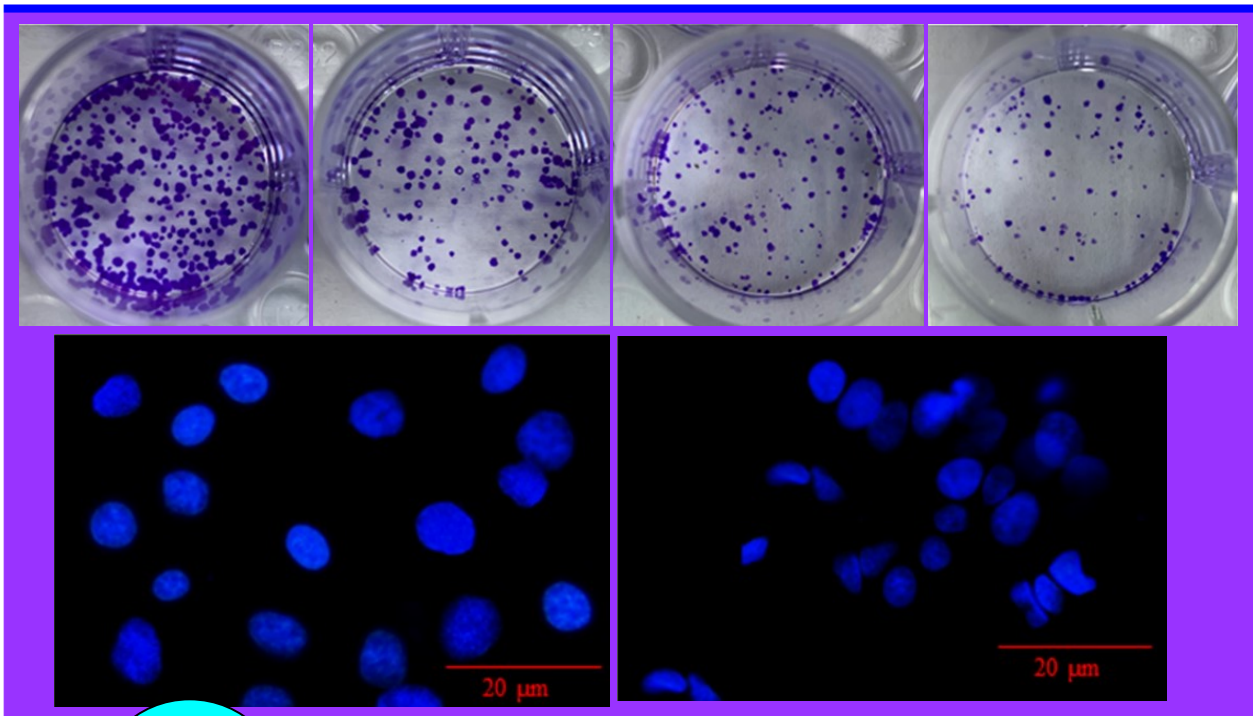

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Nigella sativa plant extract inhibits the proliferation of MDA-MB-231 breast cancer cells via apoptosis and cell cycle arrest

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Abstract

The study was designed to evaluate the antiproliferative effects of *Nigella sativa* plant ethanol extract against MDA-MB-231 triple-negative breast cancer cells. DAPI and annexin V/propidium iodide staining assays were used to examine apoptosis. Results showed that *N. sativa* extract significantly ($p<0.05$) impeded the growth of MDA-MB-231 cells, with IC_{50} of 12.5 µg/mL. The colony-forming potential of MDA-MB-231 was significantly ($p<0.05$) decreased by the *N. sativa* extract-induced apoptosis. Increased expression of Bax, caspase-3, cleaved caspase-3, and cleaved PARP was also observed in the extract-treated MDA-MB-231 cells. Moreover, flow cytometric analysis showed that *N. sativa* extract triggered G_0/G_1 phase arrest in MDA-MB-231 cells. Collectively, *N. sativa* extract exhibits potent antiproliferative activity against triple-negative breast cancer cells and may be used as a source of anti-cancer agents.

Introduction

Breast cancer is among the most prevalent types of cancer in women and caused around 0.685 million deaths in 2020 alone (Sung et al., 2021). The majority of the available chemotherapeutic agents have adverse effects that negatively impact the lives of patients. In addition, the emergence of drug resistance also forms a bottleneck in the treatment of breast cancer (Jamshidi-Kia et al., 2017).

Plants have always been a foundation for traditional medicine and have provided mankind with an unending source of remedies since early human civilizations (Laurindo et al., 2023). Several plants such as *Urtica dioica* (Fattahi et al 2013), *Smilax china* (Nho et al., 2015), *Swertia chirayita* (Barua et al., 2020), and *Panax ginseng* (Kim and Kim, 2015) exhibit anti-cancer activities against breast cancer. Medicinal plants are considered a repository of natural products with diversity in both structure and bioactivity (Laurindo et al., 2023). Consistently, many anti-breast cancer drugs have been isolated from medicinal plants such as vinblastine and vincristine from *Catharanthus roseus* (Garewal et al., 1983, Steiner et al., 1983), paclitaxel from *Taxus baccata* (Miller et al., 2007) and camptothecin from *Camptothecin acuminata* (Landgraf et al., 2020). Medicinal plant-derived phytochemicals act as drugs in their natural form or exist as significant leads for semisynthetic derivatives with high efficiency and low or no toxicity to normal cells and tissues in humans (Bahmani et al., 2014).

N. sativa belongs to the family *Ranunculaceae* and has long been used as a spice, food preservative, and health remedy in different folk and traditional medicine systems in the middle east and Asian countries (Zafar et al., 2016). The plant has been reported to contain different classes of phytochemicals including alkaloids,

carboxylic acids, flavonoids, terpenoids, glycosides, tannins, steroids, saponins, coumarins, and phenols (Reddy et al., 2018).

The seed extract of *N. sativa* was found to exert *in vivo* anti-tumor effects in P815 tumor-bearing mice (Mbarek et al., 2007). The *in vitro* anti-cancer activity of *N. sativa* seed extract against the human HepG2 hepatocellular carcinoma cells via accretion of reactive oxygen species (ROS) has also been reported (Ahmad and Tabassum, 2024). In yet another study, methanolic extract of *N. sativa* seeds was found to suppress the growth of MCF-7 cells *in vitro* via p53 and caspase pathways (Alhazmi et al., 2014). Similarly, the seed extract of *N. sativa* was found to suppress the growth of breast cancer cells *in vitro* (Dilshad et al., 2012). However, the anti-cancer activity of the *N. sativa* plant extract has not been evaluated and the underlying molecular mechanisms have not been deciphered. Moreover, the anti-cancer properties of seeds, oils, and plant extracts of *N. sativa* are mentioned in the Chinese system of medicine (Khan et al., 2011). Consistently, the present study was designed to evaluate the anti-cancer effects of *N. sativa* extract against different human cancer cell lines.

Materials and Methods

Plant material and extraction

The desiccated aerial parts of the *N. sativa* were crushed to powder in a grinder. The powder of the aerial parts was then extracted using denatured ethanol (50%) at room temperature for 48 hours. The crude extract mixture was then filtered, and the filtrate was concentrated under reduced pressure by the evaporation of excess solvent. Finally, the mixture was lyophilized to obtain a pure ethanolic extract of the aerial parts of the *N. sativa* plant. The different concentrations of the extract were made by dissolving it in dimethyl sulfoxide (DMSO).

Cell culture and conditions

SW-620 and HT-29 (colon), MDA-MB-231 and MCF-7 (breast), PC-3 (prostate), A549 (lung), and PANC-1 (pancreatic) cancer cell lines were obtained from the American Type Culture Collection (USA). Dulbecco's Modified Eagle Medium (Life Technologies, USA) supplemented with penicillin (100 units/mL), streptomycin (µg/mL), and 10%fetal bovine serum (Sigma-Aldrich, USA) was used to culture the cells. The culture was placed under moist conditions at a temperature of 37°C within a 5% CO_2 incubator.

Colony formation assay

The effect of the *N. sativa* extract to inhibit the colony generation of MDA-MB-231 cells was monitored using a clonogenic assay (Franken et al., 2006). In brief, MDA-MB-231 cells were seeded in 6-well plates with different concentrations, viz., 0, 6.25, 12.5, and 25 µg/mL, of the extract for 2 weeks at 37°C. The cells were left untouched except for medium replacement every three days. After the incubation period, cell colonies were washed using phosphate-buffered saline (PBS) and then stained with crystal violet. Lastly, the cell colonies were visualized and counted under a light microscope, considering the colonies that contained >50 cells.

Analysis of cellular and nuclear morphology

The cellular morphology and apoptotic features of treated and untreated MDA-MB-231 cells were studied using phase-contrast microscopy and fluorescence microscopy (DAPI staining), respectively. In brief, MDA-MB-231 cells were seeded in 6-well plates with

Box 1: MTT assay

Principle

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay is a rapid colorimetric assay based on the cleavage of the tetrazolium ring of MTT by dehydrogenases in active mitochondria of living cells as an estimate of viable cell number.

Requirements

Cancer cell lines (ATCC, USA); 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT); Dimethyl sulfoxide (DMSO); Incubator; Microplate reader (Biorad, USA); *N. sativa* plant extract; Sodium dodecyl sulfate buffer; 96-Well plate

Procedure

Step 1: Cells were plated in a 96-well plate for 24 hours at a concentration of 1 × 10^4 cells/well.

Step 2: Following this the old media was aspirated and fresh media containing different concentrations of ethanolic extract viz., 0-200 µg/mL, were added to each well of 96-well plants. The plates were then incubated for 24 hours at 37°C.

Step 3: Post-treatment, the medium was completely removed, followed by the addition of 20 µL of freshly prepared MTT solution (5 mg/mL) and incubation for 4 hours at 37°C.

Step 4: The formazan crystals formed in the presence of MTT were dissolved in 150 µL of DMSO and prepared for absorbance measurement using a microplate reader (Biorad, USA) set at 490 nm of wavelength. Experiments were performed in triplicate.

Calculation

The percentage of cell viability was calculated using the equation:

[mean OD of treated cells/mean OD of control cells] × 100

References

Omidi et al., 2023

References (Video)

Bahuguna et al., 2017

different concentrations, viz. 0, 6.25, 12.5, and 25 µg/mL, of the extract for 24 hours. Afterward, treated cells were first washed with PBS and then fixed in paraformaldehyde (4%). Cells for phase-contrast microscopy were loaded into an inverted phase contrast microscope (Leica, Germany) and photographed at 200x magnification. The cells for the study of apoptotic features were stained using DAPI for 10 min in a dark room, followed by washing with PBS in 3 replicas. Finally, the fluorescent MDA-MB-231 cells post-ethanolic extract treatment were examined under a Carl Zeiss fluorescence microscope (Gottingen, Germany).

Annexin V/propidium iodide

The apoptotic effects of the ethanolic extract of *N. sativa* in MDA-MB-231 cells were evaluated by the annexin-V/propidium iodide method using flow cytometry (Miller, 2004). Briefly, MDA-MB-231 cells with a concentration of 2×10^4 cells/well in 6-well plates were cultured for 24 hours, followed by incubation with the ethanolic extract (0-25 µg/mL) for an additional 48 hours. Harvesting of treated MDA-MB-231 cells was immediately followed by two times washing with PBS and then staining using annexin V-fluorescein isothiocyanate and propidium iodide binding buffer. Finally, the apoptosis percentage was detected using flow cytometry (Beckman Coulter, USA).

Cell cycle analysis

The potential of the ethanolic extract of *N. sativa* to arrest MDA-MB-231 cells was detected with the help of flow cytometry (Chen et al., 2016). The MDA-MB-231 cells were exposed to different ethanolic extract concentrations viz., 0, 6.25, 12.5, and 25 µg/mL, for 48 hours and then harvested. Post-harvest-treated MDA-MB-231 cells were washed and then fixed in 70% ethanol at -20° C overnight. Fixed cells were then again washed in PBS, followed by their suspension in 50 µg/mL propidium iodide, 0.1% triton-X-100, 0.1% sodium citrate, and 100 µg/mL RNase staining solution. Afterwards, the mixture was incubated for 30 min and then subjected to flow cytometry (Beckman Coulter, USA) for the detection of different cell cycle checkpoints.

Western blotting

The effect of *N. sativa* on the expression of different proteins was assessed by western blotting (Jia et al, 2023). In brief, MDA-MB-231 cells were treated with the ethanolic extract at various concentrations of 0, 6.25, 12.5, and 25 µg/mL for 48 hours, and then proteins were extracted by lysing the cells with RIPA lysis buffer. Denaturing SDS-PAGE (8-12%) was used to separate proteins, and then proteins were shifted to PVDF membranes (Millipore, USA). These membranes were blocked for 2 hours using 5% non-fat milk and then incubated with protein-specific primary antibodies (Bcl-2, caspase-3, cleaved caspase-3, cleaved-PARP, Bax, and actin) overnight at 4°C. After the incubation period, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG secondary antibodies for 2 hours. Finally, the protein signals were recorded with the help of an ECL kit (Amersham Pharmacia Biotech, UK).

Statistical analysis

Individual experiments for each indicated extract concentration were performed in three replicas, and the data was revealed as mean ± SD. All the statistical data was analyzed via SPSS software, and significant differences were estimated through a one-way ANOVA. The value of $p<0.05$ was taken as statistically significant.

Results

Antiproliferative effects of N. sativa extract

The effects of *N. sativa* plant extract were assessed on SW-620 and HT-29 (colon), MDA-MB-231 and MCF-7 (breast), PC-3 (prostate), A549 (lung), and PANC-1 (pancreatic) cancer cell lines. The *N. sativa* extract exhibited significant ($p<0.05$) against all the cell lines tested. However, the lowest IC_{50} of 12.5 µg/mL was observed against the MDA-MB-231 triple-negative breast cancer cells. Interestingly, the IC_{50} of *N. sativa* extract was around 5 times higher than that of normal breast epithelial FR-2 cells (Table I). Given the pronounced antiproliferative effects of the extract against MDA-MD-231 cells, further experimentation was restricted to these cells only. The effects of the extract were also investigated on the colony-forming potential of MDA-MB-231 cells. The results revealed a significant ($p<0.05$) inhibitory effect of the extract on the colony formation. Moreover, the effects of the *N. sativa* extract on the colony formation of MDA-MB-231 cells were concentration dependent (Figure 1).

Effects of N. sativa extract on cellular and nuclear morphology of MDA-MB-231 cells

After the treatment of MDA-MB-231 cells with different concentrations of *N. sativa* extract, phase-contrast microscopy was used to evaluate the effects of the extract on the morphology of the MDA-MB-231 cells. Interestingly, the untreated cells exhibited normal morphology with no significant changes, while noteworthy changes were observed in the extract-treated MDA-MB-231 cells. The *N. sativa* extract-treated cells showed a loss in cellular integrity, membrane damage, nucleus dislocation, and cell floating (Figure 2). Next, the effects of the extract were assessed on the nuclear morphology of MDA-MB-231 by DAPI staining. Results of DAPI staining indicated membrane damage, membrane blebbing, loss in cell integrity, nuclear disintegration, loss of chromatin, and DNA damage in the extract-treated cells (Figure 3). Taken together, the treatment

Table I

Effect of *N. sativa* extract on cell viability

Cell line	IC_{50} (µg/mL)
SW-620	50.5
HT-29	45.0
MDA-MB-231	12.5
MCF-7	25.0
PC-3	73.5
A549	25.0
PANC-1	50.0
FR-2	65.5

groups with exposure to different concentrations of the extract exhibited significant morphological and nuclear changes in comparison to those of the control. All these changes point towards the induction of apoptosis in extract-treated MDA-MB-231 cells.

Apoptotic effects of N. sativa extract in MDA-MB-231 cells

Taking clues from the results of scanning electron microscopy and DAPI staining, annexin V/PI staining of the MDA-MB-231 cells was carried out, and the distribution of the cells in different cell cycle phases was quantified by flow cytometry. The results showed

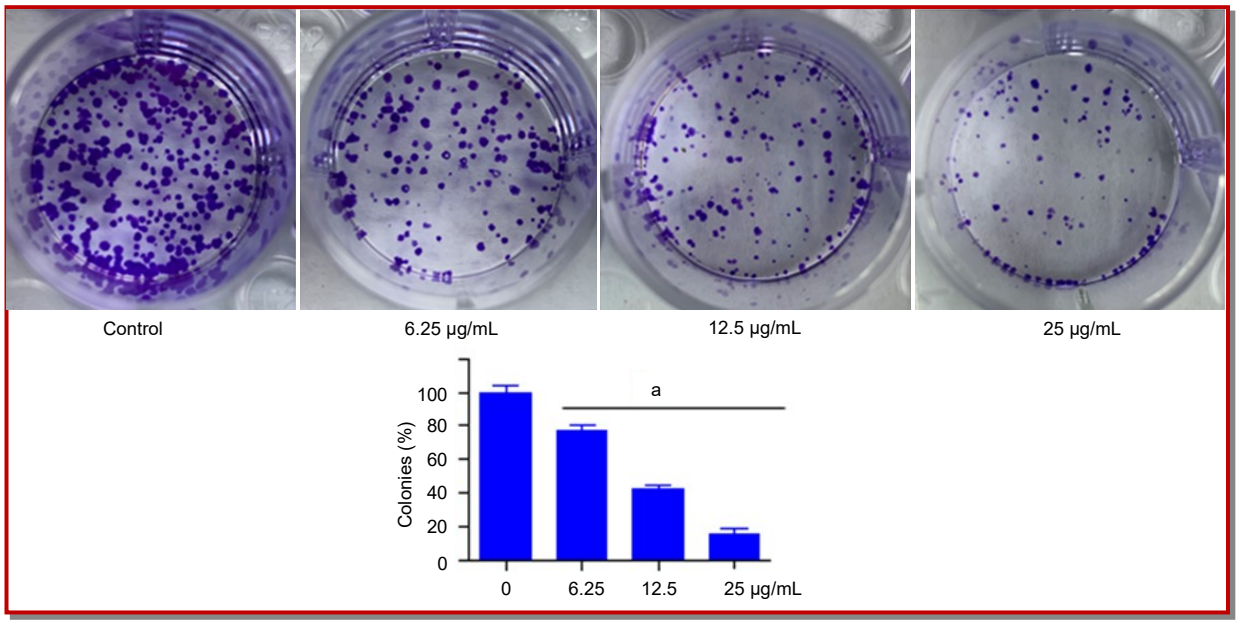

Figure 1: Concentration dependent effects of *N. sativa* extract on colony formation of MDA-MB-231 cells as revealed *by in vitro* clonogenic assay. Triplicate of each concentration was used; Data is presented as mean ± SD; [a]p<0.05

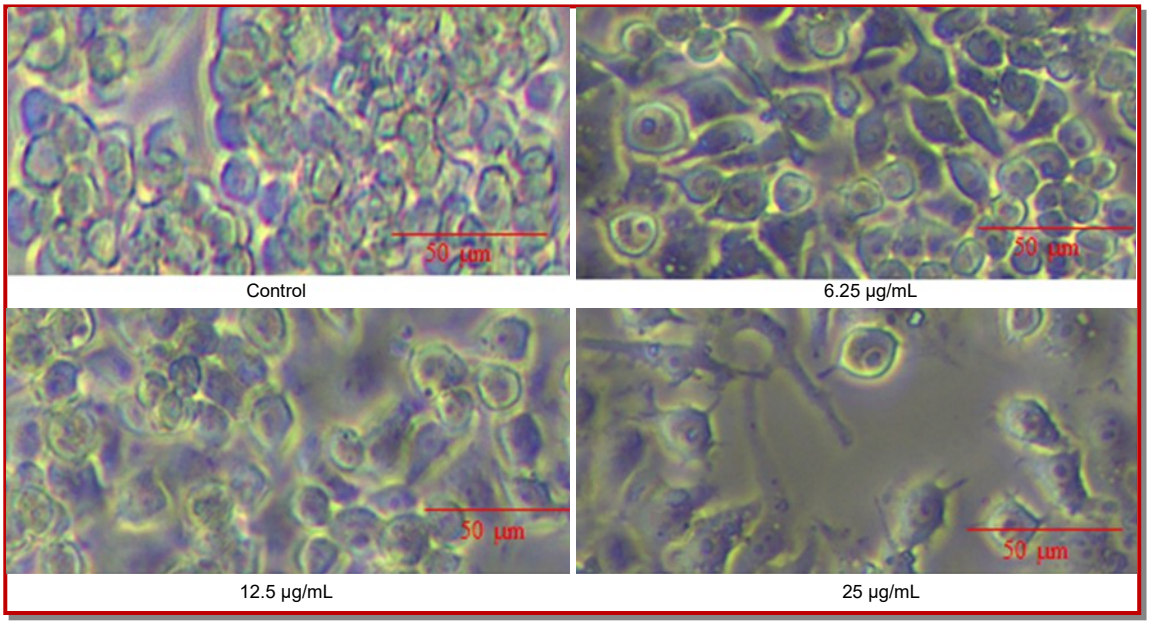

Figure 2: Phase-contrast microscopy showing the morphology of treated/control MDA-MB-231 cells. Experiments were performed in triplicate

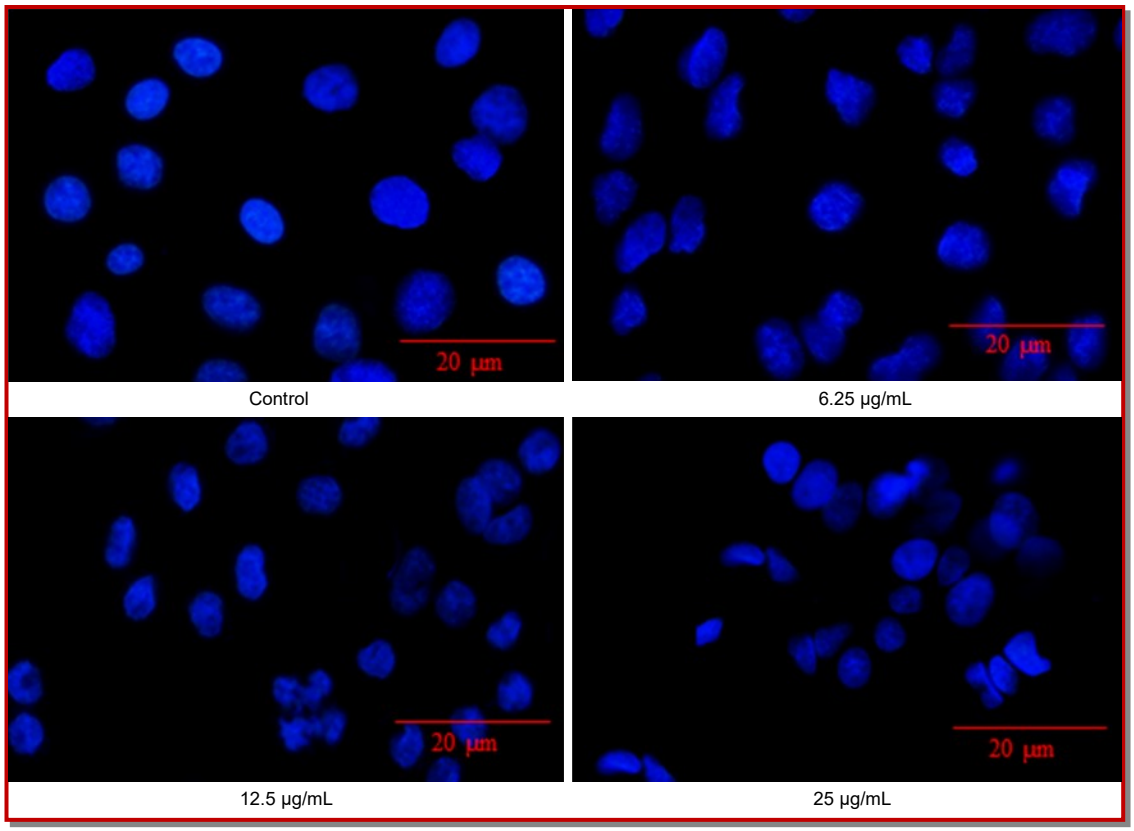

Figure 3: The DAPI staining assay showing nuclear morphology of *N. sativa* extract treated and control MDA-MB-231 cells. Experiments were performed in triplicate

that *N. sativa* plant extract induced apoptosis in MDA-MB-231 cells, and the percentage of apoptotic cells increased from 1.2% in control to 37.6% at 25 µg/mL (Figure 4A). Further confirmation of apoptosis was provided by the results of the western blotting assay, wherein the extract was found to cause a considerable increase in the expression of Bax, caspase-3, cleaved caspase-3, and cleaved-PARP. However, a concomitant decrease was seen in the case of Bcl-2 expression (Figure 4B). All these findings confirm the induction of apoptosis in extract-treated MDA-MB-231 cells.

Effect of N. sativa on cell cycle distribution of MDA-MB-231 cells

Next, the effects of the extract were also examined on the progression of MDA-MB-231 cells in different phases of the cell cycle. The results showed that upon treatment with extract, the MDA-MB-231 cells started accumulating at the G_0/G_1 phase of the cell cycle in a concentration-dependent manner. Compared to 50.1% cells in control, the G_0/G_1 phase MDA-MB-231 cells increased to 87.2% at 25 µg/mL (Figure 5). These results suggest that *N. sativa* extract induces G_0/G_1 arrest in MDA-MB-231 cells.

Discussion

The plant has tremendous phytochemical diversity, and thymoquinone is one of its key ingredients (Gali-Muhtasib et al., 2004). It is found in the stem, leaf, and seed of *N. sativa*. However, higher concentrations of thymoquinone have been reported in the seeds of *N. sativa* (Gupta et al., 2021). It has also been reported from several other plants such as *Satureja montana* (Grosso et al., 2009), *Origanum syriacum* (Zein et al., 2012), *Eupatorium ayapana* (Wanner et al., 2012) and *Monarda didyma* (Sovova et al., 2015). Thymoquinone has been reported to exhibit *in vitro* anti-cancer effects against A549 human lung cancer cells (Samarghandian et al., 2019) and MCF-7 breast cancer cells (Motaghed et al., 2013). Moreover, it was found to induce apoptosis in Jurkat lymphoblastic cells (Soltani et al., 2017). The anti-cancer activity of *N. sativa* against hepatocellular carcinoma has also been reported (Ahmad and Tabassum, 2024). In the present study, the anti-cancer activity of *N. sativa* ethanolic extract was evaluated against different human cancer cell lines. It was observed that the extract induced significant antiproliferative effects on all cell lines. However, more pronounced effects were observed against the triple-negative breast cancer cells. Triple-negative breast cancer (TNBC) cells lack three key receptors; estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2) (Zhu et al., 2023). These receptors play a crucial role in the growth and development of breast cancer cells, and their absence in TNBC makes it more challen-

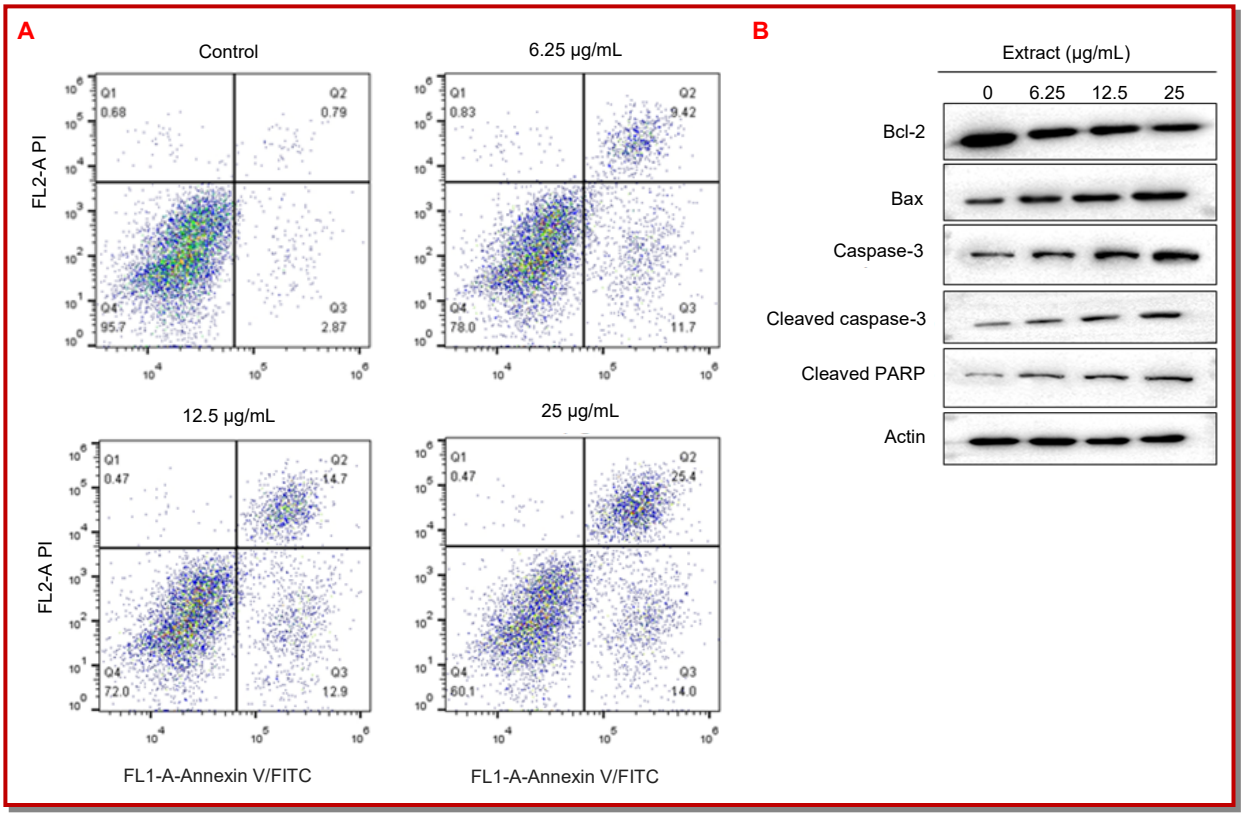

Figure 4: *N. sativa* extract induces apoptosis in MDA-MB-231 cells (A); Annexin V/PI stating assay quantifying the apoptotic effects of the extract of *N. sativa* in MDA-MB-231 cells using flow cytometry. B) Western blotting assay showing the effect of the extract on different apoptosis-related proteins. Experiments were performed in triplicate

ging to treat compared to other types of breast cancer (Maqbool et al., 2022). In this study, it was found that *N. sativa* extract could suppress the proliferation of TNBC MDA-MB-23 cells highlighting the potential of *N. sativa* as an important source of anti-cancer agents.

In previous studies the seed extract of *N. sativa* was found to trigger apoptosis in HeLa cells (Shaffi et al., 2009) and lymphoma U937 cells (Arslan et al., 2017). In yet another study seed oil of *N. sativa* was shown to induce ROS-mediated apoptosis in hepatocellular carcinoma cells (Al-Oqail et al., 2017). Taking clues from these studies, the effects of plant extract of *N. sativa* were also examined on the apoptosis of MDA-MB-231 cells. The results showed that *N. sativa* extract induced apoptosis in MDA-MB-231 cells. Apoptosis is a normal mechanism that enables cells to control their growth and eliminate abnormal cells. Apoptosis is often disrupted within cancer cells, leading to unregulated cell division and tumor development. Thus, apoptosis targeting is regarded as a viable strategy in cancer treatment (Kasibhatla and Tseng, 2003). Therapies that target apoptosis attempt either to restore cancer cells to a normal apoptotic process or increase their susceptibility to apoptotic cues (Faber et al., 2012). Thus, the current findings imply that anti-cancer effects of *N. sativa* are at least partially due to stimulation of apoptotic cell death in MDA-MB-23 cells. Bax is a pro-apoptotic protein that plays a crucial role in initiating the apoptotic pathway and Bcl-2 is an anti-apoptotic protein that counteracts the actions of Bax. The level of Bax and Bcl-2 in a cell must be balanced to determine whether or not apoptosis will occur (Czabotar and Garcia-Saez, 2023). In the present study, it was observed that in MDA-MB-231 cells, *N. sativus* extract increases the expression of Bax and decreases the expre-ssion of Bcl-2. Furthermore, *N. sativa* extract stimulated PARP and caspase-3 cleavage in MDA-MB-231 cells. In experimental studies, the presence of cleaved PARP and caspase 3 is considered to be an important indicator of apoptosis (Porter and Jänicke, 2003). Similarly, PARP is involved in DNA repair and its cleavage is triggered by caspase 3. The cleaved PARP loses its DNA repair activity and contributes to the fragmentation of DNA observed during apoptosis (Vermeulen et al 2005).

Further, defective cell cycle control in cancer cells results in higher proliferation rates, leading to the development of cancer (Williams and Stoeber, 2012). During division, cells proceed through several checkpoints and cell cycle phases. Targeting particular checkpoints in the cancer cell cycle is one common strategy (Benada and Macurek, 2015, Visconti et al., 2016). The cell assesses the integrity of its DNA and its preparedness to go on to the subsequent stage of the cell cycle at these checkpoints, which serve as control

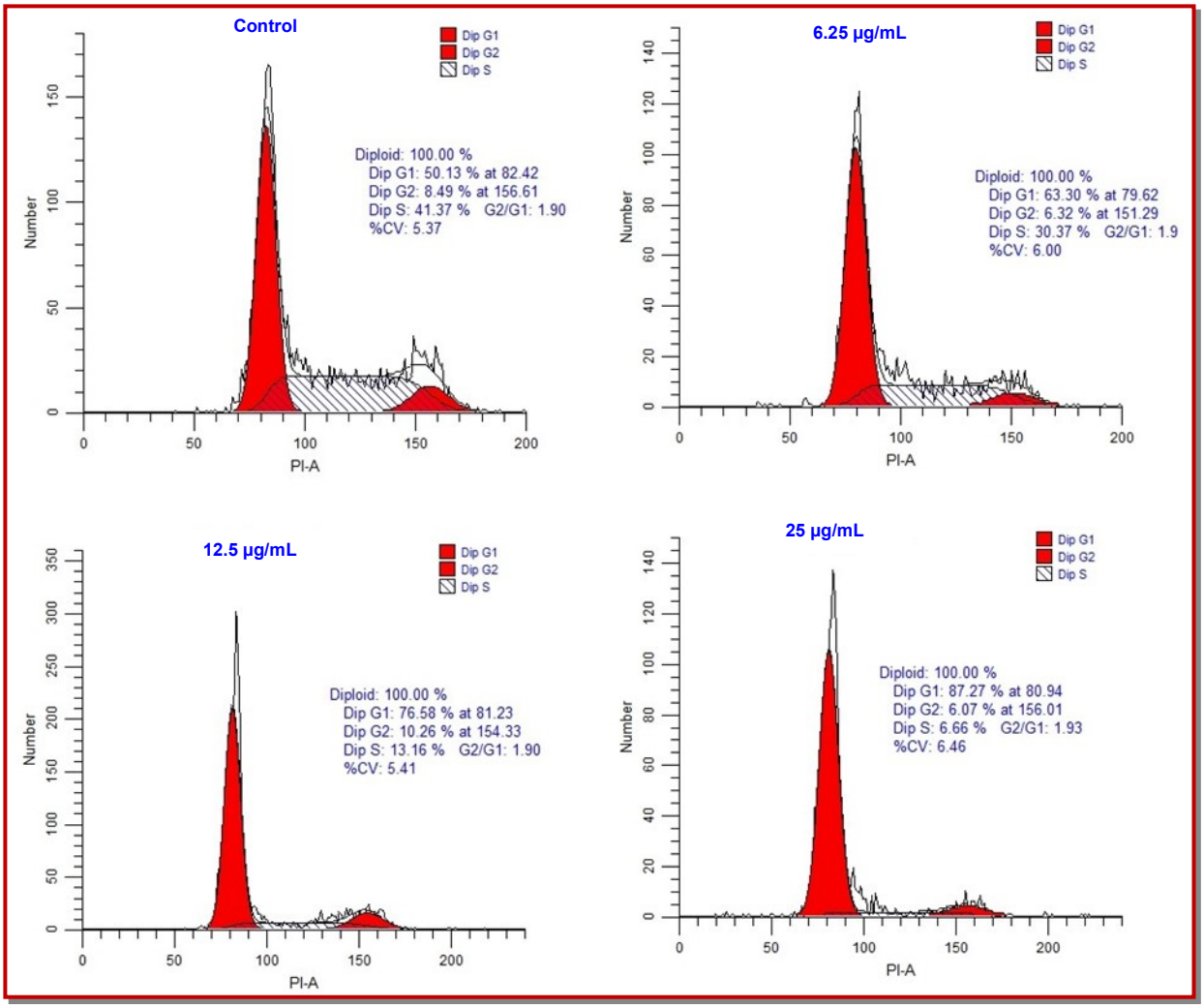

Figure 5: Effect of *N. sativa plant* extract on distribution of MDA-MB-231 cells at different phases of cell cycle. The figure depicts an increase in percentage of G_0/G_1 phase cells of MDA-MB-231 cells with increase in the concentration of the extract. Experiments were performed in triplicate

points. Cell cycle arrest can be induced at these checkpoints by activating signaling pathways or applying particular inhibitors, inhibiting cancer cells from proliferating (Benada and Macurek, 2015). In the present study, it was found that *N. sativa* extract causes arrest of MDA-MB-231 cells at G_0/G_1 checkpoint thereby inhibiting their proliferation. These results are consistent with a previous study wherein an important ingredient of *N. sativa*, thymoquinone, was found to cause G_1/S arrest of rat hepatocellular carcinoma cells (Raghunandhakumar et al., 2013). Thus, the findings of the present study indicate considerable anti-cancer potential of *N. sativa* plant extract. However, the results need to be verified in animal models of breast cancer.

Conclusion

N. sativa extract possessed remarkable potential to inhibit the proliferation of triple-negative breast carcinoma cells. The extract exerted anti-cancer effects in MDA-MB-231 cells via the induction of apoptosis and G_0/G_1 cell cycle arrest.

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Ethical Issue

The development, acquisition, authentication, cryopreservation, and transfer of cell lines between laboratories were followed according to the guidelines published in British Journal of Cancer, 2014. The research ethics committee of Jingzhou Central Hospital, Jingzhou Hospital, Yangtze University, Jingzhou, Hubei, China approved the study under approval number JCH-92CL-2023. No human subjects were used.

Conflict of Interest

Authors declare no conflict of interest

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