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ORIGINAL RESEARCH

Amentoflavone Impedes NF-κB Activation and Mediates Apoptosis Induction in Lung Cancer Cells: An in vitro and in silico exploration

Syed Shah Mohammed Faiyaz[1,2,*], Afza Ahmad[3,*], Daniya Fatima[4], Syed Monowar Alam Shahid[5], Gaurav Kaushik[6], Chaitenya Verma[7], Rohit Kumar Tiwari[8], Vinay Kumar[9]

[1]Department of Physiology, College of Medicine, University of Ha'il, Ha'il, 81451, Saudi Arabia; [2]Department of Physiology, Radha Devi Jageshwari Memorial Medical College & Hospital, Muzzafarpur, Bihar- 844127, India; [3]Department of Public Health, Dr. Giri Lal Gupta Institute of Public Health and Public Affairs, University of Lucknow, Lucknow, Uttar Pradesh, 226031, India; [4]Department of Public Health, College of Public Health and Health Informatics, University of Ha'il, Ha'il, 81451, Saudi Arabia; [5]Department of Biochemistry, College of Medicine, University of Ha'il, Ha'il, 81451, Saudi Arabia; [6]Sharda School of Allied Health Sciences, Sharda University, Uttar Pradesh, 201310, India; [7]Department of Biotechnology, Sharda University, Uttar Pradesh, 201310, India; [8]Department of Clinical Research, Sharda School of Allied Health Sciences, Sharda University, Uttar Pradesh, 201310, India; [9]College of Medicine, Pennsylvania State University Hershey Medical Center, Hershey, PA, 17033, USA

*These authors contributed equally to this work

Correspondence: Rohit Kumar Tiwari, Department of Clinical Research, Sharda School of Allied Health Sciences, Sharda University, Gautam Buddha Nagar, Uttar Pradesh, 201310, India, Email rohit.tiwari1@sharda.ac.in; Vinay Kumar, College of Medicine, Pennsylvania State University Hershey Medical Center, Hershey, PA, 17033, USA, Email vinayktyagi07@gmail.com

Context: Lung carcinoma is a major contributor to cancer incidence and mortality worldwide. Chronic activation of NF-κB triggers activation of its target genes involved in promoting malignancy, metastasis, irregular proliferation of cells, and/or their resistance to programmed cell death. Amentoflavone (AMF) is a biflavonoid with intrinsic ability to modulate key signaling pathways associated with homeostatic and non-homeostatic conditions impels its exploration as therapeutic candidate against non-small cell lung carcinoma (NSCLC) A549 cells.

Objective: This study investigates the anticancer potential of AMF in A549 cells, focusing on its unique dual role in NF-κB suppression and apoptosis induction, and compares its efficacy to the standard drug doxorubicin.

Materials and Methods: A549 cells were treated with varying concentrations of AMF for 24 h. The effects of AMF on cell proliferation, oxidative stress, mitochondrial membrane potential, caspase activation, apoptosis, and NF-κB activation was analyzed.

Results: A549 cell viability was substantially reduced ($P < 0.001$) at an AMF concentration of 60 µM. AMF exposure further increased nuclear fragmentation and condensation in A549 cells. AMF treatment induced apoptosis with concomitant augmentation intracellular production of reactive oxygen species (ROS), dissipation of mitochondrial membrane potential, and activation of the caspase cascade. Additionally, AMF mediated the inhibition of NF-κB and modulated the expression of NF-κB-associated genes involved in cell survival (Bcl-XL, Bcl-2, and survivin) and proliferation (cyclinD1). These results were further supported by in silico studies, which demonstrated a considerable binding energy score of AMF with NF-κB p65/50 compared with the standard drug (doxorubicin).

Conclusion: Thus, it was concluded that AMF exerts potent anticancer effects in NSCLC A549 cells through dual mechanism such as direct inhibition of NF-κB signaling and apoptosis induction combined with its high binding affinity, positions it as a promising therapeutic candidate for NSCLC. Further preclinical studies are warranted to validate these findings.

Keywords: anticancer, natural product, anti-inflammatory, lung cancer, apoptosis, caspases

Introduction

Lung cancer is a major public health concern in developing and underdeveloped countries. Globally, lung cancer ranks second in terms of mortality and first in terms of morbidity. Approximately 85% of all the lung cancer cases are classified as non-small-cell lung cancer (NSCLC).[1] Most patients are diagnosed with progressive stages of lung

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cancer. In case of NSCLC, the 5-year relative rate of survival among patients diagnosed with metastatic lung cancer is 6%.[2] Numerous strategies have been explored to alleviate the clinical management of lung cancer by investigating various novel pharmacological agents to overcome drug resistance, lack selectivity, and elevated therapeutic costs of therapeutics.[3] Thus, the development and formulation of an efficacious modality to overcome these challenges are prerequisites for lung cancer therapy. The use of natural compounds for the prevention and management of diverse cancer cell types has garnered considerable interest from both the pharmaceutical industry and the scientific community in recent decades owing to their intrinsically lower toxicity against normal cells.[4]

Apoptosis is an essential physiological phenomenon that facilitates homeostatic regulation and preservation of cellular populations within various tissues.[5] Owing to the significant relationship between the apoptotic mechanism and the efficacy of anticancer therapeutics, considerable scientific attention has been devoted to this mode of cellular death.[6] Augmentation of reactive oxygen species (ROS) within cancerous cells is a pivotal determinant of the initiation of apoptosis induced by phytochemical agents, as it mediates oxidative DNA damage, resulting in the dissipation of mitochondrial membrane potential ($\Delta\Psi m$) and efflux of cytochrome c, which ultimately triggers the activation of the caspase cascade.[7,8] Moreover, alterations in the expression levels of Bax and Bcl-2 proteins serve as critical indicators for assessing the sensitivity of tumor cells to anticancer treatments.[9,10]

Amentoflavone (AMF) is a naturally occurring biflavonoid compound characterized by a range of biological properties, including anti-inflammatory and antioxidative.[11,12] AMF is a member of biflavonoid family of bioactive natural compound and is thus characterized by the presence of two distinct flavonoid units known as apigenin, which are linked via C3′–C8″ bond. This peculiar structure imparts various pharmacological attributes to AMF in comparison with members of flavonoid group.[13] Notably, amentoflavone participates in anticancer mechanisms by modulating various signaling pathways, including extracellular signal-regulated kinase, NF-κB, and phosphoinositide 3-kinase (PI3K)/protein kinase B. In light of previous evidence, it can be considered that AMF possesses the intrinsic potential to exert their therapeutic effects by modulating crosstalk between different signaling molecules.[10,14] Hence, amentoflavone is viewed as a prospective therapeutic agent that requires further in-depth investigation in the field of clinical research.[15,16]

Previous evidence suggests that the NF-κB signaling pathway predominantly participates in the progression of lung tumorigenesis.[17] The NF-κB p50/p65 heterodimer is an oncogenic transcription factor that modulates the expression of proteins (JNKs and Bcl3) associated with tumor progression.[18,19] Increased activation of NF-κB, identified as an unfavorable prognostic indicator, has been demonstrated to correlate with tumor stage, lymph node metastasis, and the 5-year overall survival rates among patients diagnosed with NSCLC.[16] Moreover, it has been established that NF-κB activation plays a pivotal role in mediating acquired resistance to therapeutic interventions in lung cancer.[20,21] Consequently, the development of NF-κB signaling inhibitors may represent a promising avenue for adjuvant therapy in the treatment of NSCLC.[22] In this study, we hypothesize that AMF could reduce cell viability and induce anticancer effects in NSCLC A549 cells by modulating the expression of NF-κB and its key target genes. In the present study, we hypothesize that AMF could plausibly reduce cell viability and induce anticancer effects in NSCLC A549 cells by modulating the expression of NF-κB and its key target genes.

Materials and Methods

Materials

Amentoflavone (AMF; Cas No.: 13,190–97-1) having a molecular weight of 631.07 was procured from Sigma-Aldrich. Stock solution of AMF was prepared in 0.5% DMSO. All cell culture reagents, including the fluorescent dyes used in this study, were procured from Sigma-Aldrich (St Louis, MO, USA). Reagents and kits used for qRT-PCR were procured from ThermoScientific (Waltham, MA, USA). Colorimetric kits for caspase-8, −9, and −3, namely K113-25, K119, and K106-100, respectively, were obtained from BioVision, USA.

Methods

Cell Line

The NSCLC A549 cell line was obtained from the National Center for Cell Science (Maharashtra, India). During this study, proliferation of A549 cells took place in controlled conditions at 37°C with humidified air containing 5% CO_2. The

cells were grown in DMEM-high glucose media further enhanced with FBS (10%) and antibiotic–antimycotic cocktail (1%). All bright-field and fluorescent photomicrographs were captured at 100 μm scan area (20×) using an FLoid imaging station (ThermoScientific, Waltham, MA, USA).

Cytotoxic Effects of AMF

The cytotoxic effect of AMF on A549 cells was investigated using the MTT assay.[23] Cells (5×10^3 cells were exposed to 15, 30, 60, 120, and 240 μM AMF for 24 h. Subsequently, MTT (5 mg/mL (10 μL/well)) was added to each well, and the plate was incubated under standard cell culture conditions for 4 h. Eventually, 0.1 mL DMSO was added to each well, and the absorbance of solubilized formazan crystals was assessed at 570 nm. AMF-induced cytotoxicity against A549 cells was expressed as a percentage (%) of the cell viability, which was calculated as follows:

$$\text{Percentage(\%)cell viability} = \frac{mean\ absorbance\ of\ AMF\ treated\ A549\ cells}{mean\ absorbance\ of\ untreated\ A549\ cells} \times 100$$

Morphological Assessment Post-AMF Exposure

Morphological evaluation of A549 cells was performed to delineate the cytotoxic effects of AMF by bright-field microscopy. Then, 5×10^3 A549 cells were exposed to AMF concentrations for 24 h. Subsequently, changes in the morphology of AMF-exposed A549 cells were established by comparing them with the morphological features of untreated control A549 cells.

LDH Release Assessment

An LDH release assay was performed to assess AMF-mediated cytotoxicity in A549 cells. Briefly, A549 cells were cultured in a 96-well plate in a growth medium. The cells were treated with various concentrations of AMF for 24 h. The LDH release assay was performed according to the manufacturer's protocol, using an LDH colorimetric activity kit (catalogue no: EEA013; Invitrogen). Finally, the absorbance of the released LDH in each group was recorded at 490 nm using an ELISA reader (Bio-Rad, USA). The percentage (%) of cytotoxicity of AMF-treated cells was calculated using the following formula:

$$\%\text{Cytotoxicity} = \frac{(LDH\ activity\ in\ AMF\ treated\ cells) - (LDH\ activity\ in\ untreated\ control)}{Maximum\ LDH\ activity\ in\ AMF\ treatedcells - LDH\ activity\ in\ control} \times 100$$

Effect of AMF on Nuclear Condensation

The AMF-mediated effects on the nuclear morphology of A549 cells were investigated using DAPI staining.[24] For this, 5×10^3 AMF exposed a549 cells were fixed with ice-cold methanol for 10 min and then permeabilized using Triton X 100 and paraformaldehyde in 0.25% and 3% paraformaldehyde, respectively. The cells were then stained with 5 mg/mL DAPI for 15 min and visualized using the blue filter of a fluorescence microscope.

Assessment of Oxidative Stress Induced by AMF

AMF-mediated oxidative stress was investigated by evaluating reactive oxygen species (ROS) by DCFH-DA staining.[25] Next, 5×10^3 A549 cells were exposed to 15–240 μM AMF for 12 h. Thereafter, 10 μM DCFH-DA stain was added to each well, and the plate was left undisturbed for 30 min in the dark. The cells were carefully washed and visualized for DCF-DA-mediated green fluorescence.

For the quantitative estimation of intracellular ROS, 1×10^4 cells per well were cultured in 96-well black-bottom culture plates and allowed to adhere for 24 h in a CO_2 incubator at 37°C. A549 cells were subsequently treated with varying doses of AMF for 6 h. A549 cells were cultured for 30 min with 10 mM DCFH-DA under standard conditions. Fluorescence intensity was assessed using a multiwall microplate reader (Synergy H1 Hybrid Multi-Mode Microplate Reader, BioTek, USA) at 485:528 wavelength ratio. The resultant values are expressed as the percentage of fluorescence intensity relative to that of untreated A549 cells.

Estimation of Mitochondrial Membrane Potential (Δψm)

Rhodamine (Rh)-123 staining was used to evaluate the efficacy of AMF in dissipating ΔΨm as previously reported.[24] About, 5×10^3 A549 cells were exposed to AMF overnight. A549 cells were re-treated in the dark with 5 mg/mL Rh-123 for 30 min. Finally, AMF-treated and untreated cells were visualized for green fluorescence using a fluorescence microscope.

Assessment of AMF Mediated Apoptosis

Acridine orange (AO) and ethidium bromide (EtBr)-mediated dual staining are often used to differentiate live and apoptotic cells.[26] To evaluate the apoptosis-inducing effect of AMF, 5×10^5 A549 cells were exposed to AMF for 24 h and then pelleted. The cells in the pellet were gently mixed with 0.1 mL each of EtBr and AO and left undisturbed for 15 minutes. Both AMF-treated and untreated cells were visualized using red and green filters of the fluorescent microscope. Eventually, both live and apoptotic cells were quantified using ImageJ software (NIH, USA).

Evaluation of Caspase/s Activity

The activities of key caspases, such as caspase-3, −8, and −9, were assessed colorimetrically. 3×10^6 A549 cells pre-exposed to AMF for 24 h were briefly lysed for approximately 10 min in ice-cold lysis buffer (0.5 mL). The lysate was centrifuged (1000 rpm for 1 min) to collect the supernatant. About 0.5 mL of the cell lysate was mixed with an equal volume of reaction buffer. The reaction was briefly incubated for 10 min after supplementation with DEVD-pNA (4 mM). Finally, the absorbance of each sample was measured at 405 nm wavelength. Alterations in the levels of caspases/s were expressed as caspase activity percentage in comparison with the control or untreated A549 cells.

Attenuation of AMF-Mediated Apoptosis by Caspase Inhibitors

To characterize AMF-mediated cytotoxic effects, 1×10^4 A549 cells were pre-exposed (2 h) to 50 μM of specific inhibitors each for caspase-3, −8 and −9, namely, Z-DEVD-FMK, Z-IETD-FMK, and Z-LEHD-FMK. At the end of the incubation period, the cells were re-exposed to AMF for 24 h. The viability of A549 cells was estimated using the MTT assay as described above.

qRT-PCR Assessment

qRT-PCR was performed to evaluate the effect of AMF on the mRNA expression of Bcl2, cyclinD1, survivin, and β-actin. Followed by pre-exposing 1×10^6 A549 cells to AMF for 24 h to extract the total RNA. In the next steps, 2 μg of extracted RNA was used to synthesize cDNA using a qPCR kit according to the manufacturer's instructions. Subsequently, comparative CT methods with the β-actin housekeeping gene were used to evaluate alterations in the mRNA expression of the stated genes. Primers for the genes listed in Table 1 were designed by using the NCBI for Biotechnology Information.

Molecular Docking Studies

Molecular docking of AMF with NF-κB protein was performed using AutoDock 1.5.7, following the standard procedure. The structure of NF-κB was slightly modified by excluding the duplicate chains and water molecules. The binding pocket coordinates were selected from the centroid of the protein. Kollman charges, along with polar hydrogen atoms, were

Table 1 List of Primers

Target Gene	Forward Primer (5'-3')	Reverse Primer (3'-5')
GAPDH	GAAATCCCATCACCATCTTCCAGG	GAGCCCCAGCCTTCTCCATG
Bcl2	GATTGTGGCCTTCTTTGAG	CAAACTGAGCAGAGTCTTC
Survivin	CCTACCGAGAACGAGCCTGATT	CCATCTGCTTCTTGACAGTGAGG
CyclinD1	CCGTCCATGCGGAAGATC	GAAGACCTCCTCCTCGCACT

added to the grid box (40 × 40 × 40 Å). The structure of the molecule used for docking was saved as a pdbqt file, and the atoms were allocated as AD4 type. The Lamarckian genetic algorithm along with genetic algorithm were used for docking search parameters.

Statistical Estimations

In the current study, data are presented as mean ± SEM of at least three individual experiments performed three times. All statistical analyses were performed using GraphPad Prism (v. 5.0) software using one-way analysis of variance (ANOVA) and Dunnett's post hoc test. *Represents $P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

Results

AMF Reduced A549 Cell Viability

The cytotoxicity of AMF against A549 cells was assessed using the MTT assay. These observations indicate that AMF significantly reduced the number of viable A549 cells. As demonstrated in Figure 1A, AMF exposure reduced A549 viability to 88.13% ± 2.97%; 73.75% ± 5.47%, 55.40% ± 4.44%, 31.59% ± 4.65%, and 22.92% ± 1.91% at 15, 30, 60, 120 and 240 µM AMF concentration, respectively. Furthermore, the IC_{50} value of AMF was found to be 32.03 ± 1.51 µM after 24 h of treatment (Figure 1B).

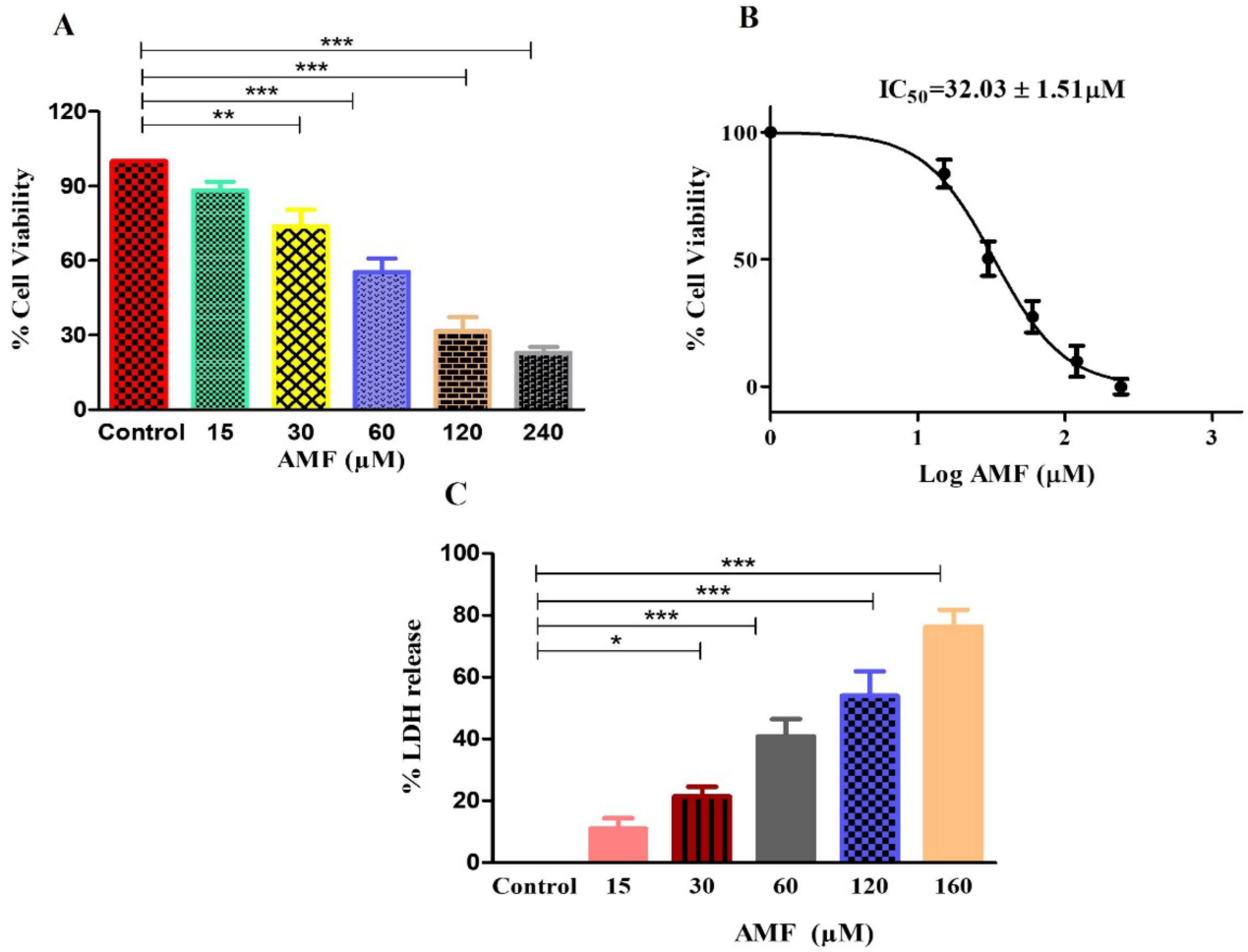

Figure 1 AMF-mediated instigation of antiproliferative effects in A549 cells (**A**) Percent (%) cell viability of A549 cells as assessed through MTT assay, (**B**) the IC_{50} concentration of AMF in treated A549 cells and (**C**) Percent (%) LDH release in AMF-treated A549 cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

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AMF Instigated Extracellular Release of LDH Release

LDH is a key enzyme that is involved in the oxidation of l-lactate to pyruvate. LDH is often released into the extracellular environment when the integrity of the cell membrane is compromised.[27] Indeed, the LDH level post treatment was found to substantially increased to 53.99 ± 3.45% (120 μM) and 76.28 ± 4.46% (240 μM). In contrast, at lower concentrations of AMF (15 μM, 30 μM and 60 μM), LDH release was found to be 10.92% ± 2.70%, 21.37 ± 2.58% and 40.89 ± 4.51%, respectively (Figure 1C). The increase in LDH release explicitly indicated that AMF caused extracellular release of LDH from A549 cells.

AMF Exposure Induced Morphological Alterations

The observations from phase contrast microscopy indicated significantly altered cell morphology in A549 cells after AMF treatment compared to that in control A549 cells. With increasing AMF concentration, significant abrupt changes were observed in A549 cells. Intriguingly, these abrupt changes included withering of the plasma membrane, increased count of floating cells, and rupture of cell constituents indicated by red, blue, and green arrows, respectively (Figure 2).

AMF Exposure Altered Nuclear Morphology

Changes in the nuclear morphology of A549 cells exposed to AMF were investigated using DAPI staining. Condensation and fragmentation of nuclear chromatin were observed concomitantly with the formation of apoptotic bodies (Figure 3). In addition, bright blue fluorescence was observed in A549 cells after treatment with 30, 60, and 120 μM AMF. Thus, AMF treatment resulted in significant alterations in the nuclear morphology of A549 cells.

AMF Elevates the Intracellular Level of ROS

To study the mode of action of AMF, we explored the AMF-mediated effects on the intracellular production of ROS using DCFH-DA staining. As shown in Figure 4A, elevated ROS levels were observed in A549 cells after AMF exposure

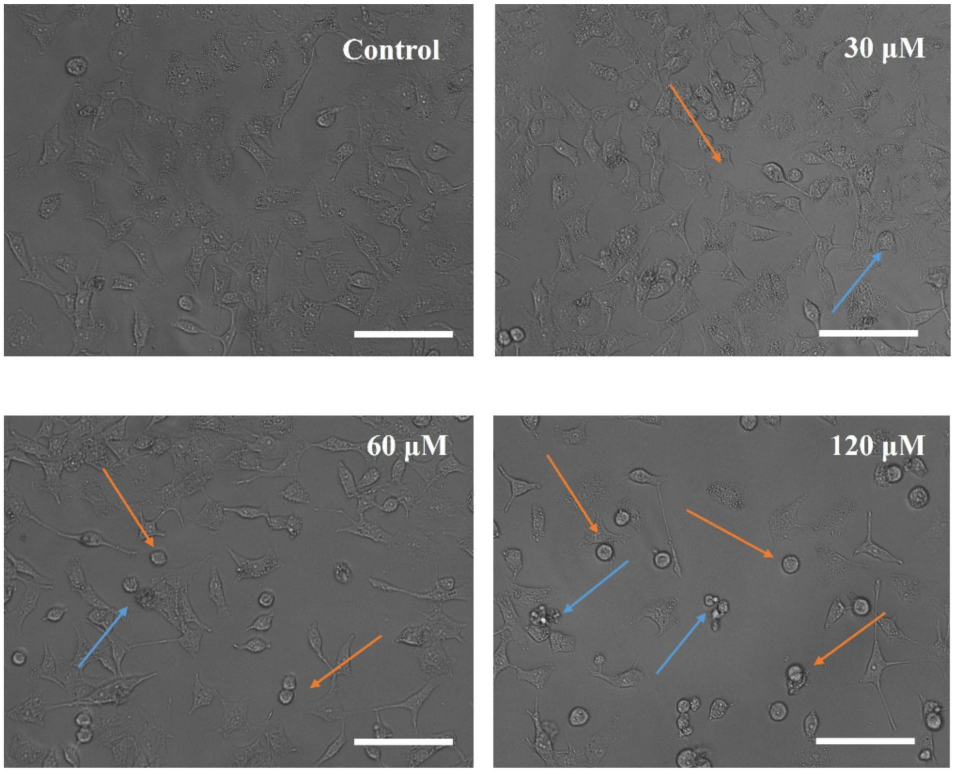

Figure 2 Altered morphology of A549 cells including rounded cells, plasma membrane blebbing and organelle disintegration as indicated by red and blue arrows respectively post-AMF exposure at various concentrations. (Magnification: 20×; Scale bar: 100 μm).

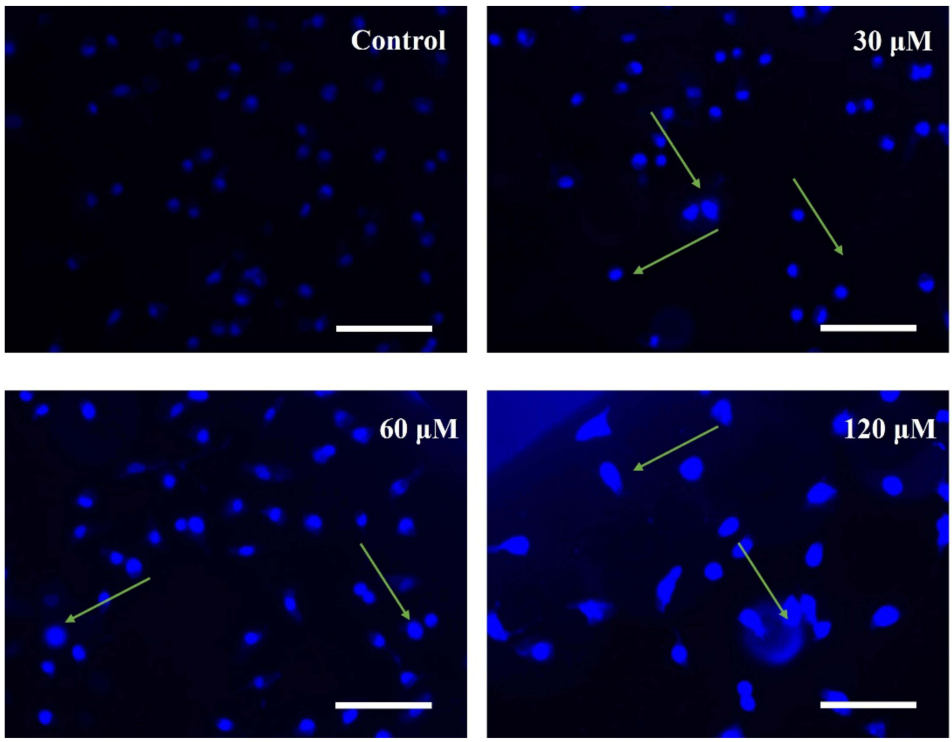

Figure 3 Instigation of nuclear fragmentation in AMF-treated DAPI-stained A549 cells as indicated through increased blue fluorescence proportionally with increase in AMF concentration as indicated by green arrows. (Magnification: 20×; Scale bar: 100 µm).

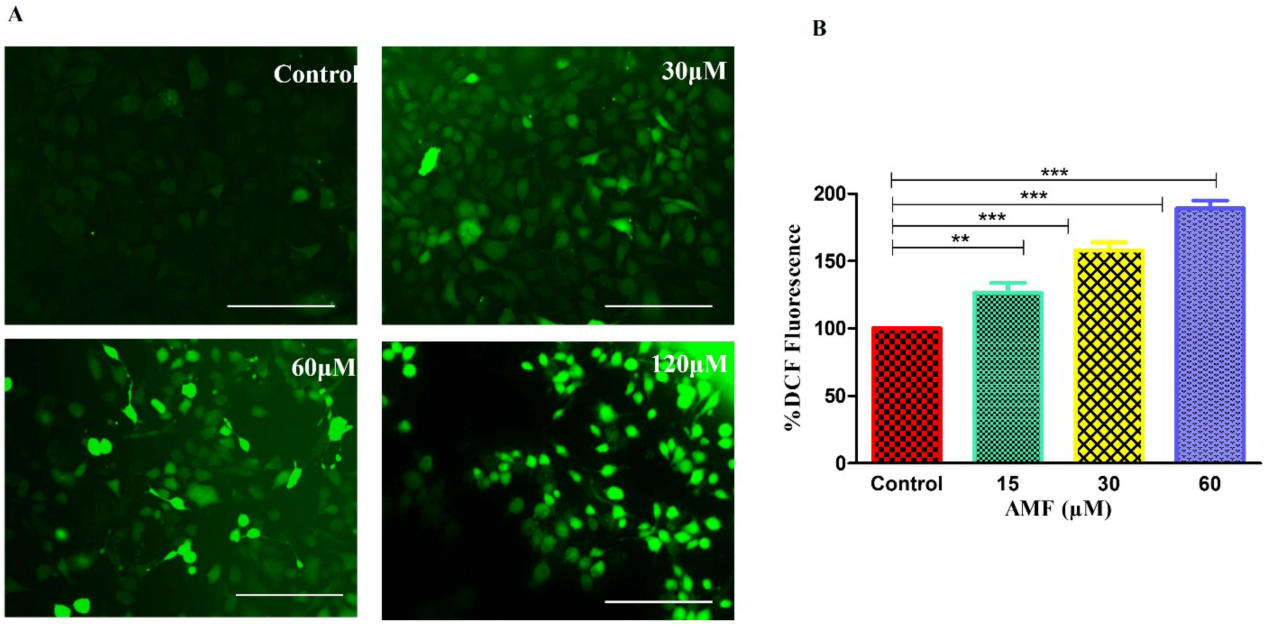

Figure 4 Effect of AMF on (**A**) intracellular ROS levels and (**B**) quantification of intracellular ROS levels within A549 cells. $^{**}P < 0.01$, $^{***}P < 0.001$. (Magnification: 20×; Scale bar: 100 µm).

at higher concentrations than those in untreated cells with low ROS levels. Furthermore, quantitative assessment of ROS revealed that increasing concentrations of AMF elevated ROS production in a dose-dependent manner. AMF treatment augments the intracellular ROS levels to 28.09 ± 4.82% (30 µM), 62.31 ± 5.80% (60 µM) and 89.08 ± 4.70% (120 µM)

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compared to untreated control (Figure 4B). Thus, the increase in fluorescence intensity was related to the enhanced ROS production in AMF-treated A549 cells.

AMF Instigated Loss in Viability of Mitochondria

Dissipation of the ΔΨm is pivotal for instigating mitochondria-mediated apoptosis.[28] Rh-123 staining was used to study the effect of AMF on the ΔΨm in A549 cells. As per the photomicrographs, it was evident that an increase in AMF concentration proportionally led to increased dissipation of ΔΨm in A549 cells (Figure 5).

AMF Exposure Increased Caspase Activity

The involvement of key caspases in AMF-mediated cytotoxicity of A549 cells was assessed colorimetrically. The findings elucidated the caspase-8 and −9 activities were 33.43% ± 5.62% (30 μM), 75.84% ± 3.24% (60 μM) and 107.92% ± 4.48% (120 μM) and 57.73% ± 4.05% (30 μM), 114.51% ± 4.20% (60 μM) and 124.88% ± 4.48% (120 μM), respectively, compared to the control cells. Moreover, caspase-3 activity was found to be 69.79% ± 4.40% (30 μM); 133.01% ± 2.19% (60 μM) and 159.46% ± 5.35% (120 μM) compared to untreated A549 cells (Figure 6A).

Caspase Inhibitors Alleviated AMF Induced Cytotoxicity

To further corroborate the cytotoxic effects of AMF on the activation of key caspases, A549 cells were first treated with specific caspase inhibitors, and an MTT assay was used to determine the viability of A549 cells. Pretreatment with caspase-8, −9, and −3 inhibitors substantially decreased the amount of cytotoxicity in lung cancer cells caused by treatment with AMF (Figure 6B–D). This, in turn, indicates that caspase-8, −9 and −3 activation are crucial for AMF-mediated apoptosis.

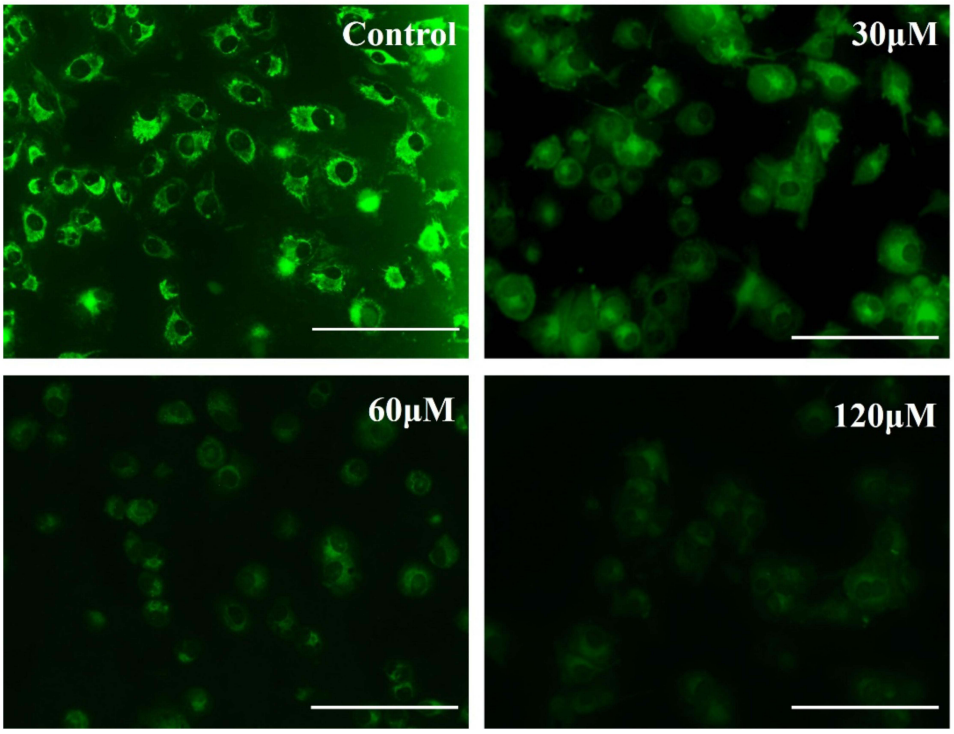

Figure 5 Fluorescent micrographs demonstrating dissipation of ΔΨm in AMF-treated A549 cells. The reducing fluorescence indicated the relative loss in mitochondrial viability due to loss of ΔΨm. (Magnification: 20×; Scale bar: 100 μm).

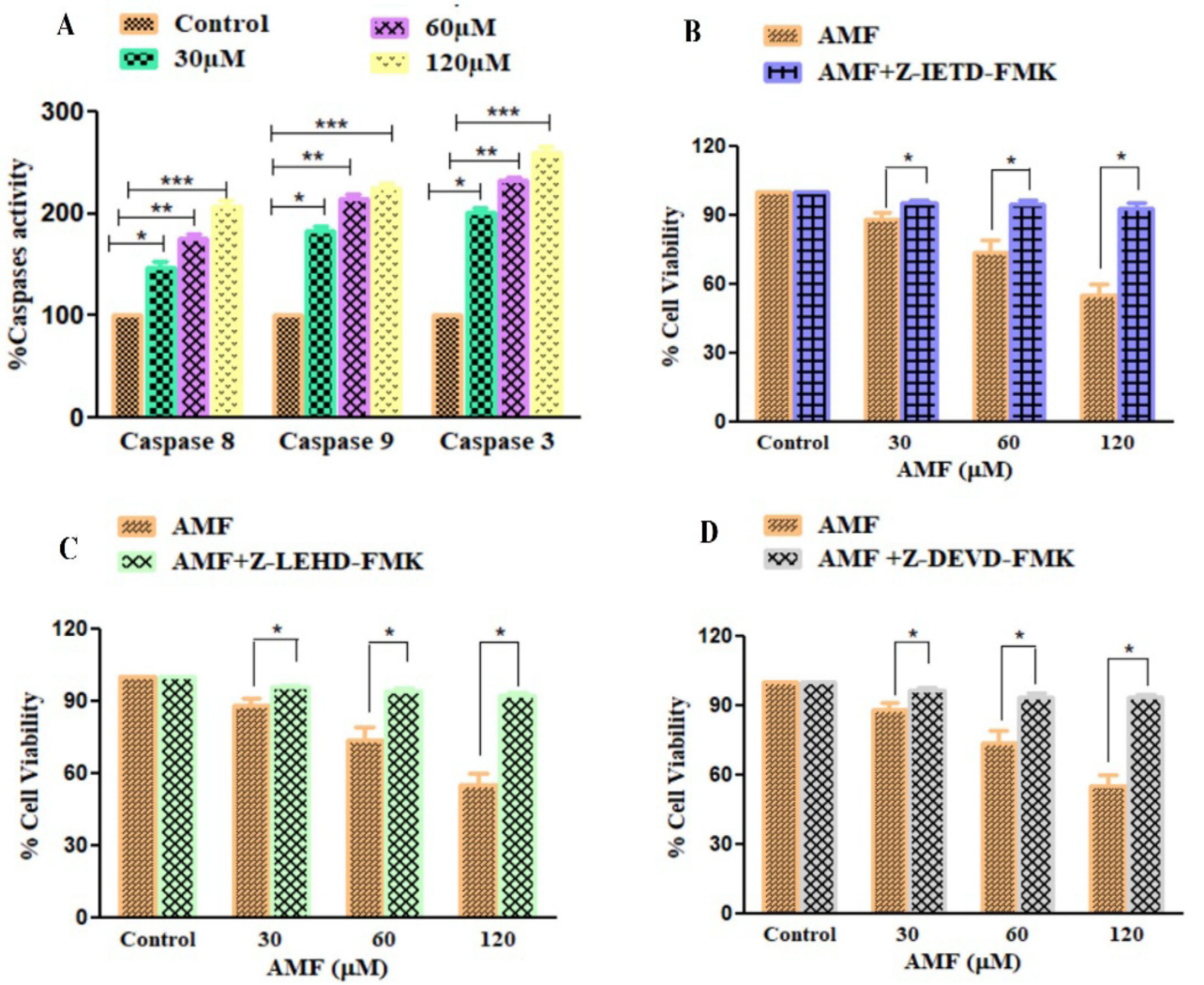

Figure 6 (**A**) Activity of caspase-8, −9 and −3 post-AMF exposure in A549 cells as evaluated colorimetrically. Percent (%) cell viability of A549 cells pretreated with (**B**) Z-IETD-FMK (caspase-8 inhibitor), (**C**) Z-LEHD-FMK (caspase-9 inhibitor) and (**D**) Z-DEVD-FMK (caspase-3 inhibitor) followed by treatment with varying doses of AMF (15–120 µM) for 24 h measured by MTT assay. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

AMF Exposure Induced Apoptotic Cell Death

EtBr/AO dual staining was performed to evaluate apoptosis in the AMF-treated A549 cells. As shown in Figure 7, the merged fluorescent photomicrographs show that live cells appeared uniformly green (marked by red arrows), whereas cells in the early phases of apoptotic cell death appeared slightly yellow. In contrast, cells in the late apoptotic phase exhibited fragmented and condensed nuclei, resulting in the incorporation of EtBr, which appeared red in color. Interestingly, early and late apoptotic A549 cells were recognized by the presence of yellow and reddish fluorescence, respectively, as indicated by the green and purple arrows. Our findings suggest that treatment with varying doses of AMF induces apoptosis in A549 cells. The number of late apoptotic cells (orange/reddish fluorescence) increased significantly after treatment with 60 µM and 120 µM AMF.

AMF-treated A549 cells in the early and late apoptotic phases were quantified. The number of early apoptotic cells (EACs) and late apoptotic cells (LACs) increased proportionally with increasing AMF concentrations. The EACs were found to be 8.80% ± 0.72% (30 µM); 14.93% ± 2.13% (60 µM); 22.62% ± 3.03% (120 µM); and 35.81% ± 3.60% (25 µM). Similarly, the LACs were found to be 14.89% ± 1.86% (30 µM); 20.43% ± 1.35% (60 µM); 28.37% ± 3.09% (120 µM); and 45.51% ± 2.81% (25 µM) indicating the competency of AMF in inducing apoptosis (Figure 8).

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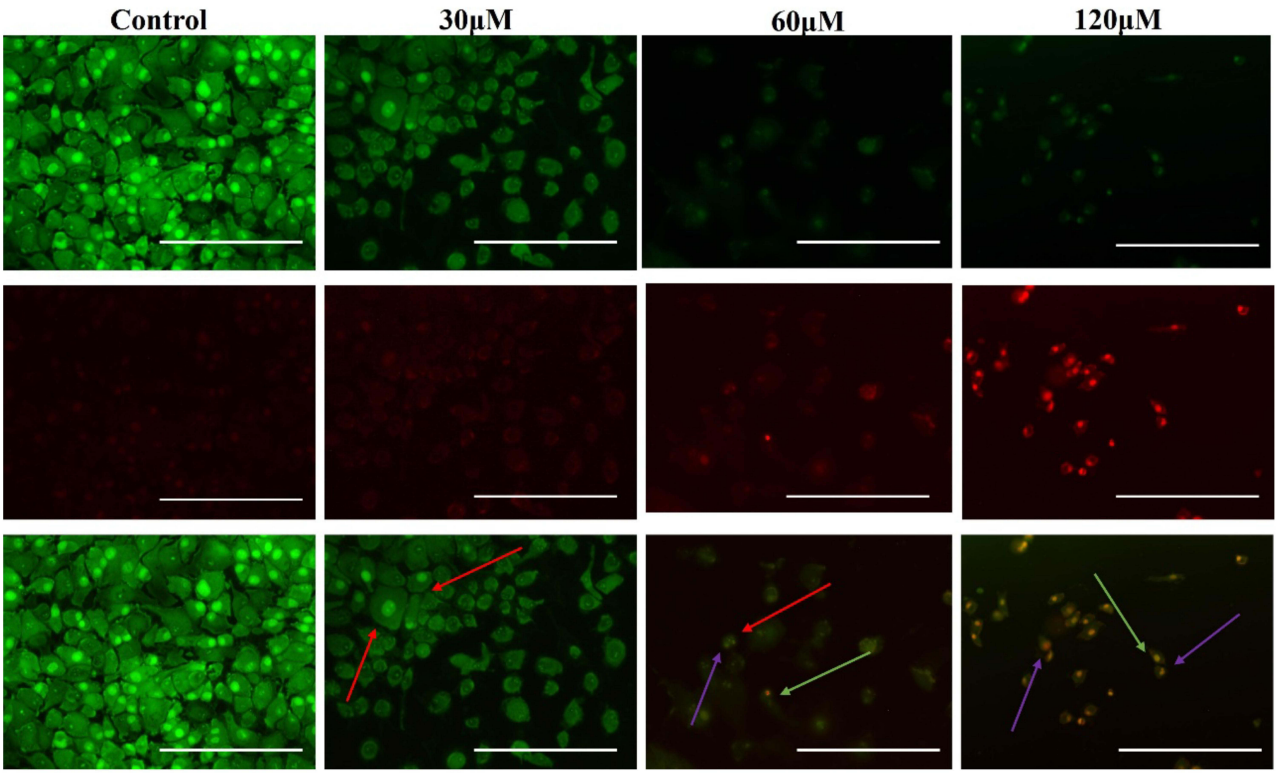

Figure 7 AMF-induced apoptotic cell death as assessed through AO/EtBr dual staining. Viable, early apoptotic and late apoptotic A549 cells post-AMF exposure at stated concentrations indicated by red, purple and green arrows respectively. (Magnification: 20×; Scale bare: 100 μm).

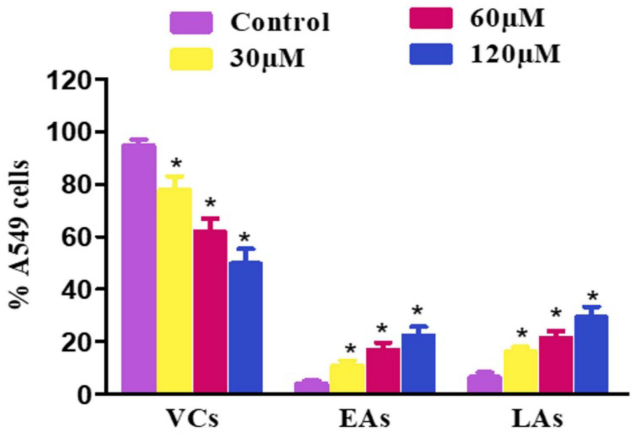

Figure 8 Quantification of viable, early apoptotic and late apoptotic cells post-AMF exposure at stated concentrations as indicated by red, green and purple arrows respectively. *$P < 0.05$.

AMF Reduced Expression of Genes Associated Proliferation and Apoptosis

Cyclin D1 plays a significant role in the cell cycle and is frequently overexpressed in lung cancer cells, including A549 cells.[29] Bcl-2 and survivin play a role in lung cancer and can therefore be targeted for therapy. NF-κB plays a complex role in cancer biology by regulating the expression of genes involved in promoting cell survival and proliferation of cells.[9] Our qRT-PCR based observations indicated that exposure of AMF reduced Bcl-2 and survivin mRNA to 0.89 ± 0.02, 0.74 ± 0.04 and 0.55 ± 0.03-fold; and 0.91 ± 0.02, 0.79 ± 0.04 and 0.58 ± 0.04-fold, respectively, in comparison with control cells (Figure 9A and B). In addition, the cyclinD1 mRNA expression was reduced to 0.85 ± 0.03, 0.72 ±

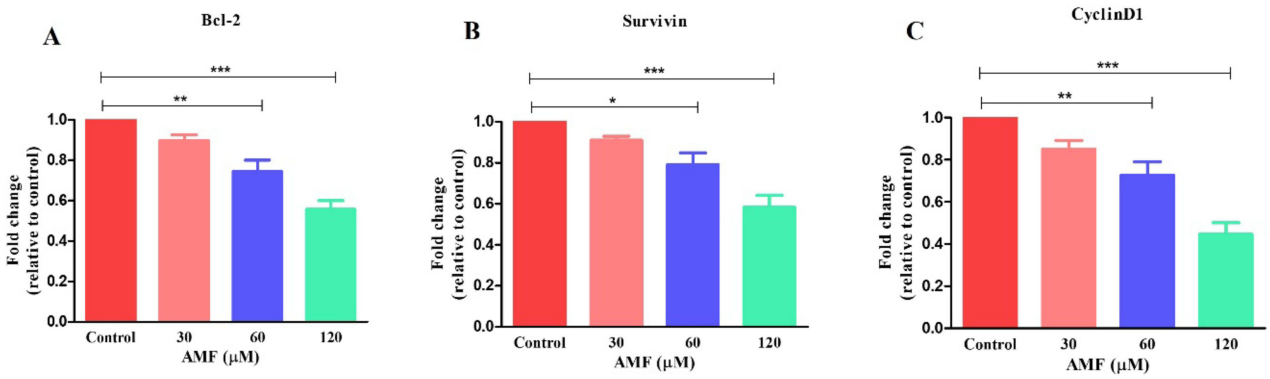

Figure 9 AMF induced modulatory effects on mRNA expression of (**A**) Bcl2, (**B**) survivin and (**C**) cyclinD1 in A549 cells. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

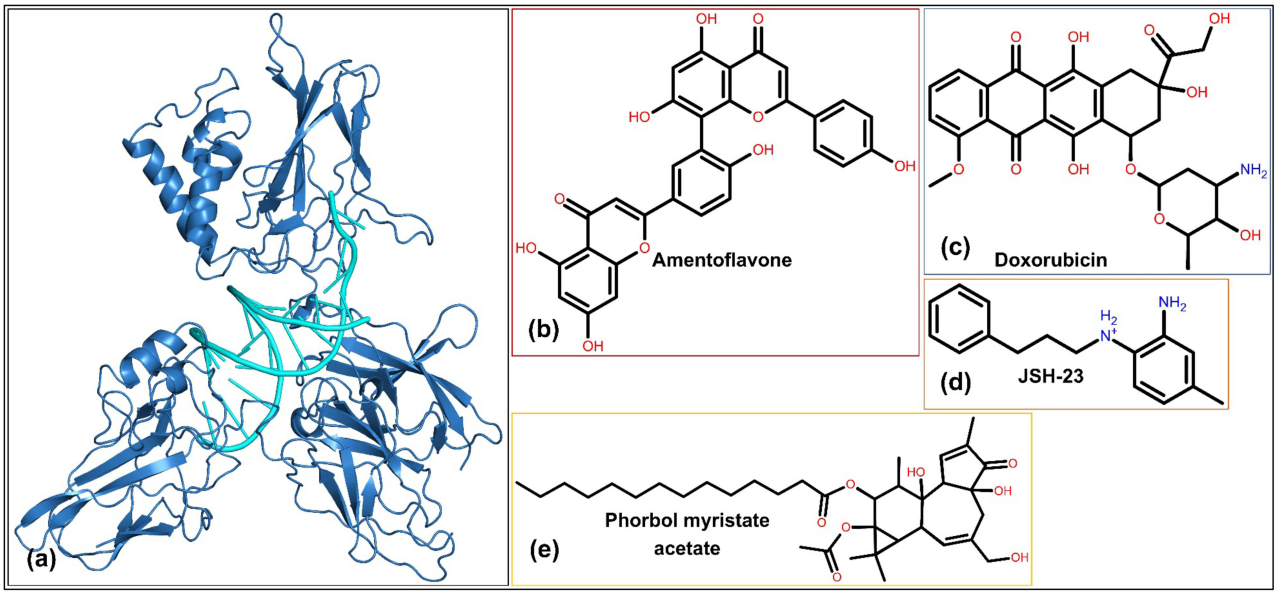

Figure 10 3D protein structure: (**a**) NF-κB p50/65. 2D chemical structures, (**b**) amentoflavone, (**c**) doxorubicin, (**d**) JSH-23, and (**e**) phorbol myristate acetate.

0.05 and 0.44 ± 0.04-fold; at 30, 60, and 120 μM AMF concentration, respectively (Figure 9C). These observational trends indicated the efficacy of AMF in altering the mRNA expression of genes associated with cellular proliferation.

Molecular Docking Studies

The 2D and 3D structures of amentoflavone, doxorubicin, and NF-κB are shown in Figure 10A–C. During docking evaluations, it was found that the binding energy of AMF with NF-κB p50/65 was –12.1 kcal/mol, whereas that of doxorubicin with NF-κB p50/65 was –10.2 kcal/mol. The residues Gln^{606}(B), dg^{5}(C), Gln^{247}(A), Phe^{607}(B), Lys^{218}(A), dt^{8}(C), and Lys^{572}(B) are involved in the hydrophobic interactions between AMF and NF-κB p50/65. However, Da^{6}(C): (Bond Length; 2.74 Å), Arg^{246}(A): (Bond Length; 3.05 Å), Arg^{605}(B: (Bond Length; 3.15 Å), Arg^{187}(A): (Bond Length; da^{18}(D): (Bond Length; 2.95 Å), and Lys^{541}(B): (Bond Length; 3.21 Å) were involved in hydrogen bonding (Figure 11A). Moreover, the hydrophobic residues interacting with doxorubicin and NF-κB p50/65 are dc^{24}(D), dg^{5}(C), Glu^{222}(A), da^{6}(C), and dc^{7}(C). However, Lys^{221}(A): (Bond Length; 3.18, 3.13, 2.97 Å), dg^{3}(C): (Bond length; 3.03, 2.85 Å), dg^{4}(C): (Bond Length; 3.00, 2.95, 2.91, 2.81 Å) were involved in hydrogen bonding (Figure 11B).

We have also compared the binding energies of molecular docked complexes of NF-κB p50/65 with JC-23 (NF-κB inhibitor) and phorbol myristate acetate (NF-κB activator). It was observed that the binding energy score of NF-κB p50/

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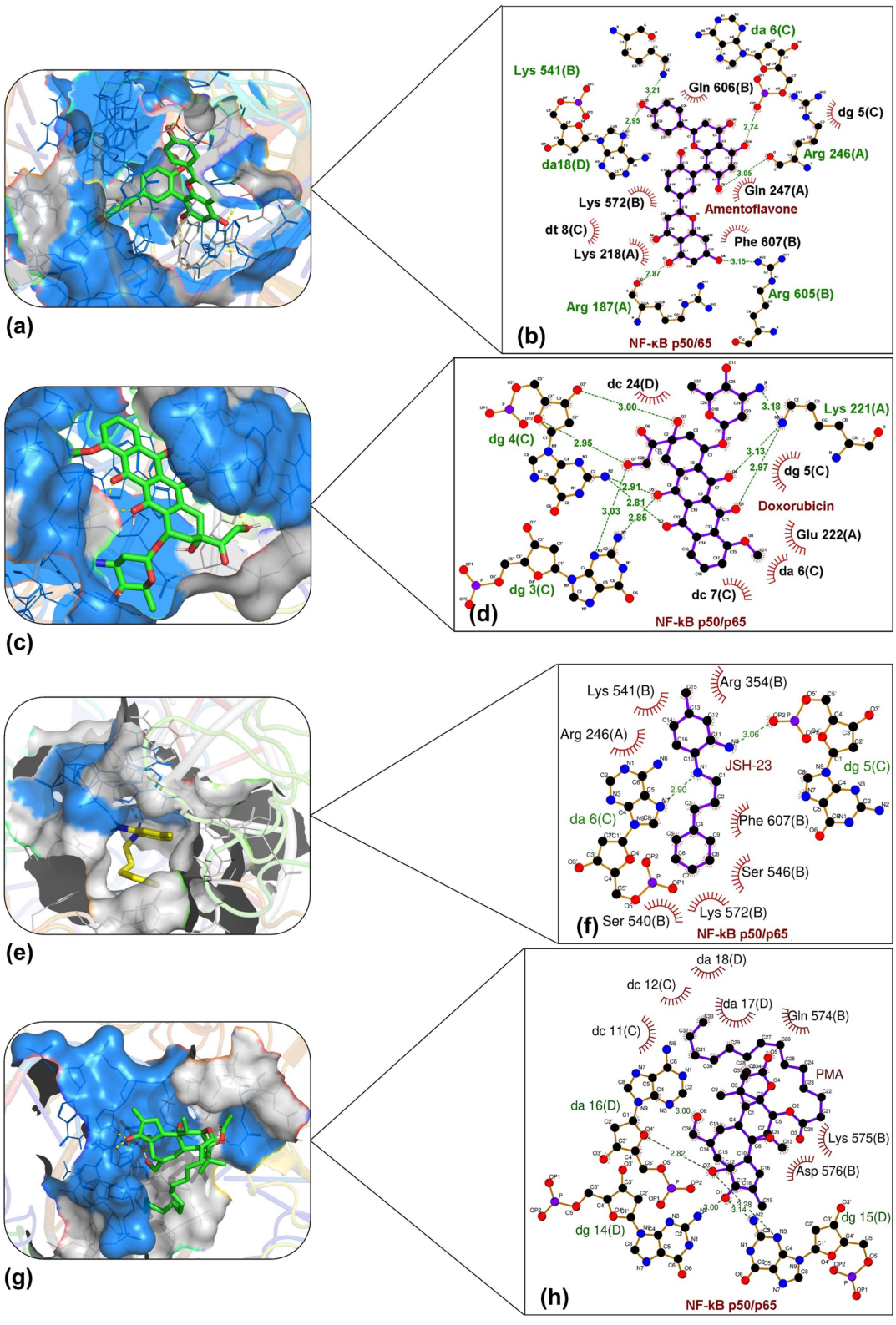

Figure 11 The three-dimensional visualization of the binding pockets of NF-κB p50/65 and the docked ligands and two-dimensional representation of the protein–ligand interactions, as generated by LigPlot is presented as follows (**a** and **b**) NF-κB p50/65-amentoflavone (**c** and **d**) NF-κB p50/65-doxorubicin, (**e** and **f**) NF-κB p50/65-JSH-23 (**g** and **h**) NF-κB p50/65-phorbol myristate acetate.

Note: In the 3D representation of the binding pocket, residues involved in hydrogen bonding are indicated in blue, while those participating in hydrophobic interactions are depicted in gray.

Table 2 Interaction Analysis of the Protein–Ligand Complexes

S. No.	Compound Name	Binding Energy	PDB ID/Protein Name	Hydrogen Bonds	Hydrophobic Residues
1.	Amentoflavone	– **12.1** (kcal/mol)	NF-κB p50/65	da^{6} (C) (Bond length: 2.74 Å), Arg^{246} (A) (Bond length: 3.05 Å), Arg^{605} (B) (Bond length: 3.15 Å), Arg^{187} (A) (Bond length: 2.87 Å), da^{18} (D) (Bond length: 2.95 Å), Lys^{541} (B) (Bond length: 3.21 Å)	Gln^{606} (B), dg^{5} (C), Gln^{247} (A), Phe^{607} (B), Lys^{218} (A), dt^{8} (C), Lys^{572} (B)
2.	Doxorubicin	– **10.2** (kcal/mol)		Lys^{221}(A) (Bond length: 3.18, 3.13, 2.97 Å), dg^{3}(C) (Bond length: 3.03, 2.85 Å), dg^{4} (C) (Bond length: 3.00, 2.95, 2.91, 2.81 Å)	dc^{24} (D), dg^{5} (C), Glu^{222} (A), da^{6} (C), dc^{7} (C)
3.	JSH-23	– **6.6** (kcal/mol)		dg^{5} (C) (Bond length: 3.06 Å), da^{6} (C) (Bond length: 2.90 Å)	Arg^{246} (A), Arg^{354} (B), Ser^{540} (B), Lys^{541} (B) Ser^{546} (B), Lys^{572} (B), Phe^{607} (B)
4.	Phorbol myristate acetate (PMA)	– **8.1** (kcal/mol)		dg^{14} (D) (Bond length: 3.00 Å), dg^{15} (D) (Bond length: 3.00, 3.28 Å), da^{16} (D) (Bond length: 2.82 Å)	dc^{11} (C), dc^{12} (C), da^{18} (C), da^{17} (D), Gln^{574} (B), Lys^{575} (B), Asp^{576} (B)

65 with JC-23 (NF-κB inhibitor) is –6.6 kcal/mol (Figure 11C) whereas the binding energy score of amentoflavone with phorbol myristate acetate is –8.1 (kcal/mol) (Figure 11D). The residues of AMF, doxorubicin, JC-23 and Phorbol myristate acetate with NF-κB p50/65 are summarized in Table 2. It can be substantiated that the binding energy scores of amentoflavone with NF-κB p50/65 are comparable with the binding energy scores of JC-23 and phorbol myristate acetate with NF-κB p50/65, respectively. Thus, on the basis of these findings, it can be concluded that amentoflavone possesses the potential to be employed as a NF- κB inhibitor.

Discussion

In the last few decades, substantial efforts have been made to explore novel therapeutics for the management of lung cancer. In addition, considerable emphasis has been placed on investigating combinatorial, repurposed drugs, and less invasive therapies against traditional methods for the treatment of cancers, including lung cancer. Currently, available therapeutic options have significant drawbacks, including no selectivity, occurrence of therapeutic resistance, and varying responsiveness.[30] The application of natural compounds for the prevention and treatment of different cancers has attracted significant attention from scientific and pharmaceutical communities over the past few decades.[31] In this study, we investigated the therapeutic potential of AMF in lung cancer NSCLC using A549 cells as an in vitro model. These results explicitly indicated that AMF have the potential to inhibit the growth and proliferation of lung cancer cells. During the initial screening, AMF exposure led to a significant decrease in the number of viable A549 cells. This observation could have resulted only from the intrinsic cytotoxic potential of AMF in A549 cells. In the present study, we did not evaluate the toxicity of AMF against normal cells because previous reports have substantiated the low cytotoxic effects of AMF against normal cells.[32] In A549 cells, LDH is involved in the conversion of lactate to pyruvate, which is a crucial step in the Warburg effect. The cytotoxicity of AMF was further established when the LDH assay showed an increased LDH release from A549 cells in the extracellular environment. These cytotoxic findings further corroborated the results of the morphological investigations conducted in this study. At high AMF concentrations, morphological alterations such as blebbing of the plasma membrane and rounding of A549 cells with an increased number of floating cells were observed and recorded.

Previously published reports have established that enhanced production of intracellular ROS directly generates oxidative stress, leading to the destruction of vital cell components such as nucleic acids, proteins, and lipids. The loss of functionality of these components ultimately leads to the onset of programmed cell death or apoptosis.[33] DCFH-DA staining has been widely used to estimate ROS generation in biogenic models. The results of this study showed augmented DCFH-DA-mediated fluorescence. This elevated fluorescence implies that AMF successfully induced ROS generation in A549 cells in a concentration-dependent manner.

Several plant-based compounds are known for their apoptotic potential and are used in the clinical management of various cancers. Reports have established that biological compounds capable of inducing apoptosis are promising anticancer agents.[34,35] Chromatin condensation and nuclear fragmentation are the initial yet well-known morphological trademarks of apoptosis. Our findings suggested that condensed and fragmented A549 nuclei stained with DAPI indicated that AMF induced significant alterations in the normal nuclear morphology of A549 cells. These observations indicated that AMF could trigger apoptotic cell death in A549 cells by reducing nuclear integrity.

Oxidative stress is a dual-edged sword in the field of cancer biology. Chronically active ROS are regarded as potential instigators of mutations in the genetic constituents of mammalian cells.[36] Intriguingly, cancer cells are also vulnerable to ROS-mediated apoptotic cell death, making them potential therapeutic targets for the clinical management of cancer.[37] The present report also undoubtedly indicates that AMF exposure induces a spike in ROS generation in NSCLC A549 cells. Elevated ROS levels eventually trigger a loss of mitochondrial viability resulting from $\Delta\Psi m$ dissipation.[38] Our study led us to conclude that AMF significantly reduces mitochondrial viability by promoting $\Delta\Psi m$ dissipation in A549 cells. This loss of mitochondrial viability may have initiated an apoptotic cascade in A549 cells.

Caspases are cysteine proteases that play pivotal roles in the regulation of apoptosis.[39] Later stages of apoptosis are marked by the cytosolic release of cytochrome c, which could result from the activation of caspase-8 mediated extrinsic apoptotic pathway or the intrinsic apoptosis pathway.[40] Intriguingly, either of the two apoptotic pathways result in downstream activation of caspase-3. In this study, AMF exposure was found to be correlated with elevated expression of caspases associated with both extrinsic and intrinsic apoptotic pathways in NSCLC A549 cells. Evading apoptosis is regarded as one of the peculiar hallmarks of cancer development. The induction of apoptosis is a crucial target for cancer therapeutics.[41] AO/EtBr dual staining was performed to establish the apoptosis-inducing efficacy of AMF in A549 cells. The results obtained from EtBr/AO staining explicitly indicated an increased number of cells with reddish/orange fluorescence, indicating that AMF exposure caused dose-dependent apoptosis in A549 cells.

Cyclin D1 is a pivotal protein involved in homeostatic division and proliferation of cells. In cancer biology, cyclin D1 is associated with the proliferation of tumor cells via complex interactions with other cellular proteins, leading to a G1 to S phase transition during the cell cycle.[29] Bcl-XL and survivin are important contributors to apoptosis of lung cancer cells is Bcl-X_L and survivin protein. Bcl-X_L and survivin promote evasion of apoptotic cell death by acting as anti-apoptotic proteins.[42] The bioflavonoid used in this study successfully reduced the mRNA expression of key regulators of cell proliferation and apoptosis in lung cancer cells. AMF showed competence in instigating apoptosis by reducing the expression of anti-apoptotic Bcl-X_L concomitantly with cyclin D1 and survivin proteins. Indeed, flavonoids have been previously reported to modulate the expression of key regulatory molecules through various mechanisms, such as impeding the phosphorylation of transcription factors, modulating the p53 signaling pathway, and altering the expression of different miRNAs.[43–46]

NF-κB is an important transcription factor that is considered to be an important therapeutic target for cancer treatment.[16] As it also plays a pivotal role in various other homeostatic signaling processes, careful modulation of NF-κB is very important. NF-κB is also an important player in mediating tumor progression and plays a significant role in imparting resistance to chemotherapy and radiotherapy. Several reports have recently outlined the correlation between NF-κB and lung cancer using in vitro and in vivo models.[47] In the context of our study, previous evidence has shown that NF-κB positively modulates the growth and proliferation of cancer cells by evading apoptotic cell death.[48] Therefore, owing to its important role, we studied the effects of AMF on NF-κB expression using in silico approach. Molecular docking studies confirmed that AMF docked with NF-κB p50/65 at binding energies of −12.1 kcal/mol. This molecular interaction was compared by docking doxorubicin (a standard drug) with NF-κB p50/65 at binding energies of –10.2 kcal/mol. Furthermore, we have also compared the molecular docking of NF-κB p50/65 with JC-23 and phorbol

myristate acetate, respectively. This, in turn, predicted that AMF have nearly comparable competency in modulating the expression of NF-κB, similar to that of the standard chemotherapeutic doxorubicin. Importantly, our in silico findings were corroborated by the RT-PCR results. NF-κB is reported to be an important inducer of Bcl-X_L expression and is concomitantly associated with the increased production and transcription of cyclin D1 and survivin.[29,49] Thus, it may be inferred that the reduction in the mRNA expression of cyclin D1 and Bcl-X_L after AMF exposure resulted in reduced NF-κB expression. Therefore, based on these results, it can be concluded that AMF mediated the downregulation of the NF-κB signaling pathway by targeting various downstream genes such as Bcl-2, survivin and cyclinD1 in lung cancer cells.

Conclusion

In summary, the current study has substantiated that AMF exerted anticancer effects by inhibiting proliferation and mediating cell death in A549 cells by targeting the NF-κB signaling pathway. The results for A549 cells were also supported by the molecular docking studies. The present report is still a preliminary study and demands subsequent preclinical and clinical explorations regarding the plausible use of AMF as an efficacious targeted drug for the treatment of lung cancer. Furthermore, the molecular mechanisms underlying the anticancer efficacy of AMF should be explored in detail.

Data Sharing Statement

All data generated or analyzed during this study are included in this article.

Acknowledgment

Afza Ahmad is currently affiliated with the Department of Biochemistry, Babu Banarasi Das College of Dental Sciences, Babu Banarasi Das University, BBD City, Ayodhya Road, Lucknow – 236028, India.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

No potential conflict of interest was reported by the authors.

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