

Growth inhibition and induction of apoptosis in estrogen receptor-positive and negative human breast carcinoma cells by *Adenocalymma alliaceum* flowers

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Abstract

Adenocalymma alliaceum (*A. alliaceum*) is well known for its traditional medicinal uses and as a substitute for garlic. The methanol extract from *A. alliaceum* flowers (*AAF*) was investigated for its growth inhibitory activity on the estrogen receptor positive MCF-7 and estrogen receptor negative MDA-MB231 breast cancer cells by MTT assay. Treatment of breast cancer cells with different concentrations of *AAF* resulted in dose dependent growth inhibition with a growth inhibitory concentration (GI_{50}) of $53.1 \pm 4.1 \mu\text{g/mL}$ in MCF-7 and $23.9 \pm 3.7 \mu\text{g/mL}$ in MDA-MB-231 cells. Treatment of breast cancer cells with *AAF* resulted in time-dependent sequence of events marked by apoptosis, as shown by translocation of phosphatidylserine and activation of caspase-3. Analysis of data suggests that *AAF* exerts growth inhibition on both breast cancer cells through apoptosis induction, and that it may contain potent anticancer secondary metabolites valuable for application in drug products.

Keywords : *Adenocalymma alliaceum*, breast cancer cells, apoptosis, enrichment factor, sulphur compounds

1. Introduction

Breast cancer is the second leading cause of cancer-related deaths (1). The treatments

include surgery, radiation, and in some cases, drugs that have a specific target such as tamoxifen in estrogen-dependent tumours (2). However, the majority of cases, especially those that result in metastasis, are still treated with conventional chemotherapy. The problem in drug resistance is a major obstacle in chemotherapeutic treatment, therefore, there is a great need for the development of new therapeutic drugs that will be more efficient or will synergise with existing ones.

There has been a growing interest in the use of herbs as a potent source of new therapeutic anticancer drugs. Plants contain a wide variety of secondary metabolites that have potent biological effects, including anticancer activity (3). In this research we focused on the growth inhibitory effect of the *Adenocalymma alliaceum* flowers on breast cancer cells and its mode of action.

Adenocalymma alliaceum Miers. (family: Bignoniaceae), commonly known as 'garlic creeper', is native to the Amazon rain forests of South America. The leaves and flowers are widely consumed by Brazilians as a substitute for garlic (4). The plant has a number of traditional medicinal properties such as antimycotic, analgesic, antiarthritic, anti-inflammatory, antipyretic, antirheumatic, antitussive, depurative, purgative, and vermifuge

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properties (5). It contains nonacosane, oct-1-en-3-ol, octacosan-1-ol, pentatriacont-1-en-17-ol, hentriacontane, hexacosan-1-ol, hexatriacontane, trithiacyclohexene, n-triacontane, triacontan-1-ol, 24-ethyl-cholest-7-en-3 β -ol, 3 β -hydroxy-urs-18-en-27-oic acid, β -sitosterol, stigmasterol, fucosterol, glycyrrhetol, daucosterol, dotriacontan-1-ol, β -amyrin, β -peltoboykinolic acid, ursolic acid, α -4-hydroxy-9-methoxy-lapachone, α -9-methoxy-lapachone, apigenin, cosmosiin, cyanidin-3-O- β -D-rutinoside, luteolin, n-scutellarein-7-O- β -D-glucuronide, aspartic acid, glutamic acid, leucine, 1-2:3-vinyl-dithi-4-ene, 1-2:3-vinyl-dithi-5-ene, dithiacyclopentene, allin, allyl sulfides, diallyl sulfides and triallyl sulfides (5-11).

As part of our continuing search for bioactive natural products, in the present study we examined the effect of methanol extract from *A. alliaceum* flowers (*AAF*) on estrogen receptor positive (ER +ve) MCF-7 and estrogen receptor negative (ER -ve) MDA-MB-231 human breast cancer cells, including the mode of cell death. Results of the present study show that *AAF* has potent growth inhibitory effect on both cells. Its activity is through apoptosis as detected by the adhesion of annexin V to phosphatidylserine on the outer leaflet of the cell membrane and activation of caspase-3.

2. Material and Methods

2.1 Plant material

Fresh flowers of *Adenocalymma alliaceum* was collected in the city of Tirupati, Andhra Pradesh state and identified by Dr. Madhava Chetty, Department of Botany, Sri Venkateswara University, Tirupati. The flowers were dried in hot air oven at a temperature not more than 50°C. The dried flowers were powdered using an electric blender. Powdered flowers (50 g) were extracted with methanol using soxhlet extractor. The methanol extract was concentrated in rotary evaporator at a temperature

not more than 50°C. The concentrated methanol extract was dried using freeze dryer at -33°C.

According to the National Cancer Institute (NCI), USA; a crude extract may be considered as potent cytotoxic if its $IC_{50} \leq 20 \mu\text{g/mL}$ (12). So, in the present study, the highest concentration of extract used was 150 $\mu\text{g/mL}$. Dried extract was dissolved in 50% (v/v) methanol in ultra pure water to obtain the final concentrations of 150, 140, 130, 120, 110, 100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 2.5, 1.25 and 0.625 $\mu\text{g/mL}$. For proliferation and apoptosis assays, the solutions of different concentrations of extract were sterilised by passing them through 0.22 μm membrane filters.

2.2 Reagents

MTT assay kit and Dual Apoptosis assay kit were purchased from Biotium, USA. Cell Death Detection ELISA PLUS kit was purchased from Roche Applied Sciences, Germany.

2.3 Cells and culture condition

Breast-adenocarcinoma cells MCF-7 (ER +ve) and MDA-MB-231 (ER -ve) from ATCC (Rockville, MD) were cultured in Dulbecco's Modified Eagle's medium (DMEM) with 10% FBS and 2 mM L-glutamine, 1% penicillin/streptomycin (PenStrep) under a fully humidified atmosphere, 5% CO_2 at 37°C. For experiments, cells were collected from subconfluent monolayers with accutase. The studies were carried out using cells from passages 3-7.

2.4 Proliferation assay

The effect of *AAF* on the viability of the cancer cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazoliumbromide) assay. Briefly, 99 μL of the cell suspensions were plated in 96-well flat-bottomed tissue culture plates (Nunc, Denmark) at a concentration of $1 \leftrightarrow 10^4$ cells/well. After incubating the plates for 24 hours at 37°C in a

humidified incubator, different concentrations of sterilised *AAF* solutions were added to the respective wells of the plate. The final volume in each well was 100 μ L. Each concentration of *AAF* was repeated in minimum of three wells in each plate and the assay was repeated in at least two plates. The plates were further incubated for 48 hours. Five microliters MTT reagent was added to each well and incubated for 4 h after which the plates were centrifuged at 600g for 5 min at 4°C. MTT solution and medium were aspirated from the wells and 100 μ L of buffered DMSO was added to each well. The plates were shaken for five minutes and the absorbance (OD) was recorded on a microplate reader at the wavelength of 570 nm and a reference wavelength of 630 nm. The effect of *AAF* on growth inhibition was assessed as percent cell viability where vehicle (0.5%v/v methanol in water)-treated cells were taken as 100% viable. Percentage of cell viability in each well was calculated using the formula:

Percentage of viable cells =

$$\frac{-OD \text{ of the extract} - OD \text{ medium control}}{OD \text{ vehicle control} - OD \text{ medium control}} \sqrt{\leftarrow 100}$$

The GI_{50} value, the concentration of *AAF* required to reduce the cell growth by 50%, was evaluated from the dose-response curve.

Untreated and vehicle treated cells were incubated as controls. The final concentration of methanol in each well did not exceed 0.5% (v/v). This concentration did not affect the apoptosis or cell proliferation of the investigated cells.

2.5 Apoptotic death assays

Apoptotic death assays were carried out at only one concentration of *AAF*, GI_{50} , on MCF-7 and MDA-MB-231 cancer cells. Mode of cell death (apoptosis) was qualitatively determined using Dual Apoptosis assay and quantified using Cell death detection by enzyme-linked immunosorbent assay (ELISA).

2.5.1 Dual apoptosis assay

This assay was carried out using Dual Apoptosis Assay Kit with NucView™ 488 caspase-3 substrate & sulforhodamine 101-annexin V (Texas Red)-annexin V according to instructions in the product protocol. This kit detects two important apoptosis events, caspase-3 activation and phosphatidylserine (PS) translocation in a single experiment. The MCF-7 and MDA-MB-231 cells were incubated for 24 hours in a humidified CO₂ incubator on coverslips, which were previously coated with poly-L-lysine. The cells were challenged with the extract and the negative control cells were challenged with the 0.5% (v/v) methanol in water. The cells were incubated further for 6 hours and 12 hours to observe the apoptotic changes. After the respective incubation periods, the culture medium was aspirated and the cells were washed with annexin V binding buffer. Then annexin binding buffer (100 μ L), 0.2 mM NucView™ 488 caspase-3-substrate (5 μ L) and sulforhodamine 101-annexin V (5 μ L) were added to each cover slip and incubated for another 45 minutes. The coverslips were washed with annexin V binding buffer and mounted in annexin V binding buffer on to slides. The apoptotic events in the stained cells were observed under a fluorescence microscope using FITC and Texas-Red filters. The positively stained apoptotic cells were counted and the apoptotic index was calculated as the number of apoptotic cells relative to the total number of cells.

2.5.2 Cell death detection by enzyme-linked immunosorbent assay (ELISA)

The mechanism of cell death, i.e. apoptosis or necrosis was quantitatively determined using the Cell Death Detection ELISA^{PLUS} assay (13) as recommended by the manufacturer. This kit can detect and quantify both apoptosis and necrosis. Briefly, 99 μ L of the cells suspensions (MCF-7 and MDA-MB-

231) were plated in 96-well flat-bottomed tissue culture plates at a concentration of $1 \leftrightarrow 10^4$ cells/well. After incubating for 24 hours at 37°C in a humidified incubator, sterilised solutions of *AAF* were added to the respective wells of the plate. The final volume in each well was 100 μ L. Each solution was repeated in minimum of three wells. The plate was centrifuged at 600g at 4°C for 10 minutes. The DNA fragments released from the cells due to necrosis were present in the supernatant layer. The supernatant was carefully transferred without disturbing the cell pellets into a glass vial and stored in a refrigerator at 4°C until further analysis for necrosis. The cell pellet containing the apoptotic bodies was resuspended in lysis buffer and incubated for 30 minutes at room temperature. The plate was centrifuged and cell lysate was transferred into a glass vial and stored in a refrigerator at 4°C until further analysis for apoptosis.

Supernatant and cell lysate solutions (20 μ L) were placed in triplicate into wells of streptavidin coated microplate and added 80 μ L of the immunoreagent, containing a mixture of anti-histone-biotin and anti-DNA-POD. The plate was covered with an adhesive cover foil and incubated for 2 hours at 25°C in a shaking incubator at 300 rpm. During the incubation period, the anti-histone antibody binds to the histone-component of the nucleosomes and simultaneously captures the immunocomplex to the streptavidin-coated microplate via its biotinylation. At the same time, the anti-DNA-POD antibody reacts with the DNA-component of the nucleosomes. The unbound antibodies were washed with incubation buffer. The amount of nucleosomes retained by the POD in the immunocomplex, corresponding to the extent of apoptosis and necrosis, was quantitatively determined photometrically with ABTS as substrate using microplate reader at a wavelength 405 nm and reference wavelength of 490 nm.

2.6 Statistical analysis

All the data represented were mean \pm S.D (standard deviation) of triplicate. Statistical analyses were conducted using SPSS ver. 15.0 software. The significance between control and treated groups was performed by student's t-test and p values less than 0.05 were taken as significant.

3. Results

To evaluate the activity of *AAF* on the growth of mammary cancer cells, the MCF-7 cells, a well established model for the in vitro investigation of estrogenic activities and MDA-MB-231 (ER -ve) cells were employed.

3.1 Growth inhibitory activities of *A. alliaceum* flowers

The effect of *AAF* was studied as a dose-response experiment for 48 h at the dilutions of 0.25-150 μ g/mL. Both cells exhibited significant *AAF*-induced suppression of growth. A dose-dependent inhibition of cell growth was observed between 4-80 μ g/mL (Fig. 1). The growth inhibitory effect of *AAF* was more pronounced in MDA-MB-231 cells, which was mirrored in its GI₅₀ concentrations (Fig. 1). The 50% inhibitory

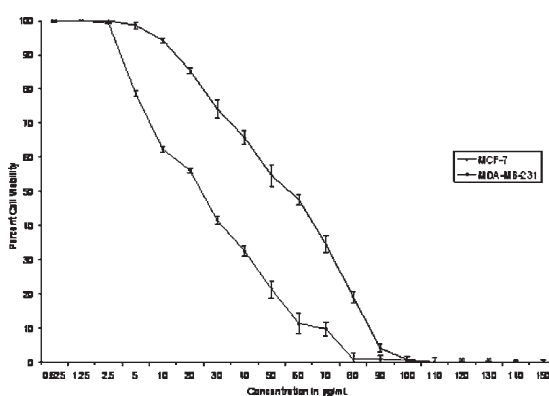


Fig. 1 Growth inhibitory activities of methanol extract from *A. alliaceum* flowers on MCF-7 and MDA-MB-231 breast cancer cells. The percentage cell viability was determined in triplicate. Data represent the mean \pm SD. The percent cell viability was significantly ($P < 0.05$) different from control at concentrations; \oplus 5 μ g/ml in MDA-MB-231 and \oplus 10 μ g/ml in MCF-7.

concentration of *AAF* was $53.1 \pm 4.1 \mu\text{g/mL}$ (MCF-7) and $23.9 \pm 3.7 \mu\text{g/mL}$ (MDA-MB-231). The growth of both cells was inhibited almost completely by $100 \mu\text{g/mL}$ of *AAF*. At concentration of extract of up to $2 \mu\text{g/mL}$ no effect was observed on the proliferation of both cells.

3.2 *Adenocalymma alliaceum* flowers induced apoptosis in MCF-7 and MDA MB 231 cells

In principle, a reduction of cell growth can reflect either a decreased proliferation rate or an enhanced cell death by either necrosis or apoptosis or a combination of these two

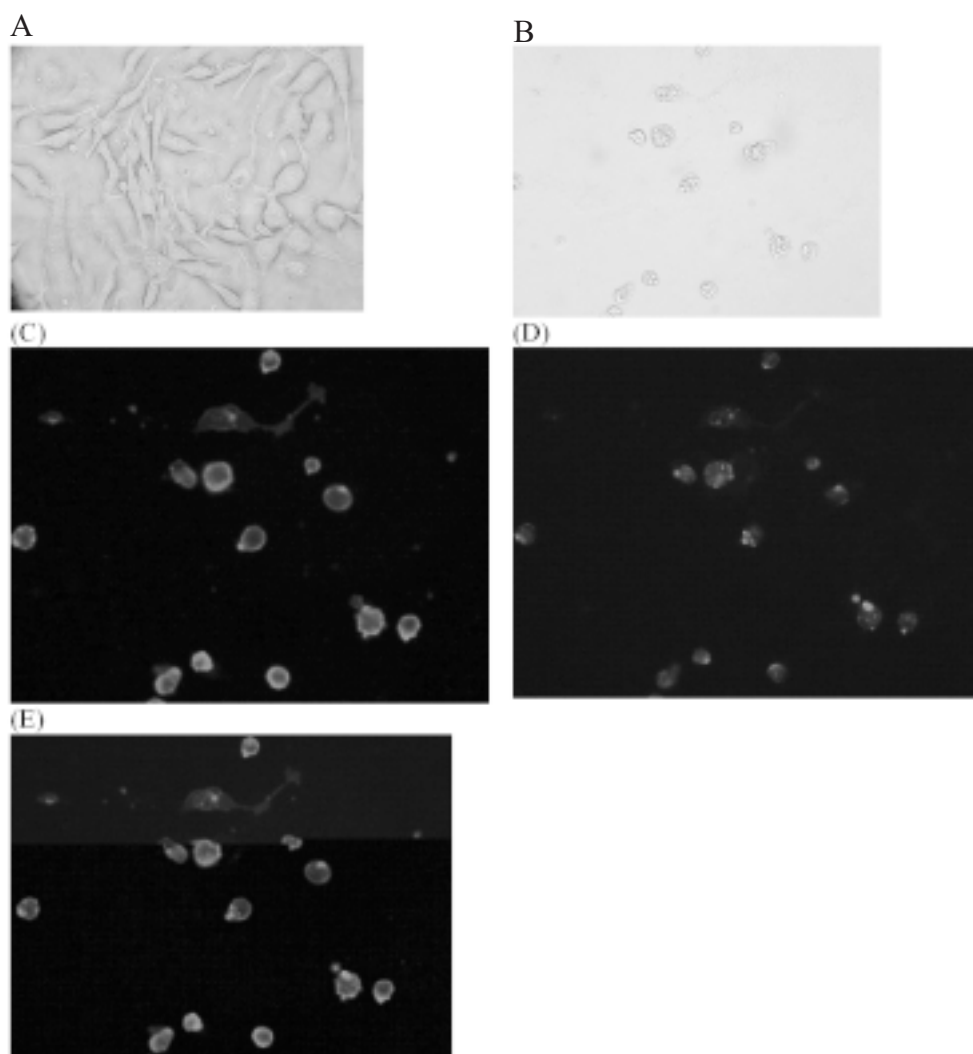


Fig. 2 Morphological changes of the MDA-MB-231 cells after 12 h treatment (A) solvent (0.5% v/v methanol) only; (B) with $25 \mu\text{g/mL}$ of *AAF* viewed under bright field and stained cells viewed under FITC & Texas-red filters using fluorescent microscope (C) red border around the cell indicating PS translocation; (D) green nucleus in the cell indicating caspase-3 activation; (E) green nucleus surrounded by red border indicating the apoptotic cell.

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mechanisms. We also investigated whether the extracts can induce apoptosis by monitoring the two important apoptosis markers: phosphatidylserine (PS) translocation on cell membrane (identified by sulforhodamine 101-Annexin V, 14) and caspase-3 activation (identified by NucView™ 488 caspase-3 substrate, 15). The morphological changes were inspected by microscopy. Some cells were beginning to detach from the plate and becoming rounded after 6 h treatment of *AAF* (50µg/mL for MCF-7 and 25µg/mL for MDA-MB-231). Because loss of adhesion to the culture dishes of tumoral epithelial cells has been described as an apoptosis-related event (16), we examined the morphological apoptotic changes on slides under bright field after 6 h and 12 h. In opposite to good spreaded cells in the negative control, a morphological change with cell shrinkage was detected in cells treated with *AAF* extract. Necrosis was less prominent than apoptosis. In Fig. 2 the representative photomicrographs of MDA-MB-231 cells after treatment with 25 µg/mL *AAF* extract in comparison to solvent treated control are shown.

To further substantiate the growth inhibitory effects of *AAF*, the apoptotic cells were monitored by Annexin V adherence and caspase-3 activation. In viable cells, PS is located on the cytoplasmic surface of the cell membrane; in apoptotic cells, PS is translocated from the inner to the outer leaflet of the plasma membrane, thus exposing PS. Annexin V is a Ca²⁺ dependent phospholipids-binding protein with high affinity for PS. The binding of sophorodamine101-AnnexinV probe to PS that has translocated to the outer membrane cell produces red border around the cell under fluorescent microscope using red filter (Fig. 2). Caspase-3 (CPP32) is a cytosolic protein that normally exists as a 32-kDa inactive precursor. It is cleaved proteolytically into a heterodimer when the cell undergoes apoptosis (17). The cleavage of NucView™488 caspase-3 substrate by activated caspase-3 stains the cell nucleus green (Fig. 2). The induction of apoptosis by *AAF* was time-dependent (Fig. 3). In MCF-7, there were fewer apoptotic cells, *AAF* at concentration of 50µg/mL inducing apoptosis in 18.3 and 30.7% after 6 and 12 h treatment. In MDA-MB-231 cells an elevation in apoptosis

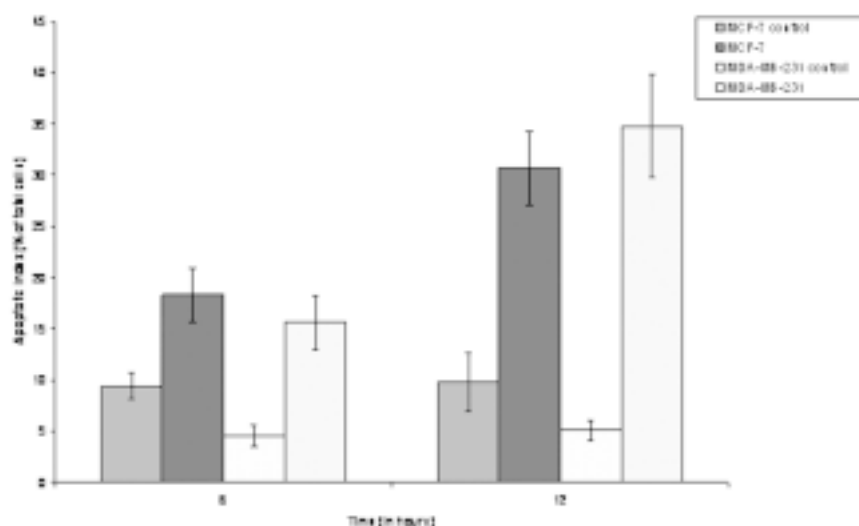


Fig. 3 Time-dependent apoptosis of MCF-7 and MDA-MB-231 breast cancer cells induced by methanol extract from *A. alliaceum* flowers. The apoptotic indices were determined in triplicate. * indicates that the results are significantly different ($P < 0.05$) from respective controls.

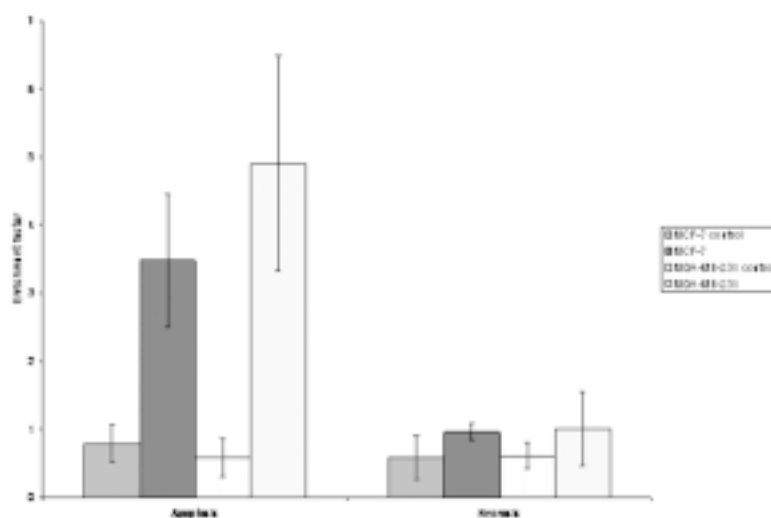


Fig. 4 Quantification of apoptosis and necrosis in MCF-7 and MDA-MB-231 breast cancer cells induced by methanol extract from *A. alliaceum* flowers. The enrichment factor was determined in triplicate. The results represent mean \pm SD. * indicates that the results are significantly ($P < 0.05$) different from respective controls. NS, indicates the results are not statistically significant.

positive cells up to 15.6% was found after 6 h and reached 34.8% after 12 h exposure to 25 μ g/mL *AAF*. The solvent controls did not increase the spontaneous apoptotic rate in the two malignant cells tested.

To quantitate and further support the finding that *AAF* exposure causes apoptosis in cancer cells we performed cell death detection by ELISA. Compared to solvent treated control, exposure of MCF-7 and MDA-MB-231 cells to *AAF* at 50 and 25 μ g/mL concentrations resulted in 3.5- and 4.9-fold increases in induction of apoptosis while necrosis induced by *AAF* is negligible (Fig. 4).

4. Discussion

The results reported herein reveal that the methanol extract of *A. alliaceum* exerts growth inhibitory action on MCF-7 and MDA-MB-231 breast cancer cells. The dying cells exhibit the ultrastructural and biochemical features that

characterise apoptosis, as shown by the loss of viability, PS translocation and capase-3 activation. Like many other plants, *AAF* extract is a multicomponent mixture with pharmacologically active substances. The growth inhibitory activity may be attributed to a number of sulphur containing compounds.

We first established the 50% growth inhibitory concentration using MTT assay. The MDA-MB-231 (ER -ve) cells were significantly more sensitive than MCF-7 (ER +ve) cells with an approximately 2-fold variation in the GI_{50} concentration of the *AAF*. The growth inhibitory action of *AAF* on breast cancer cells is dose-dependent and probably evoked by ER-mediated and non-ER-mediated mechanisms because of the various physico-chemical properties of individual components of *AAF*. Discovery of active compounds from natural products with apoptosis-inducing ability rather than cytotoxic ability is of great interest for cancer treatment. Screening for anti-cancer substances is commonly

conducted using viability assays. An inherent problem with this approach is that all compounds that are toxic and growth inhibitory, irrespective of their concentration-dependent mechanism of action, will score positive. Apoptosis is essential for normal physiological development but is also critical in eliminating any abnormal cells after exposure to genotoxic or DNA-damaging agents.

Therefore, we investigated the apoptotic changes in mammary cancer cells induced by *AAF*, using dual apoptosis assay kit. The morphological changes occurred early, after 6 h treatment with, with loss of adhesion. The apoptotic changes characterised by PS exposure (detected by Annexin V adherence) and caspase-3 activation, as shown in Fig. 2 on MDA-MB-231 cells are representative for both cells. The initial apoptotic rate of MCF-7 cells in comparison to MDA-MB-231 was higher (Fig. 3) probably due to the estrogen deprived test conditions. About 31% of ER +ve cells and about 38% ER -ve cells were undergone apoptosis after 12 h treatment with 50 and 25 µg/mL *AAF* respectively. These findings correspond with the results from proliferation assays and suggest that *AAF* cytotoxicity appears to be explained in part by the induction of apoptosis. Further, it was interesting to note that there was a significant increase in the level of apoptosis compared to necrosis in both mammary cancer cells induced by *AAF* (Fig. 4). To our knowledge this is the first report showing the growth inhibitory activity of *A. alliaceum* on cancer cells.

5. Conclusion

In conclusion, to our knowledge this is the first report showing that *A. alliaceum* flowers exhibits an growth inhibitory effect by induction of apoptosis that is associated with phosphatidyl serine translocation and caspase-3 activation in MCF-7 and MDA-MB-231 cancer cells. As apoptosis has become a promising therapeutic target in cancer research, these results confirm

the potential of *A. alliaceum* flowers as an agent of chemotherapeutic and cytostatic activity in human breast cancer cells. However, more detailed studies are required to determine the exact mechanism(s) of action of *A. alliaceum*, specifically evaluating its effects on epigenetic and signal transduction pathways.

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References

1. Ahmedin, J., Rebeca, S., Elizabeth, W., Yongping, H., Jiaquan, Xu. and Michael, J. Thun. (2009). Cancer Statistics, 2009. A Cancer Journal for clinicians, 59: 225-249.
2. Carolin, K.A. and Pass, H.A. (2000). Prevention of breast cancer. Critical Reviews in Oncology/Hematology, 33: 221-238.
3. Sporn, M.B. (2000). Chemoprevention of cancer. Carcinogenesis, 21: 525-530.
4. Zoghbi, M.G.B., Ramos, L.S., Maiya, J.G.S., De Silva, M.L. and Lun, A.I.R. (1984). Volatile sulfides of the Amazonian garlic bush. Journal of Agricultural and food chemistry, 32: 1009-1010.
5. Ravindra, S., Ashok Kumar, Chandra Shekhar Prasad, Bhawana Srivastava and Nawal Kishore Dubey. (2008). Antimycotic and antiaflatoxicogenic potency of *Adenocalymma alliaceum* Miers. on fungi causing biodeterioration of food commodities and raw herbal drugs. International Biodeterioration & Biodegradation, 62: 348-351.

6. Triguna, N. Misra., Ram. S. Singh and Hari, S. Pandey. (1991). Aliphatic compounds from *Adenocalymma alliaceum* leaves. *Phytochemistry*, 30: 541-543.
7. Apparao, M., Kjer, A., Olsen, O., Venkata Rao, E., Rasmussen, K.W. and Sorensen, H. (1981). Alliin in the garlicky taxon *Adenocalymma alliaceum* (Bignoniaceae). *Phytochemistry*, 20: 822-823.
8. Triguna, N.Misra., Ram, S. Singh., Hari, S. Pandey. and Sekhar C. Sharma. (1989). Aliphatic hydroxy-ketones from *Adenocalymma alliaceum* leaves. *Phytochemistry*, 28: 933-936.
9. Zoghbi, M.G.B., Andrade, E.H.A. and Maja, J.G.S. (2002). Volatile constituents from *Adenocalymma alliaceum* Miers. and *Petiveria alliacea* L., two medicinal herbs of the Amazon. *Flavour and Fragrance Journal*, 17: 133-135.
10. Apparao, M., Kjer, A., Madsen, J.O. and Venkata Rao, E. (1978). Diallyl Di-, tri- and tetrasulphide from *Adenocalymma alliaceae*. *Phytochemistry*, 17: 1660-1661.
11. Pandey, H.S., Sharma, S.C., Singh, R.S. and Misra, T.N. (1992). Glycyrrhetol and β -Peltoboykinolic Acid from *Adenocalymma alliaceum*, 58: 225
12. Cordell, G.A., Kinghorn, D. and Pezzuto, J.M. (1993). Separation, structure elucidation, and bioassay of cytotoxic natural products, in Colegate, S.M. and Molyneux, R.J. (eds): *Bioactive natural products*. Boca raton, CRC Press, pp: 195-216.
13. Hui Meng Er, En-Hsiang Cheng. and Ammu Kutty Radhakrishnan. (2007). Anti-proliferative and mutagenic activities of aqueous and methanol extracts of leaves from *Pereskia bleo* (Kunth) DC (Cactaceae). *Journal of Ethno pharmacology*, 113: 448-456.
14. Vermes I, Haanen, C. and Steffens-Nakken H. (1995). A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. *J Immunol Meth.*, 184: 39-51.
15. Mac Farlane, M., Merrison, W., Dinsdale, D. and Cohen GM: (2000). Active caspases and cleaved cytokeratins are sequestered into cytoplasmic inclusions in TRAIL-induced apoptosis. *J Cell Biol.*, 148: 1239-1254.
16. Desjardins, L.M. and MacManus, J.P. (1995). An adherent cell model to study different stages of apoptosis. *Exp Cell Res.*, 216: 380-387.
17. Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K. and Gallant, M. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature*, 376: 37-43.