significant morphological alterations, including cell shrinkage and membrane blebbing, indicative of programmed cell death. These findings align with the MTT assay results, reinforcing the potential of *Sesamin* as an anti-cancer agent. Further molecular studies are warranted to elucidate the precise apoptotic pathways involved.

3.3 Cell Cycle Analysis by Flow Cytometry

Flow cytometry analysis was performed to evaluate the effects of *Sesamin* on the cell cycle distribution of SKOV3 human ovarian cancer cells. The control group (right panel) exhibited a high proportion of cells in the G0/G1 phase (82.00%), with lower percentages in the S phase (11.55%) and G2/M phase (6.46%). However, upon treatment with *Sesamin* (left panel), a significant reduction in the G0/G1 phase population (58.77%) was observed, accompanied by an increase in the S phase (19.73%) and G2/M phase (21.01%) (Figure 3). This redistribution suggests that *Sesamin* induces cell cycle arrest at the G2/M phase, thereby preventing cellular proliferation and promoting apoptosis. The increase in S phase cells further implies impaired DNA synthesis or replication stress, reinforcing *Sesamin*'s role as an effective anti-cancer agent. These findings indicate that *Sesamin* may exert its cytotoxic effects through disruption of cell cycle progression, making it a promising therapeutic candidate for ovarian cancer treatment.

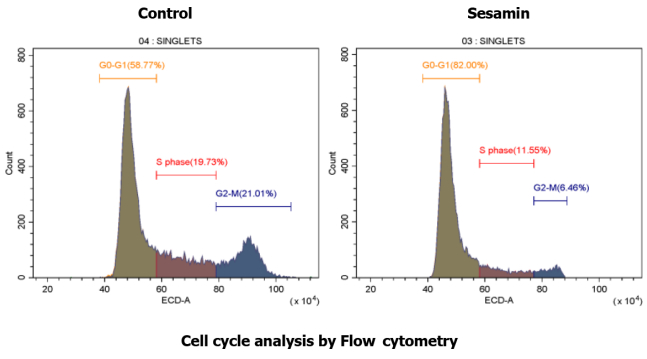


Figure 3. Sesamin effects on cell cycle arrest in human SKOV3 cells: SKOV3 treated with Sesamin were subjected to cell cycle distribution analysis.

3.4 Cell Death Analysis by Flow Cytometry

The presented results demonstrate the apoptotic effect of *Sesamin* on SKOV3 ovarian cancer cells. The flow cytometry analysis using Annexin V-FITC staining reveals a significant shift in cell populations following *Sesamin* treatment. In the control group (left panel), the majority of cells (99.65%) are in the Lower-Left quadrant (LL), indicating a predominantly viable cell population. However, after *Sesamin* treatment (right panel), there is a substantial increase in early apoptotic (UL: 10.22%), late apoptotic (UR: 3.42%), and necrotic (LR: 5.20%) cell populations, confirming the cytotoxic effect of *Sesamin* (Figure 4). The fluorescence microscopy images further support this finding, showing increased nuclear fragmentation and apoptotic bodies. These findings suggest that *Sesamin* effectively induces apoptosis in SKOV3 cells, making it a promising candidate for ovarian cancer therapy. Statistical validation using the t-test confirms the significance of these changes.

3.5 Molecular Docking Analysis

Molecular docking simulations were done to analyze the binding interactions of *Sesamin* with major apoptotic regulators, such as Caspase-3, Caspase-9, BAX, BCL-2, P53 and BCL-xL (Figure 5). The docking results showed favourable binding affinities, especially with Caspase-9

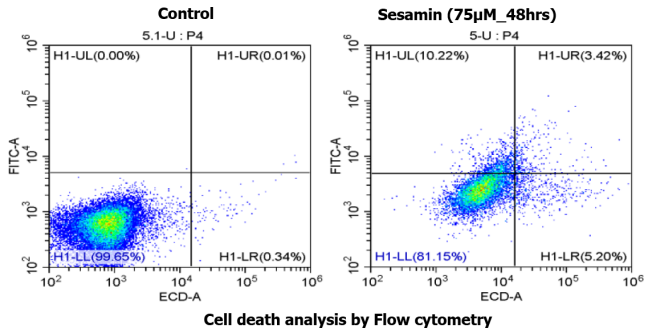


Figure 4. Sesamin causes cell death in SKOV3 cells. SKOV3 cells were treated with sesamin and their cell cycle distribution and apoptosis were determined by Annexin - FITC using flow cytometry. (LL-Live cells; UL-early apoptosis; UR-Late apoptosis; LR-Necrosis). Statistical significance was tested using the t-test.

(-8.34 kcal/mol), Caspase-3 (-7.62 kcal/mol), and P53 (-7.02 kcal/mol) (Table 1), suggesting that *Sesamin* could directly contribute to the activation of apoptotic pathways. Interestingly, *Sesamin* established hydrogen bonding interactions with GLN-381 of Caspase-9, indicating its potential in the induction of the intrinsic apoptotic pathway. Additionally, *Sesamin* established interactions with BCL-2 (-7.59 kcal/mol) and BCL-xL (-6.04 kcal/mol), indicating its inhibitory action on anti-apoptotic proteins, hence promoting apoptosis.

3.6 Gene Expression

3.6.1 Bcl-xL mRNA Expression

The effect of *Sesamin* (75 µM, 48 hours) on SKOV3 ovarian cancer cell mRNA expression of Bcl-xL, an anti-apoptotic gene, was evaluated by real-time PCR (qPCR) with SYBR green dye. Amplification curve: The plot for amplification shows successful and exponential amplification of the target gene, verifying successful qPCR performance. Melt curve analysis: One sharp peak in the melt curve establishes the specificity of the

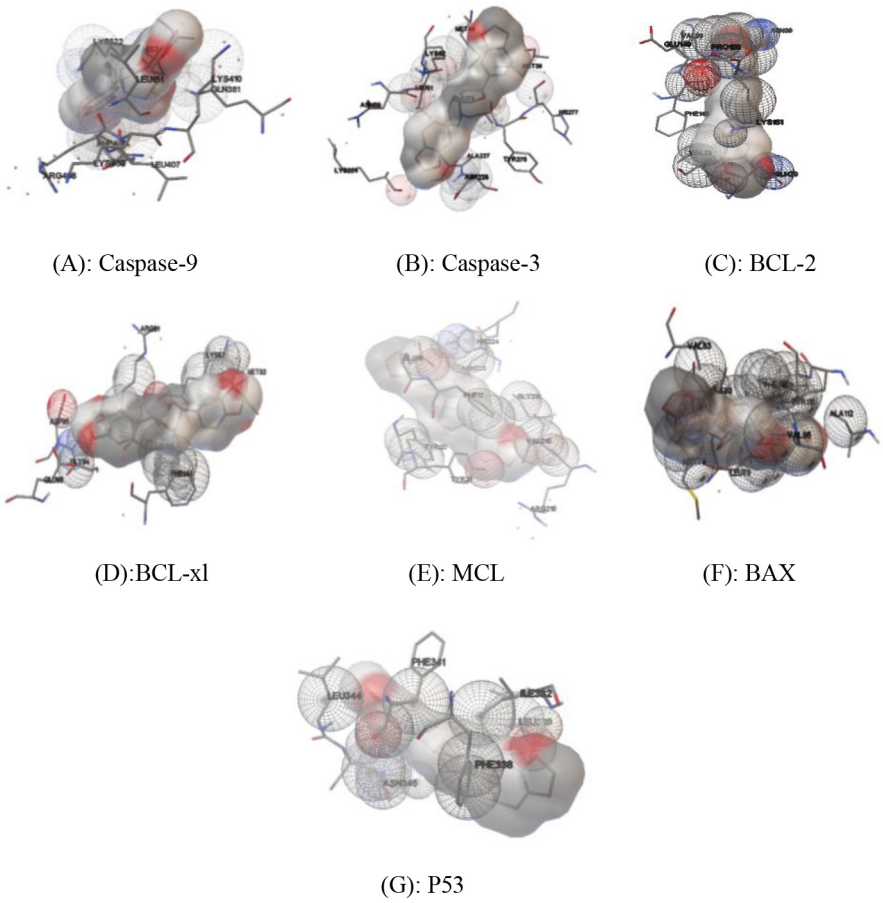
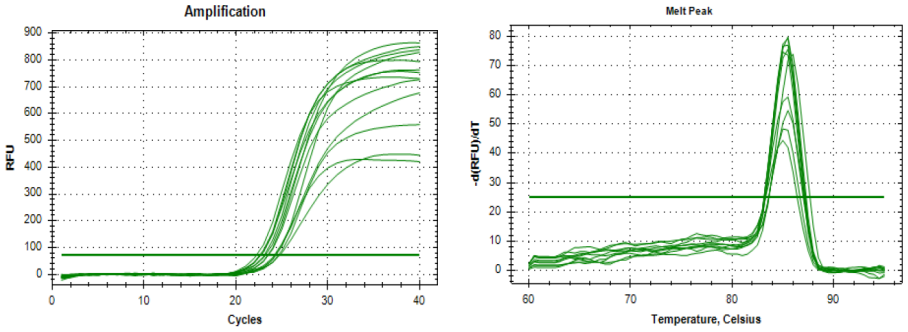


Figure 5. Molecular docking study showing the binding interactions of sesamin with major apoptotic and anti-apoptotic proteins. (A) Docking pose of sesamin in the active site of caspase-9, showing hydrogen bonds, hydrophobic contacts, and van der Waals interactions with catalytic residues important in apoptotic signaling. (B) Sesamin interaction profile with caspase-3, demonstrating stabilization by hydrogen bonding and π-π stacking with residues critical to executioner caspase function. (C) Sesamin binding conformation within active pocket of Bcl-2, with interactions that might interfere with anti-apoptotic function and induce apoptosis. (D) Docking visualization of sesamin complexed with Bcl-xL, exposing important interactions with residues that cause inhibition of pro-apoptotic signaling pathways. (E) Sesamin's molecular binding with Mcl-1, highlighting hydrogen bonding and hydrophobic interactions potentially to destabilize its anti-apoptotic shield. (F) Model of docking between sesamin and Bax, demonstrating stabilization in pro-apoptotic domains, hinting at mitochondrial apoptosis activation. (G) Mapping interaction of sesamin with the tumor suppressor protein p53, pointing to potential stabilization of p53's DNA-binding domain and its modulation of transcriptional activity towards inducing apoptosis.

Table 1. The target molecule and compound binding energies and amino acid interaction

TARGET MOLECULES	COMPOUND	BINDING ENERGY	AMINO ACID INTERACTED	BOND FORMED
CASPASE-9	SESAMIN	-8.34	LEU-151, LYS-322, HIS-346, GLN-381, PHE-406, LEU-407, ARG-408, LYS-409, LYS-410	1H-GLN-381
CASPASE-3	SESAMIN	-7.62	MET-39, MET-44, ASN-80, LEU-81, LYS-82, LYS-224, ALA-227, ASP-228, PHE-275, TYR-276, HIS-277	-
BCL-2	SESAMIN	-7.59	GLN-20, VAL-21, VAL-36, ASN-39, PHE-148, GLU-149, PRO-150, LYS151	-
BCL-xL	SESAMIN	-6.04	MET-83, LYS-87, LEU-90, ARG-91, GLY-94, ASP-95, GLU-98, PHE-144	-
MCL-1	SESAMIN	-6.06	GLU-16, PHE-17, TYR-20, TYR-21, ARG-215, VAL-216, GLY-219, ASN-233, HIS-244	-
BAX	SESAMIN	-6.95	LEU-76, MET-79, ILE-80, VAL-83, VAL-95, ALA-112, TYR-115, PHE-116,	-
P53	SESAMIN	-7.02	LEU-330, ILE-332, PHE-338, PHE-341, LEU-344, ASN-345	-



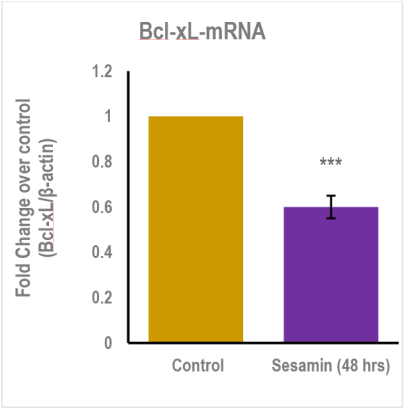


Figure 6. Quantitative real-time PCR (qPCR) analysis of BCL-xL gene expression. Results represent the relative mRNA levels of the anti-apoptotic gene BCL-xL, normalized to the housekeeping gene, to assess its modulation following treatment. A decrease in BCL-xL expression indicates a potential induction of apoptotic pathways.

PCR reaction, excluding non-specific amplification or primer-dimer: mRNA expression analysis. The bar graph shows a significant down regulation of Bcl-xL mRNA expression in the *Sesamin*-treated group when compared to the control (Figure 6). Gene expression was normalized to β-actin, and statistical analysis showed a highly significant reduction ($p < 0.05$). These data indicate that *Sesamin* suppresses Bcl-xL expression,

resulting in a switch in the apoptotic balance toward pro-apoptotic signalling and cell death in SKOV3 ovarian cancer cells. Inhibition of Bcl-xL, a major survival factor, further indicates the pro-apoptotic and anti-cancer activity of *Sesamin* in ovarian cancer treatment.

3.6.2 Bcl-2 mRNA Expression

The impact of *Sesamin* on the mRNA level of Bcl-2, a gene involved in anti-apoptosis, in SKOV3 ovarian cancer cells was examined through real-time PCR. The amplification plot verifies successful amplification of the target gene, and the melt curve analysis demonstrates the specificity of the PCR reaction, with one sharp peak indicating no primer-dimer formation or non-specific amplification. The qPCR quantitative real-time PCR results, shown as a bar graph (Figure 7), show that 48-hour treatment with 75 µM *Sesamin* decreases Bcl-2 mRNA expression significantly compared to the control. The relative expression levels of Bcl-2 were normalized to β-actin as an internal control. The results show a statistically significant reduction ($p < 0.05$) in the expression of Bcl-2 mRNA after treatment with *Sesamin*, indicating that *Sesamin* induces apoptosis through the down regulation of anti-apoptotic signals in SKOV3 cells. This decrease in the expression of Bcl-2 confirms the premise that *Sesamin* induces apoptosis in ovarian cancer cells by tilting the balance in favour of pro-apoptotic pathways. These results are in agreement with past research indicating the promise of *Sesamin* as a cancer agent to induce apoptotic responses in cancer cells.

3.6.3 p53 mRNA Expression

The qRT-PCR melt curve analysis and amplification proved that the p53 gene was successfully and specifically amplified, yielding precise gene expression results. The bar graph expression analysis representation showed that the p53 mRNA levels were highly upregulated after *Sesamin* treatment for 48 hours. The induction of p53 expression ($p < 0.01$) indicates that *Sesamin* is involved in the activation of the tumour suppressor pathway, a major cellular defence mechanism against cancer. The p53 gene is a recognized tumour suppressor protein that controls cell cycle arrest and apoptosis following

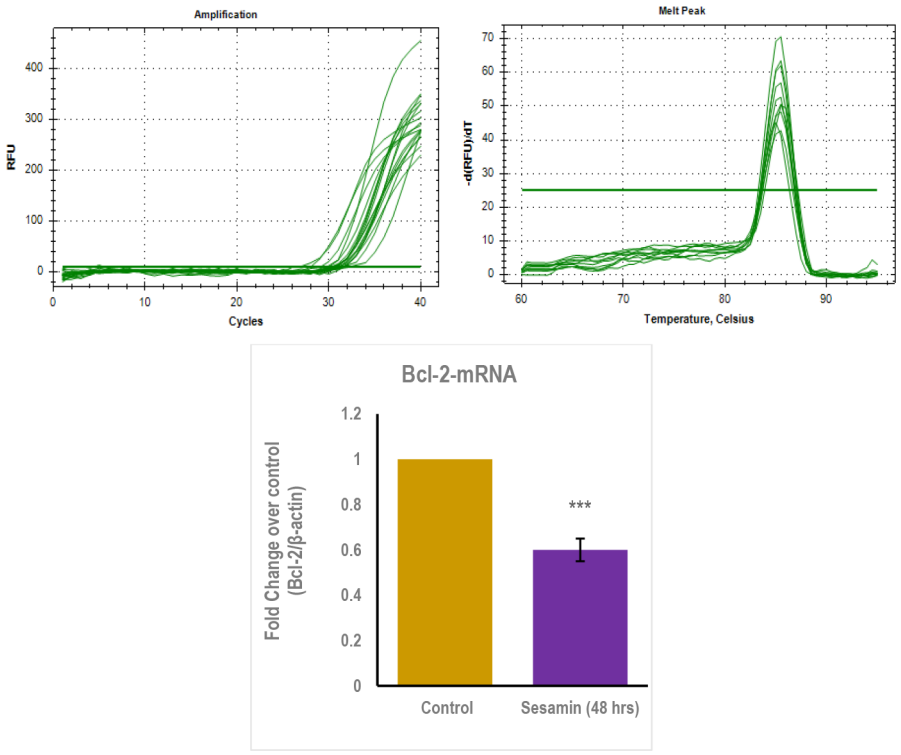


Figure 7. Quantitative real-time PCR (qPCR) analysis of BCL-2 gene expression. The relative expression of the anti-apoptotic BCL-2 gene was evaluated to determine changes in cell survival regulation. Downregulation of BCL-2 suggests enhanced apoptotic susceptibility.

DNA damage and oncogenic stress. The upregulation observed underscores the promise of *Sesamin* in initiating anti-cancer pathways through p53 activation, which might result in cell cycle regulation, enhancement of DNA repair, and induction of apoptosis (Figure 8). These results indicate that *Sesamin* may prove to be an effective natural therapeutic agent in the treatment of cancer by activating the p53-mediated tumour suppression pathway.

3.6.4 CASPASE-9 mRNA Expression

Similarly, the qRT-PCR test confirmed the specific and effective amplification of the Caspase-9 gene, assuring its consistent detection. Bar graph analysis confirmed statistically significant enhancement in Caspase-9 mRNA expression ($p < 0.01$) following 48-hour *Sesamin* treatment, showing a strong activation of apoptotic signalling. Caspase-9 is one of the major initiator caspases of the intrinsic mitochondrial pathway of apoptosis, which plays a central role in programmed cell death. Its up-regulation indicates that *Sesamin* boosts apoptotic signalling, possibly for the clearance of cancer cells through mitochondrial-mediated apoptosis. This induction of Caspase-9 expression is also consistent with the action of *Sesamin* as an apoptosis-inducing agent, thus further solidifying its potential to be an anti-cancer drug (Figure 9). The evidence suggests that *Sesamin* may induce cancer cell death *via* intrinsic apoptotic pathways, and it presents a promising lead for further studies in targeted therapies for cancer.

3.7 Protein Expression

3.7.1 Bax Protein Expression Analysis

Western blotting indicates that *Sesamin* treatment increases protein expression of Bax to a significant level in comparison to the control. Bax (Bcl-2-associated X protein) is an established pro-apoptotic protein of the Bcl-2 family, essential for mitochondria-mediated apoptosis. It promotes Mitochondrial Outer Membrane Permeabilization (MOMP), resulting in cytochrome c release, activation of caspase cascades, and eventual

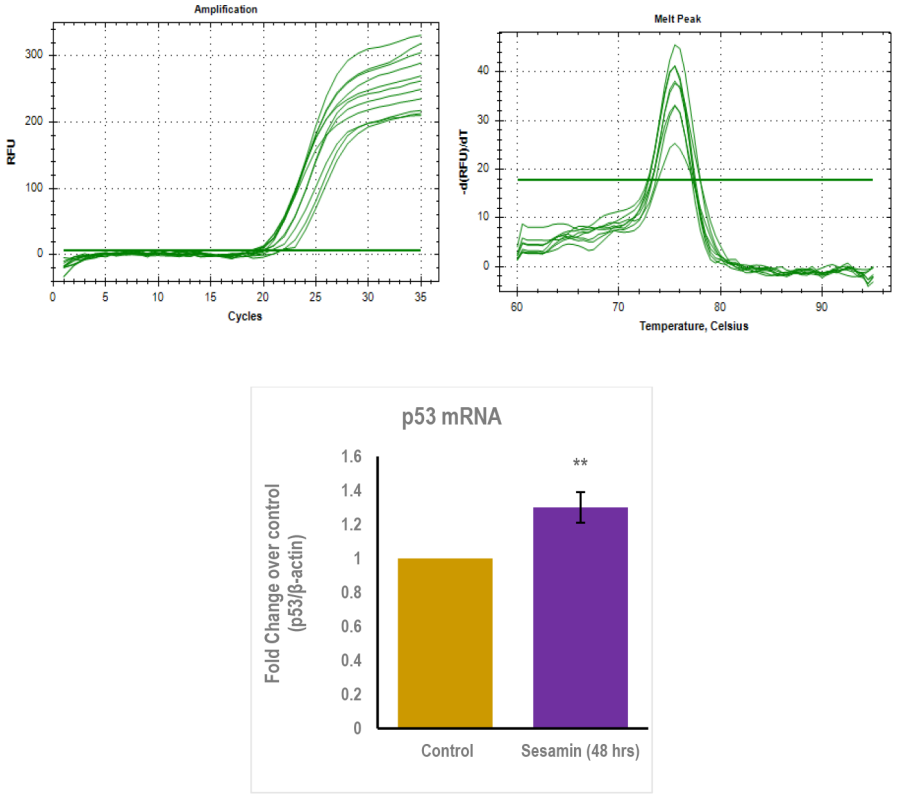


Figure 8. Quantitative real-time PCR (qPCR) analysis of P53 gene expression. The expression levels of the tumor suppress or gene P53 were measured to investigate its activation in response to treatment. Upregulation of P53 expression reflects activation of the intrinsic apoptotic pathway and potential DNA damage response.

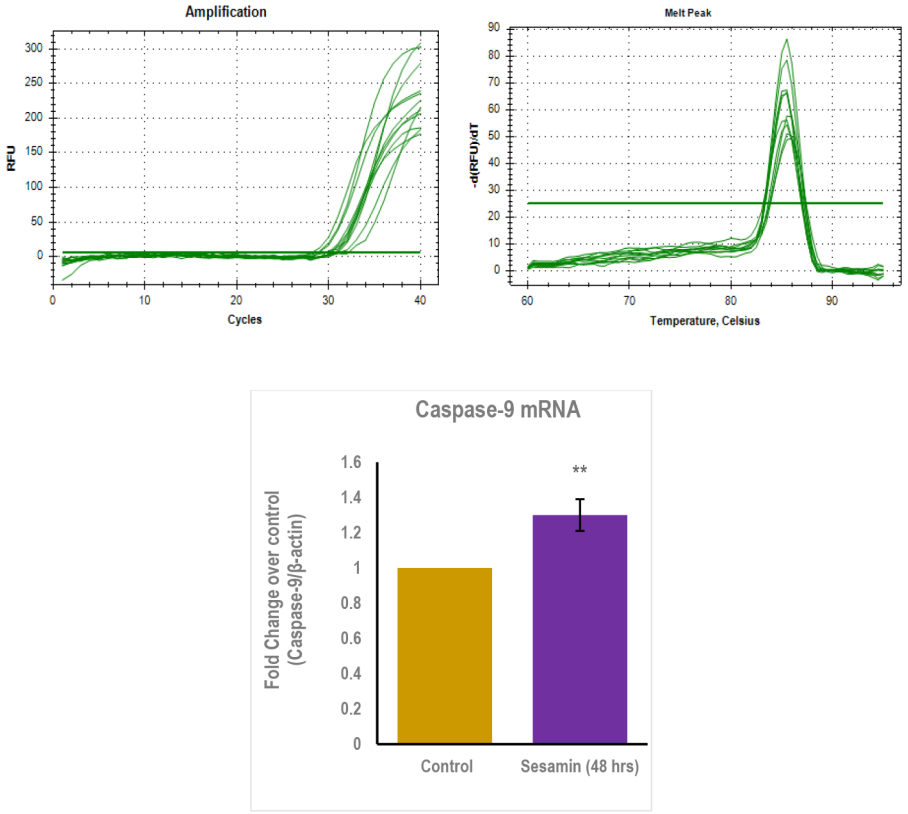


Figure 9. Quantitative real-time PCR (qPCR) analysis of Caspase-9 gene expression. Caspase-9 mRNA levels were quantified to evaluate the initiation of the intrinsic apoptosis cascade. An increase in Caspase-9 expression indicates activation of apoptosis via the mitochondrial pathway.

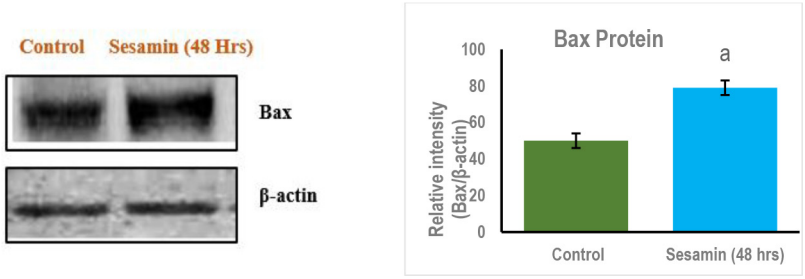


Figure 10. Impact of *Sesamin* on Bax protein expression in human SKOV3 cells: SKOV3 cells were treated with 75μM of *Sesamin* for 48 hrs. Protein expressions were detected by western blotting. Protein levels were analyzed by densitometry and shown as relative intensity. β-Actin was used as an internal control. a- Indicates statistical significance between control vs. *Sesamin* treatment group at $p < 0.05$ level using Student's–Newman–Keul's test.

programmed cell death. The upregulation of Bax after *Sesamin* treatment is highly indicative that *Sesamin* increases apoptosis *via* the intrinsic mitochondrial pathway. This process is crucial in inducing cancer cell killing, as it reverses the action of anti-apoptotic proteins such as Bcl-2, tilting the scale towards cell death instead of survival. Upregulated Bax is a key determinant of mitochondrial-induced apoptosis, ascertaining that *Sesamin* indeed kills cancer cells effectively *via* intrinsic apoptotic pathways. These results are in line with the increasing evidence for *Sesamin*'s anti-cancer potential as a natural compound (Figure 10). Through the augmentation of Bax-mediated apoptosis, *Sesamin* could be used as a therapeutic agent for cancer treatment, especially in cancers where apoptotic resistance is a problem. The findings highlight *Sesamin*'s

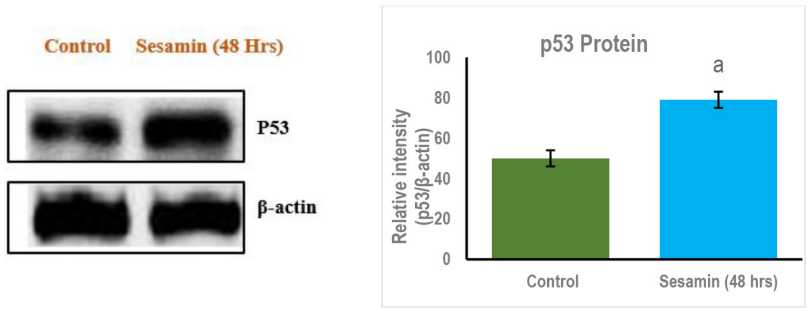


Figure 11. Impact of *Sesamin* on p53 protein expression in human SKOV3 cells: SKOV3 cells were treated with 75μM of *Sesamin* for 48 hrs. Protein expressions were analyzed by western blotting. Protein contents were quantified by densitometry analysis and expressed in relative intensity. β-Actin was considered as internal control. a- Represents statistical difference between control vs *Sesamin* treatment group at $p < 0.05$ level using Student's–Newman–Keul's test.

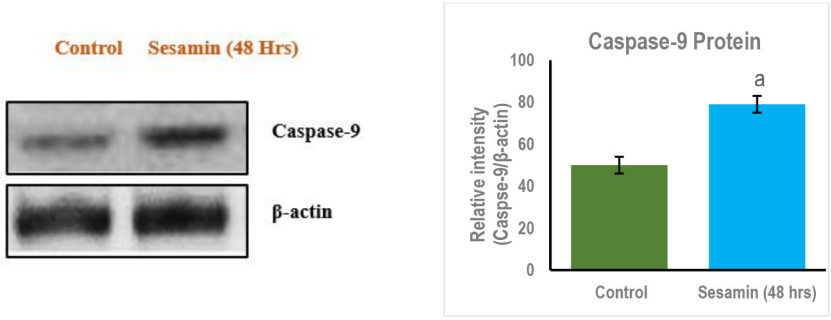


Figure 12. Effect of *Sesamin* on Caspase-9 protein expression in human SKOV3 cells: SKOV3 cells were exposed to 75μM of *Sesamin* for 48 hrs. Protein expressions were examined by western blotting. Protein content was measured by densitometry analysis and presented as relative intensity. β-Actin was taken as internal control. a- Shows statistical significance between control vs. *Sesamin* treatment groups at $p < 0.05$ level by Student's–Newman–Keul's test.

ability to target mitochondrial pathways and thus its potential as a lead compound for future cancer therapy research and clinical development.

3.7.2 p53 Protein Expression Analysis

Western blot analysis verifies increased expression of p53 protein after treatment with *Sesamin* versus control. p53 is an important tumour suppressor protein that maintains cell cycle arrest, DNA repair, and apoptosis due to cellular stress and DNA damage. Increased expression of p53 indicates that *Sesamin* boosts tumour-suppressive mechanisms and can initiate growth inhibition and programmed cell death in cancer cells. Since the activation of p53 is central to its anti-proliferative activity, the enhanced p53 expression following *Sesamin* treatment is evidence that the compound is capable of regulating oncogenic signalling pathways (Figure 11). The discovery enhances the support for the contention that *Sesamin* is a natural cancer agent with potential in anti-cancer therapeutics via induction of p53-dependent tumour suppression.

3.7.3 Caspase-9 Protein Expression Analysis

The Western blot also shows the dramatic upregulation of Caspase-9 protein expression in reaction to *Sesamin* treatment. Caspase-9 is an initiator of the intrinsic mitochondrial apoptotic pathway and is central to programmed cell death. Its upregulated expression indicates that *Sesamin* induces apoptosis by initiating intrinsic apoptotic signalling cascades, which may result in cancer cell destruction (Figure 12). The induction of Caspase-9 generally leads to caspase-3 induction, finally inducing apoptosis and inhibiting tumour development. The results suggest that *Sesamin*- induced apoptosis is probably attributed to mitochondrial-dependent pathways, further validating its potential as a pro-apoptotic and anti-cancer agent. By inducing the expression of Caspase-9, *Sesamin* could sensitize cancer cells to

apoptosis, rendering it a useful natural compound for targeted cancer therapy.

4. Discussion

The present study provides compelling evidence of the anti-cancer activity of *Sesamin* in SKOV3 human ovarian cancer cells. The MTT assay data indicated a marked inhibition of cell viability in a time- and dose-dependent fashion, indicating that *Sesamin* can suppress the growth of SKOV3 cells effectively. Our result is consistent with earlier reports of the anti-proliferative activity of *Sesamin* in different cancer cell lines, such as breast and colorectal cancers[23,24]. The cytotoxicity was also supported by morphological changes like cell shrinkage, membrane blebbing, and chromatin condensation, which are characteristic of apoptosis. The AO/EB dual staining immunofluorescence assay validated apoptotic progression in *Sesamin*-treated cells. Presence of early and late apoptotic cells, as seen by green and orange/red fluorescence, respectively, confirms the assumption that *Sesamin* causes programmed cell death. The findings of our study agree with previous study, who proved *Sesamin* induces apoptosis by oxidative stress-mediated pathways in hepatocellular carcinoma cells[25]. Flow-cytometry analysis also disclosed the mechanism behind *Sesamin* cytotoxicity by showing cell cycle arrest at the G2/M phase. The cell accumulation in the G2/M phase indicates that *Sesamin* inhibits mitotic progression, a proven approach to stopping cancer cell growth[26]. The rise in the S phase population also indicates that *Sesamin* can cause replication stress, which may lead to apoptosis induction. Comparable results were also observed, who showed that *Sesamin* modulates the major regulators of the cell cycle, resulting in inhibition of growth in lung cancer cells[27]. The results of Annexin V-FITC staining from flow cytometry also supported the apoptotic character of *Sesamin*-induced cell death, as indicated by the dramatic increase in early and late apoptotic cell populations. Our results are consistent with earlier research showing that *Sesamin* induces apoptosis *via* intrinsic and extrinsic pathways in various models of cancer[28]. Molecular docking analysis offered further insights into the molecular basis of *Sesamin*'s pro-apoptotic activities. *Sesamin* showed high binding affinities with the crucial apoptotic regulators Caspase-9, Caspase-3, and p53, indicating its possible involvement in initiating apoptotic pathways. Notably, the interaction of *Sesamin* with Caspase-9 *via* hydrogen bonding with GLN-381 validates its potential to trigger the intrinsic apoptotic pathway. Earlier computational research has also shown the binding tendency of *Sesamin* towards apoptotic and anti-apoptotic proteins in favour of sustaining its role as a cell death modulator[29]. Gene expression analysis *via* qPCR also confirmed *Sesamin*'s role in apoptosis regulation. The pronounced downregulation of anti-apoptotic genes Bcl-xL and Bcl-2 indicates that *Sesamin* tips the balance towards pro-apoptotic signalling, resulting in cell death. Our finding is in agreement with the earlier reports showing that natural phytochemicals like *Sesamin* are capable of potently suppressing Bcl-2 family proteins, hence inducing mitochondrial-mediated apoptosis[30]. In addition, the increase in p53 mRNA levels following *Sesamin* treatment suggests that *Sesamin* is capable of triggering tumour-suppressive pathways, an important factor in determining its potential as an anti-cancer drug. The increase in Caspase-9 mRNA expression further attests to the activation of the intrinsic apoptotic pathway, affirming *Sesamin*'s involvement in mitochondrial-mediated apoptosis. Upregulation of pro-apoptotic proteins, Bax, p53, and Caspase-9, in *Sesamin*-treated cells was confirmed by Western blot analysis. The induction of Bax expression is important, as this allows Mitochondrial Outer Membrane Permeabilization (MOMP) to occur, resulting in the release of cytochrome c and the activation of caspase cascade. Our findings support observations, which stated that apoptosis induced by *Sesamin* in colorectal cancer cells is conducted through the intrinsic mitochondrial pathway[31]. Furthermore, the enhanced p53 protein expression also emphasizes the tumour-suppressive activity of *Sesamin* in accordance findings in breast cancer models. Our research yields strong evidence that *Sesamin* has powerful anti-cancer properties in SKOV3 ovarian cancer cells through several mechanisms, involving cell cycle arrest, induction of apoptosis, and regulation of major apoptotic regulators. The remarkable downregulation of anti-apoptotic proteins and upregulation of pro-apoptotic markers reinforce *Sesamin*'s value as a natural drug candidate for the treatment of ovarian cancer. Moreover, the identification of the specific molecular

mechanisms responsible for the apoptotic and anti-proliferative activities of *Sesamin* will increase our knowledge of its therapeutic value for targeted cancer therapy.

5. Conclusion

Our study demonstrates that *Sesamin* has a powerful anti-cancer activity in SKOV3 ovarian cancer cells through induction of apoptosis, suppression of cell growth, and interference in vital survival networks. The combination of molecular docking, gene profiling, and protein expression offers an insights into the apoptotic mechanism of *Sesamin* by activation of caspases and modulation of mitochondrial pathway. In comparison with the previous research, our results provide additional knowledge of the cytotoxic activity of *Sesamin* and demonstrate its potential as a targeted therapy.

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7. Author Contribution

Performed experiments, data collection, and manuscript drafting- Heera Maheswari Jayaveeran; Conceptualization, supervision, and critical revision of the manuscript - Ponnulakshmi Rajagopal; Assisted in experimental design, methodology, and data validation - Manju Parthiban; Conducted bioinformatics, data interpretation, statistical analysis and critical revision of the manuscript- Selvaraj Jayaraman; Contributed to literature review - V. Sureka Varalakshmi and Krithika Chandrasekaran. All authors have read and agreed to the published version of the manuscript.

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