



## Anticancer Activity of Ethyl-acetate Fraction of *Allium sativum* in Breast Cancer MCF-7 Cells

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### ABSTRACT

Garlic (*Allium sativum*) is a medicinal plant used as a food spice with numerous ethnopharmacological applications for treating illnesses. The study intended to determine whether the major phytochemicals found in active fraction of garlic extract have the ability to inhibit the growth of breast cancer MCF-7 cells *in vitro*. Different fractions of *Allium sativum* extract were subjected to MTT assay, and the most active *Allium sativum* ethyl acetate fraction (ASEAF) was selected for the examination of ROS estimation, apoptosis study, mitochondrial membrane potential (MMP), and cell cycle analysis by flow cytometer. MCF-7 cells treated with ASEAF exhibited apoptotic-like morphology; however, there was no substantial cytotoxicity towards the normal Vero cell line. The confirmation of apoptotic cell death and necrosis was achieved by the use of a double staining kit. Notably, ASEAF showed an increase in ROS level and a reduction in MMP. A cell-cycle investigation revealed that the ethyl acetate fraction arrested the cells in the S phase resulting in cellular apoptosis. In conclusion, this study supports the therapeutic application of ASEAF that could be effective against human breast cancer.

**Keywords:** *Allium sativum* ethyl acetate fraction; Anticancer; Breast cancer, GC-MS analysis

Volume 6, Issue 15, Nov 2024

Received: 15 Aug 2024

Accepted: 25 Oct 2024

Published: 15 Nov 2024

## INTRODUCTION

The low survival rate and spreading behavior of the cancer have resulted in a rampant and continuous global health burden (Miller et al., 2022). Breast cancer, being the most prevalent malignancy among Indian women and the second most common malignancy among women worldwide, presents as a leading cause of mortality due to cancer disease. Apoptosis is a process of cell death biologically, and it is more protective for humans since it does not show any kind of inflammatory reaction; thus, it can be used as a viable target for anticancer treatment (Yuan et al., 2022, Suraweera et al., 2020, Pfeffer et al., 2018). One main function of apoptosis is to fight against cancer and the process involves several biological processes including cell contraction, nuclear condensation, chromatin aggregation, production of reactive oxygen species (ROS), modulation of MMP, apoptotic body formation, and DNA destruction (Upadhyay et al., 2024, Lopez et al., 2015, Roos et al., 2013). The currently available treatments mainly chemotherapy and radiation therapy may damage the normal cells leading to adverse side effects in patients. Despite significant advancements in the diagnosis and cure of breast cancer, the disease cannot be cured with the medications currently available on the marketplace. Thus, plant-based substances may be an intriguing approach to improving the therapy of breast cancer by inducing apoptosis of cancer cells but avoiding the healthy cells. Plant-based substances such as flavonoids, alkaloids, phenylpropanoids, and terpenoids are being employed in the treatment of infectious disorders and breast cancer considering their beneficial effectiveness along with minimal toxic effects (Yuan et al., 2022, Kushwaha et al., 2020, Sindhu et al., 2021).

Because of its therapeutic qualities, garlic (*Allium sativum*) has been used in the medical field to treat and prevent a wide range of illnesses (Tesfaye et al., 2021). Furthermore, due to its carminative, antipyretic, sedative, aphrodisiac, and diuretic properties, garlic has long been used as a traditional home medicine for heart problems, stomach, respiratory, and urinary tract infections (Cho et al., 2009). Garlic and its associated chemicals have long been known to possess a wide range of biological actions, such as antihypertensive, antibacterial, antifungal, renoprotective, anticarcinogenic, antioxidant, and antidiabetic effects (Block. E., 2010, Ilić et al., 2011). *A. sativum* has been identified with its medicinal properties as antimicrobial, antiseptic, antiviral, antioxidant, anticancer, immunostimulant, cardioprotective, and

hypoglycemic (Botas et al., 2009). White *A. sativum* bulb extract has shown a range of pharmacological and biological properties, such as anti-inflammatory, anti-microbial, and anti-tumor properties (Park et al., 2012). Garlic consumption and the development of cancer are inversely correlated by epidemiological research (Galeone et al., 2009). The main components of *A. sativum* i.e. organo-sulfur compounds have an anticancer effect against a large variety of cancer cells (Li et al., 2018). Out of 33 raw vegetable extracts, a previous study found that raw garlic extract was the most potent and highly selective anticancer agent, exhibiting activity against a variety of cancer cells while sparing non-cancerous cells (Li et al., 2018). Moreover, a growing body of research indicates that garlic inhibits the growth and metastasis of gastric, liver, prostate, and lung malignancies by triggering apoptosis and blocking cell cycle progression (Tang et al., 2013, Wen et al., 2004, Shin et al., 2012, Cao et al., 2008). Surprisingly, the process of apoptosis employing *A. sativum* crude extract more importantly *Allium sativum* ethyl acetate fraction (ASEAF) against breast cancer cells MCF-7 has not yet been studied.

The present study was focused on investigating the anticancer effect of ASEAF against MCF-7 cells by employing MTT assay, flow cytometry, and fluorescence microscopy for MMP analysis, ROS generation, and cell cycle arrest. Additionally, gas chromatography-mass spectrometry (GCMS) testing was also performed to find out the major chemical compounds in ASEAF.

## **MATERIAL AND METHOD**

### **Chemicals and Reagents**

Sigma Aldrich, USA supplied antibiotics (P/S) solution, DMEM/F-12 media, FBS, and DCFH-DA and Rhodamine-123 dyes. DAPI and MTT dyes were acquired from Himedia, India, and Annexin V FITC apoptosis kit was obtained from Biovision, USA. Analytical-grade reagents were used in the experiment.

### **Extract preparation and fractionation of Garlic bulb**

White garlic bulbs were bought from a nearby vegetable market in Lucknow, India. The garlic plant sample was recognized (specimen no. IU/PHAR/HRB/20/04) and deposited at the Pharmacognosy and Phytochemistry Department, Integral University, Lucknow. Garlic bulbs

were crushed into a fine, homogeneous gel. The 95% ethanol solution (1:4, volume weight) was allowed to soak into the homogenized gel for 72 hours at room temperature. The filter paper called Whatman No. 1 (125 mm) was used to filter and integrate the resulting solvents. Employing a Rotavapor (BuchiRotavapor R-205, Switzerland), the resultant filtrate was concentrated under vacuum at 45°C. To create a semi-solid paste, the extracted material was additionally concentrated in a water bath at 45°C. It was then sealed in an airtight container and kept at 4°C until needed for additional investigations. The ethanolic extract of the garlic bulb is made up of a diverse range of phytochemical components with varying polarity. By fractionating using solvents such as n-hexane, chloroform, ethyl acetate, acetone, and water of different polarities, these ingredients were divided into multiple groups of compounds of distinct polarities as previously mentioned (Babbar et al., 2014). Till further analysis, the acquired sub-fractions were kept in a sealed airtight container. Based on the initial cytotoxicity investigation, the most effective fraction was analyzed for GC-MS characterization and subsequent in vitro cell death research.

### **Cell line and culture**

Breast cancer cell MCF-7 and normal kidney epithelial cell line Vero were procured from the NCCS, Pune, India. Cells were cultured in culture flasks containing DMEM: F12 (1:1) medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 1% antibiotic solution in an incubator (Model-371, FORMA-STERI CYCLE, Thermo Scientific, USA) at 37°C and 5% CO<sub>2</sub>.

### **MTT assay**

According to a standard protocol, the screening MTT test of different fractions from *A. sativum* ethanolic extract was conducted against MCF-7 cells (Siddiqui et al., 2014). MCF-7 cells were seeded containing  $1 \times 10^4$  cells/mL in a 96-well plate overnight. Stock solution of different fractions was made in culture medium and was further diluted in the same media to 5, 10, 15, 18, and 20 mg/mL concentrations to treat cultured cells for 24 hours. After the incubation time, the absorbance values were determined by passing a stock of MTT dye with a concentration of 5 mg/mL through an ELISA plate reader (Bio-Rad PW41, USA) set to 550 nm. The IC<sub>50</sub> value of each fraction was determined with the help of the GraphPad Prism 5.1 version. An inverted

microscope (Nikon Eclipse TS100, Japan) was used to detect structural alterations at the cellular level. The most active fraction was also tested against normal kidney epithelial cell line Vero.

#### **Nuclear condensation assay**

The most active fraction, ASEAF was further subjected to apoptotic cell death analysis. The apoptosis-inducing impact of ASEAF was investigated at three doses, which include 11mg/mL as low dose (LD), 13.57mg/mL as IC<sub>50</sub> value, and 16mg/mL as high dose (HD). Nuclear condensation was detected using DAPI staining according to a previously described method (Siddiqui et al., 2019). Images of the dyed cells were captured using a fluorescence microscope, Zeiss AxioVert 135, US.

#### **Double staining assay**

The apoptotic cells were counted using an Annexin V-FITC Apoptosis Kit through flow cytometry, FACS Lyric, BD Biosciences, USA. Briefly, MCF-7 cells at a density of  $1 \times 10^6$  cells/mL were subjected to ASEAF LD, IC<sub>50</sub>, and HD treatments for 24 h. Upon collection of cells, the pellets were suspended in a binding buffer and stained in the dark with 5 µl of Annexin V-FITC and 5 µl of PI at 25°C for 15 minutes. Flow cytometry was utilized to determine the percent of apoptotic, live, and necrotic cells.

#### **Analysis of intracellular ROS level**

The levels of ROS within the cells were quantified using DCFH-DA dye and assessed through fluorescence microscopy photography and flow cytometry after a 12-hour incubation period (Siddiqui et al., 2015). The fluorescence intensity within the individual cells was examined using an inverted fluorescent microscope. Both the treated and untreated cells were gathered, rinsed with PBS, and placed in PBS solution containing 10 µM DCFH-DA dye at a temperature of 37°C for 20 minutes to measure the level of ROS. Subsequently, the cells were rinsed two times with PBS and then made ready for analysis using flow cytometry.

### **Evaluation of MMP**

Changes in MMP were determined by the fluorescent probe Rh-123, as described previously (Fan et al., 2015). Fluorescence microscopy images of the treated and untreated cells were taken. Cells incubated with 10  $\mu$ M Rh-123 in the dark for 30 minutes were prepared for flow cytometry analysis. The washed cells were suspended in 500  $\mu$ l of PBS and then prepared for the flow cytometric analysis.

### **Analysis of cell cycle checkpoints**

After seeding the cells at a density of  $1 \times 10^6$  cells/mL in a T-25 flask, ASEAF was employed at different doses for 24 hours. Cells were stained with PI dye and flow cytometry analysis was used to examine the cell cycle checkpoints as explained previously (Ahamad et al., 2014).

## **RESULTS**

### **Effect of ethyl acetate fraction on cellular morphology and cell viability**

MCF-7 human breast cancer cells were exposed for 24 h to increasing concentrations (5–20 mg/mL) of ASEAF. The calculated  $IC_{50}$  values of ethyl acetate were found to be 13.57 mg/mL of concentration. ASEAF treatment gave noteworthy structural differences when compared to the untreated MCF-7 cells. Untreated cells exhibited the usual characteristics of attached homogenous and even cell surface whereas treated breast cancer cell MCF-7 cells became nonattached and spherical in morphology (Figures 1A). As apparent in Figures 1A and 1D, ASEAF reduced the cell viability of the MCF-7 cells to 88.37, 68.60, 46.50, 32.56%, and 25.58% at 5, 10, 15, 18, and 20 mg/mL concentrations, respectively. Figure 1D shows the percent cell viability of MCF-7 cells at various concentrations of the most active ASEAF after 24 hours. Figure 1B shows the normal Vero epithelial cells, after 24-hour treatment with ASEAF. Treated cells did not display the characteristic morphological change in the same dose-dependent manner compared to MCF-7 cells. Only a few numbers of normal cells showed a non-adherent, unattached upon higher ASEAF doses after 24-hour exposure. Vero cells showed % cell viability at 96.92, 96.71, 96.33, 97.20, and 96.42%, respectively, at the dose level of 5, 10, 15, 18, and 20 mg/mL (Figure 1C). Based on  $IC_{50}$  value of ASEAF against-

MCF-7 cells, three effective doses- low dose (LD=11 mg/mL), IC50 dose (13.57 mg/mL), and high dose (HD=16 mg/mL)- were selected for further *in vitro* cell death analysis.

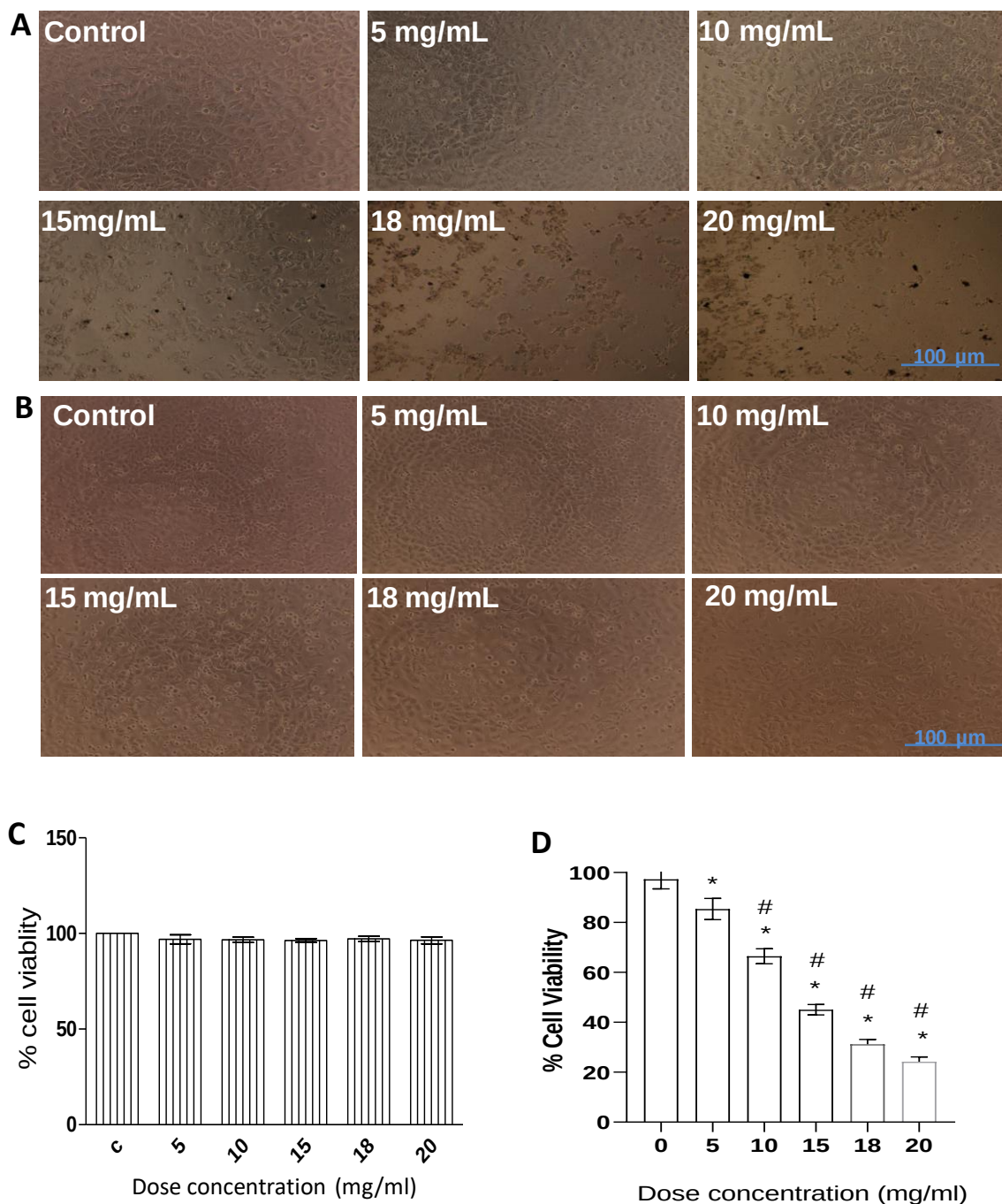


**Figure 1.** Microscopic observation and cytotoxic activity of different concentrations (5–20 mg/mL) of ethyl acetate fraction=EAF of Ethanolic garlic extract against MCF-7 and normal Vero cells. (A & B) Photomicrograph of breast cancer cells MCF-7 and normal cell Vero

respectively, treated with 5 to 20 mg/mL concentrations of ASEAF. Photomicrographs were taken with an inverted phase contrast microscope. Scale bar = 100  $\mu$ m. (C & D) Percent cell viability of Vero cells and MCF-7 at various concentrations of ASEAF after 24 hours. Values are expressed as mean  $\pm$  SD of three independent experiments. \* $p < 0.05$  as compared to control and # $p < 0.05$  as compared to low dose 5 mg/mL

### Chromatin condensation assay

As observed through fluorescence microscopy, ASEAF caused chromatin condensation in treated cells relative to control cells over a 24-hour incubation period (Figure 2). ASEAF boosted chromatin condensation in a concentration-dependent manner. At HD of ASEAF, the greatest nuclear condensation was noted.

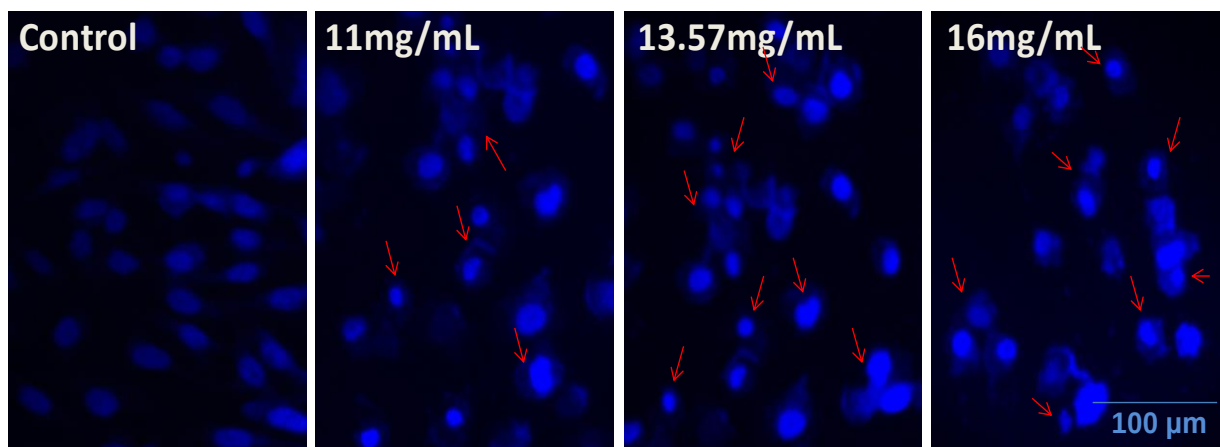


**Figure 2.** Apoptosis-inducing activity of ASEAF (A) Photomicrographs shows the chromatic condensation in MCF-7 treated cells at LD, IC<sub>50</sub> and HD of ASEAF after 24 h. Control breast cancer cells depict healthy structure while ASEAF exposure showed chromatin condensation and membrane blebbing. Scale bar = 100  $\mu$ m.

### Quantification of apoptosis

To confirm apoptosis induction by a statistical method, the MCF-7 cells were further analyzed using an Annexin V-FITC Apoptosis Detection Kit. The survival rate of the untreated cells was 97.51%, and the cells were considered healthy and viable. ASEAF exhibited cell death to 9.16% early apoptotic and 6.89% late apoptotic at 11 mg/mL concentration, 12.24% early apoptotic and 32.75% late apoptotic at 13.57 mg/mL, and 22.40% early apoptotic and 67.78% late apoptotic at 16 mg/mL concentrations (Figure 3). These results represent that at LD, ASEAF induced early apoptosis but at HD, late apoptotic features were observed.

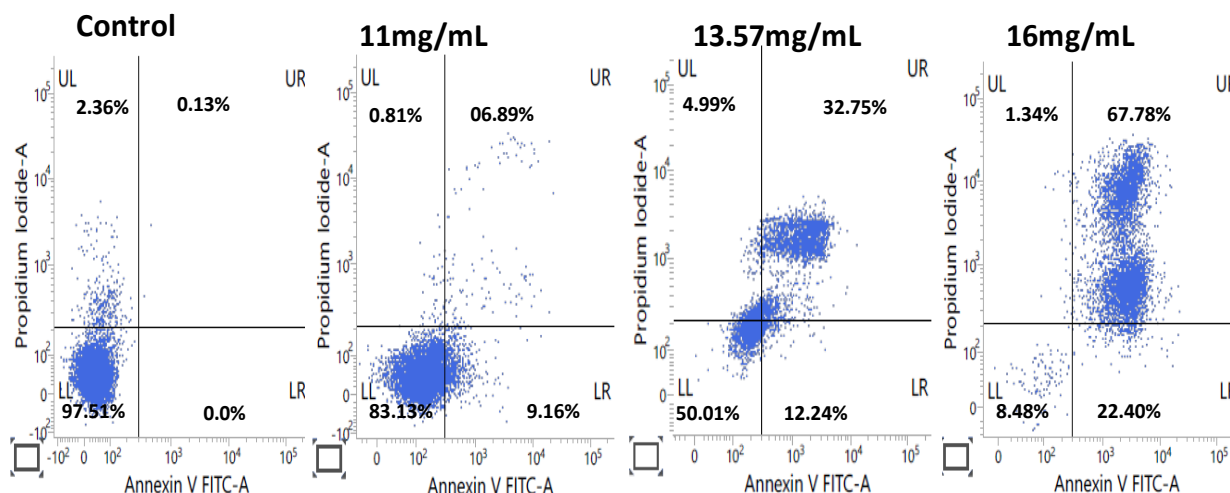


**Figure 3.** ASEAF mediated induction of apoptosis in MCF-7 cells. Flow cytometry analysis after 24 hours of treatment with three effective doses of ethyl acetate fraction using annexin V/FITC & PI double stain. Representative figures showing the population of viable (annexin V- PI-), early apoptotic (annexin V+ PI-), late apoptotic (annexin V+ PI+) and necrotic (annexin V- PI+) cells.

### ASEAF induces intracellular ROS generation

Photomicrographs indicate that, unlike untreated MCF-7 cells, treated cells showed notably higher intensities of intracellular ROS (Figure 4A). The quantitative estimation of ROS generation using a flow cytometer revealed that in control cells, the level of ROS was 0.16%, consistent with low ROS intensity typically seen in healthy cells. However, ASEAF induced ROS levels significantly to 37.95, 47.98, and 59.53% at 11, 13.57, and 16 mg/mL concentrations of ASEF, respectively (Figure 4B).

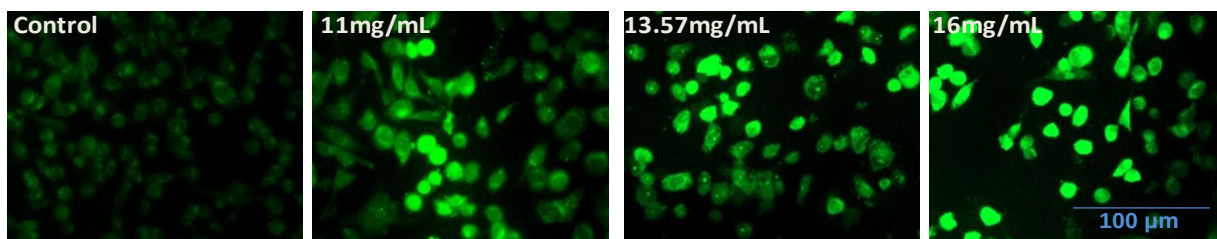
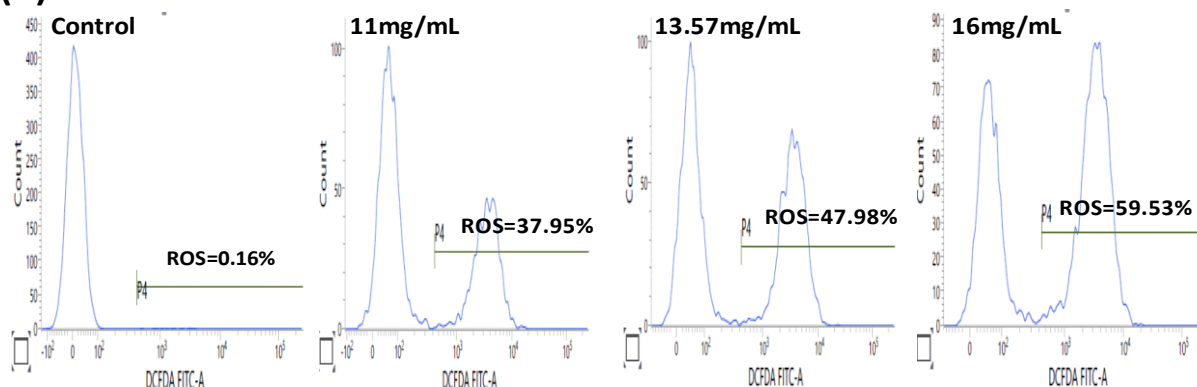
**(A)****(B)**

**Figure 4.** Intracellular ROS generation of MCF-7 cells (A) Photomicrographs showing intracellular ROS generation induced by three effective concentrations of ASEAF after 12 hours incubation. Photomicrographs were taken with a fluorescence microscope. Scale bar=100  $\mu$ m. (B) The fluorescence in the cells is represented as the percentage of ROS production analyzed using flow-cytometry.

#### Analysis of MMP loss by ASEAF

Flow cytometric and fluorescence microscopic studies showed that treated MCF-7 cells had higher MMP loss compared to the untreated ones. The loss of MMP is indicated as decreased green fluorescence of Rh 123 dyes presented in microscopic photographs. Figure 5B represents the flow cytometry result for the percent MMP activity. The findings showed a decrease in the percentage of MMP in the treated MCF-7 cells with increased concentrations of ASEAF. The green fluorescence of untreated breast cancer cells was 7.43%, while ASEAF treatments at doses of 11, 13.57, and 16 mg/mL to MCF-7 cells have been showing 72.30%, 77.80%, and 86.43% of green fluorescence, respectively.

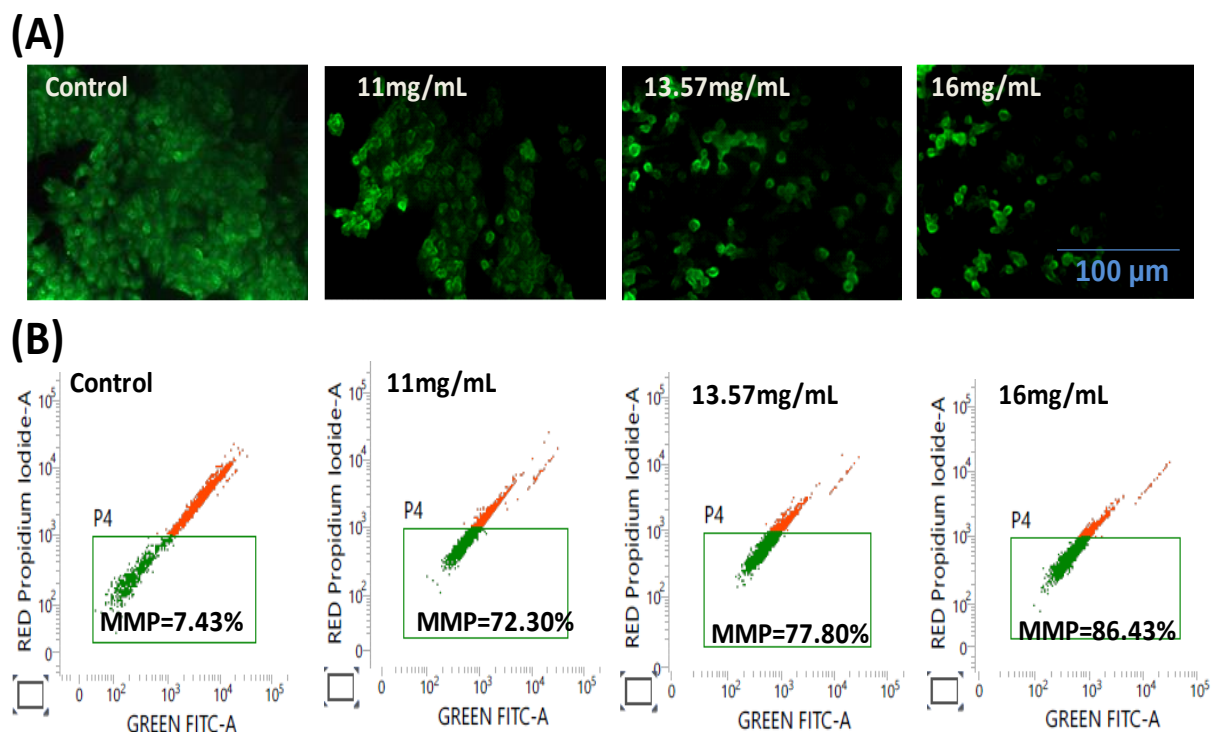


**Figure 5.** Mitochondrial membrane potential loss of breast cancer cells MCF-7 stained with Rh 123 dye. **(A)** Microscopic photomicrographs under a fluorescence microscope (scale bar = 100 μm) indicate a decrease in MMP (an early event in apoptosis) with increasing concentrations of ASEAF **(B)** Representative profile of flow-cytometer evaluation exhibits the percentage of cell population with mitochondrial membrane potential loss.

### ASEAF induces S and G2/M phase arrest

The representative cell cycle distribution at different concentrations of ASEAF is depicted in Fig. 6. The ASEAF treatment at desired doses increased the S and G2/M phase distribution of MCF-7 cells while decreasing the number of MCF-7 cells in the G0/G1 phase. These results suggested that ASEAF induced S and G2/M phase checkpoints of the cell cycle in MCF-7 cells. Cells arrested in cell cycle checkpoints may enter into either DNA repair or cellular apoptosis.

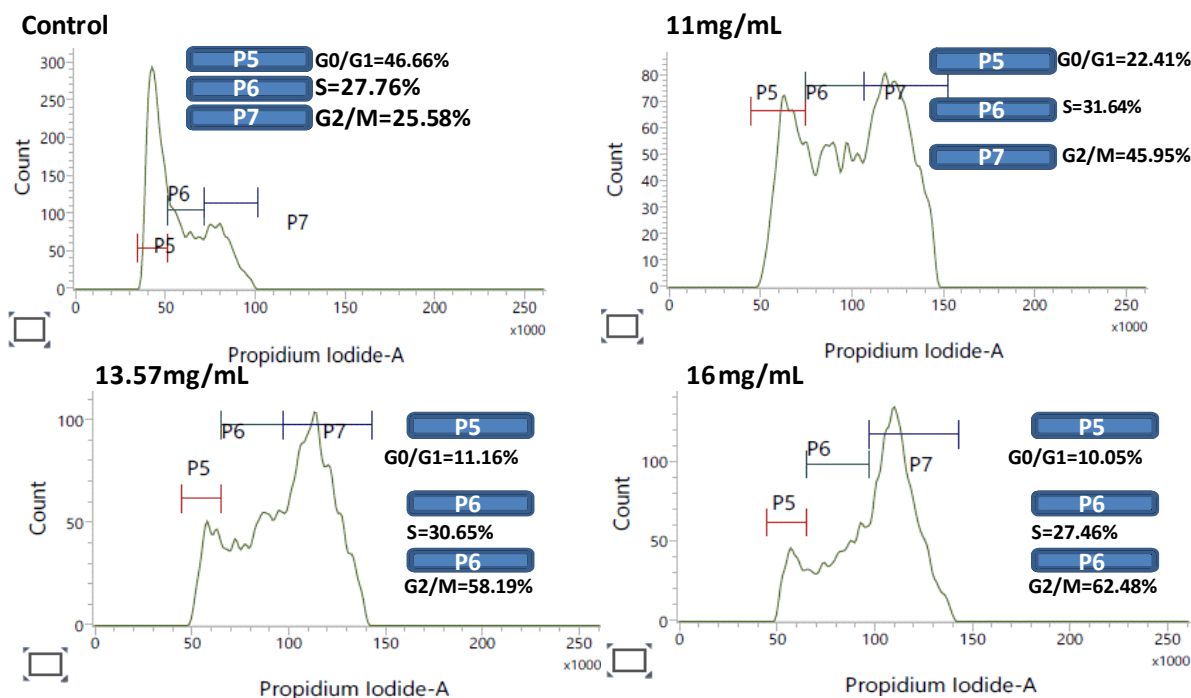


**Figure 6.** DNA content analysis of MCF-7 cells by flow-cytometry. Pictorial graph showing the mean proportion of cells in different phases of cell cycle following treatment with three effective concentrations of ASEAF for 24 hours.

## DISCUSSION

Breast carcinoma is the most common cause of mortality in cancer among women and affects nearly two million women annually (Youlden et al., 2014). Natural treatments by herbs and phytochemicals in particular have drawn much attention due to their application in the treatment and prevention of cancer-related problems along with available chemotherapy drugs (Haiaty et al., 2020). Much of the research that might have nailed drugs to fight cancer has emanated from the ethno-traditional use of medicinal plants (Gonzales et al, 2006). In a previous study, crude garlic extract was found to exhibit the most potent and highly selective anticancer therapeutic action following its comparison against 33 other natural extracts on different cancer cells without causing damage to the benign cells (Li et al., 2018).

The finding of the present study revealed that ASEAF exhibited anticancer potential against breast cancer MCF-7 cells and induced apoptotic cell death in cancer cells leaving healthy cells unaffected. A study has reported that the sulfide constituent occurring in oils is due to allicin

degradation. In garlic, the formation of allicin occurs when the garlic bulb is crushed secreting an enzyme alliinase which converts it into alliin, as allicin is a very unstable substance, it undergoes quick reactions that result in the formation of sulfur derivative components (Banerjee et al., 2003). In the cytotoxic study, ASEAF did not cause any significant change in morphology and deleterious effects on the survival of the normal cell line Vero (Figure 1B). ASEAF-treated breast cancer cells showed spherical, shrink in size, and detachment of cells from cultured plate surface (Figure 1A). This investigation provided early evidence of programmed cell death. The ASEAF treatment against the MCF-7 cells had an  $IC_{50}$  value of 13.57 mg/mL which showed prominent cytotoxicity. A recent study has shown that the  $IC_{50}$  values of Ajwa date pulp extract against MDA-MB-231 cells at 24 and 48 h were 17.45 and 16.67 mg/mL, respectively, while garlic crude extract exhibited  $IC_{50}$  values of 13.69 mg/mL against the human breast cancer cells MDA-MB-231 cells (Upadhyay et al., 2024Khan et al., 2021). It has been noted that the effective concentration of pulp extract from the plant is comparatively high which is due to the large content of polysaccharides. Nuclear condensation results showed typical features of apoptosis, viz. fragmentation of nuclei, chromatin condensation, shrinking of cells, and dispersed apoptotic cells in ASEAF-treated MCF-7 cells (Figure 2). Further, Annexin-V/PI dual staining showed early and late apoptosis in ASEAF-treated MCF-7 cells as analyzed by flow cytometry to provide statistical evidence for these findings. These results depicted that though there was a moderate increase in early apoptosis, late apoptosis was drastically increased as the percentage viability of cells was reduced. Early apoptosis was induced by ASEAF and late apoptosis was forced by ASEAF which resulted in the death of cancer cells.

To provide more evidence that the death of cells occurs through oxidative stress, the production of intracellular ROS in ASEAF-treated MCF-7 was investigated. Data on increased ROS production showed that MCF-7 cells were subjected to mitochondrial oxidative stress destabilization, leading to cell death upon the treatment with ASEAF (Figure 4A &B). ROS has been regarded as one of the major regulators of both the extrinsic and intrinsic pathways of cell survival and apoptotic mechanisms (Davalli et al., 2016, Siddiqui et al., 2019). The overwhelming production of ROS brings about the disruption of the plasma membrane and cytoskeleton which ultimately leads to chromosomal destruction (Guo et al., 2013). Moreover,

it was found that there was a loss of MMP as evidenced by Figure 5A & B, indicating the mitochondrial membrane disruption. Qualitative and quantitative data from flow cytometry and fluorescence microscopy showed the impairment of mitochondrial membrane structure and the induction of intrinsic apoptosis in ASEAF-treated MCF-7 cells. However, mitochondria also play important roles in many cellular processes unrelated to the production of ATP, including the control of membrane capacity, calcium signaling, metabolism, and apoptotic cell death (Khan et al., 2021). Facilitation of MMP depletion can be associated with enhanced cancer treatment efficacy (Khan et al., 2021, Delierneux et al., 2020). Cell cycle investigation of current research indicated that a higher proportion of cells in the S and G2/M phases equated to that of control cells, although the proportions of cells in the G0/G1 phases were reduced (Figure 6). The current results are consistent with a recently published study, which showed that exposure to ajwa dates pulp extract in HCC cells and garlic crude extract in MDA-MB-231 cells promoted the percentage of cells in the S and G2/M phases while decreasing the number of cells in the G0/G1 phase (Upadhyay et al., 2024, Siddiqui et al., 2019).

## CONCLUSION

This study examined the inhibitory effects on growth and the induction of cell death caused by active ASEAF in MCF-7 cells. GC-MS analysis indicated the presence of 2,4-Dimethyl-5,6-dithia-2,7-nonadienal (8.82%), followed by Diallyldisulphide (5.70%) and 2-Heptadecanol (4.65%), predominantly in ASEAF, which may contribute to the induction of apoptotic cell death. The observed effect correlated with ROS production and MMP loss in MCF-7 cells. Furthermore, ASEAF induced DNA damage that halted the cell cycle at the S and G2/M phases of MCF-7 cells and subsequently led to apoptosis. In summary, ASEAF has the potential to emerge as a groundbreaking and efficacious treatment for breast cancer in the future; however, further animal and clinical investigations are necessary to justify its therapeutic use.

**Declarations****Ethical Approval**

Not applicable

**Consent to Participate**

All authors agree to participate in and publish this work, and they affirm that it is original research.

**Consent to Publish**

If the manuscript accepted, the term and condition of the publisher will be followed.

**Authors Contributions**

Shivbrat Upadhyay, Rumana Ahmad, Mohsin Ali Khan, and Sahabjada Siddiqui conceived and designed the study; Shivbrat Upadhyay, Raushan Kumar, and Ishrat Husain performed the experiment; Shivbrat Upadhyay, RumanaAhmad, Mohsin Ali Khan, Alok Singh, Raushan Kumar, Sneha Ghildiyal, Manjari Baluni, Ishrat Husain, Tanveer Ahamad, Indrajeet Singh and Sahabjada Siddiqui analyzed the data. All authors contributed to the manuscript writing, editing and final proof.

**Funding**

Not applicable

**Competing Interests**

There are no related financial or non-financial interests to disclose.

**Availability of data and materials**

All data were obtained from the experiments and third party is not involve in any data or materials in this manuscript.

**Acknowledgments**

The authors acknowledge to Department of Biotechnology and Department of Biochemistry, Era University, Lucknow, India for the cell culture facility. The authors would also like to acknowledge Mr. Dayanand Vishwakarma, and Mr. A.L. Vishwakarma, senior technicians, CDRI, Lucknow, India for their support in GC-MS and flow-cytometry analysis. The authors also extend their appreciation to Taif University for supporting the work.

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