



Autophagy Inhibition Enhances the Mitochondrial-Mediated Apoptosis Induced by Mangrove (*Avicennia marina*) Extract in Human Breast Cancer Cells

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SS and MK designed the study. Authors SS, MK and LE wrote manuscript and performed experiments. Author VBB provided general coordination of the study. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Avicennia marina* (AM) is a widely distributed mangrove plant that has been used in traditional medicine for centuries for the treatment of a number of diseases. The objective of the present study was to evaluate the leaf ethyl acetate extract of AM for its cytotoxic and apoptotic potential along with in-depth investigations of its mechanism of action in breast cancer MCF-7 cells.
Study Design: The ethyl acetate extract of leaves and stems of AM was tested against estrogen positive breast cancer cell line MCF-7 using various assays.
Place and Duration of Study: The study was carried out at King Abdullah University of Science and Technology, Thuwal, Saudi Arabia, from July 2013-June 2014.
Methodology: Dose- and time-dependent growth inhibition of cancer cells was measured using MTT assay. The mechanisms of apoptosis induction were determined using various assays: phosphatidylserine exposure, caspase-3/7 activation, mitochondrial membrane potential disruption,

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reactive oxygen species (ROS) production, cell cycle analysis, autophagy, and protein expression using western blotting. The modulation of apoptotic genes (p53, Mdm2, NF-kB, Bad, Bax, Bcl-2 and Casp7) was also determined using real time PCR.

Results: The AM extract inhibited breast cancer cell growth and induced apoptosis in a concentration dependent manner. We demonstrated a non-classical mode of apoptosis induction in MCF-7 cells by AM extract, where ROS production altered the mitochondrial membrane potential to induce apoptosis. Breast cancer cells treated with 200 µg/ml concentration of AM extract showed increased ROS production and disrupted MMP but no PARP-1 cleavage and a marked decrease in Caspase-7 protein levels (24 and 48 h) were detected. A significant amount of autophagy was also observed at the same concentration. However, treatment of MCF-7 cells with 200 µg/ml of AM extract along with the inhibition of autophagy by chloroquine, significantly increased the apoptosis from 20% to 45%.

Conclusion: Our data provide evidence that AM extract triggers ROS-mediated autophagy as well as caspase-independent apoptosis. The results also strengthen the view that concurrent targeting of apoptotic and autophagic pathways may provide effective therapeutic strategy against cancer.

Keywords: Mangrove; breast cancer; apoptosis; autophagy; reactive oxygen species; caspases.

1. INTRODUCTION

Breast cancer remains to be one of the deadly diseases in the world and is a leading cause of death among woman globally. According to World Health Organization (WHO), 508000 women died worldwide in 2011 due to breast cancer (<http://www.who.int/cancer/detection/breastcancer/en/index1.html>). Due to high rate of breast cancer and its ability to develop drug resistance, there is an urgent need to find novel drugs effective against cancer. Natural plants have been considered as an important source of molecules active against cancer and are considered as a primary source of choice for identifying new lead molecules because of their low reported toxicities and long-term use in traditional medicine.

Avicennia marina (Forsk.) Vierh., (Acanthaceae family) is an evergreen, salt-tolerant mangrove tree widely distributed along the tropical and subtropical coastlines, including the coast of the Gulf region. The earliest records of the medicinal uses of mangroves date back to year 1230. The extracts of the barks, leaves and fruits of the plant have also been used to treat the skin disorders in an ancient Egypt [1]. The plant's wax was used as an aphrodisiac and to alleviate tooth ache in the ancient times [2]. Some of the reported traditional medicinal uses of *A. marina* (AM) extract include the astringent and anti-fertility effects [2], and treatment of rheumatism, small pox and ulcers [3]. The consumption of the leaves of the plant as an animal feed has been reported to result in some mineral deficiency in camels [4]. Toxicological studies of leaf extract of AM regarding haematological, biochemical and

pathological effects in rats [2] have reported only minor adverse effects, and hence validated the use of plant leaves as a herbal remedy and an animal feed in drought stricken areas.

Since Bell et al. [5] first reported the isolation of triterpenoids from different parts of AM, more than sixty compounds have been isolated from this plant species [6]. The versatility and adeptness to grow in conditions of high salinity, high temperatures, anaerobic soil, strong winds and changing ocean tides allow mangroves to produce a rich source of interesting metabolites. The chemical investigations of the plant have revealed the presence of terpenoids and steroids, naphthalene derivatives, flavones, iridoid glucosides, phenyl propanoid glucosides, flavonoids and abietane diterpenoid glucosides. Biological activities of the different extracts or isolated molecules from the plant species include antimicrobial [7,8,9], anti-inflammatory [10], antiplasmodial [11], antioxidant [12], antifouling [13] and anticancer effect [6,14,15,16,17,18].

Despite known anticancer properties of AM, studies focusing on the molecular mechanisms of cell death are lacking. In the present study, we evaluated the molecular mechanisms underlying the apoptotic effects of ethyl acetate extract from leaves and stems of AM (referred as AM extract) against MCF-7 (breast adenocarcinoma) cancer cell line. Since autophagy involves the recycling of macromolecules and damaged organelles and serves as a survival mechanism for cancer cell, while the excessive autophagy leads to cell death [19], we also aimed at studying the interplay of autophagy and apoptosis in AM extract treated cancer cells.

2. MATERIALS AND METHODS

2.1 Plant Material and Extract Preparation

A. marina was obtained from the coast of the Red Sea, Thuwal, Kingdom of Saudi Arabia. The coordinates of the location are 22.314865, 39.090640. Ethyl acetate extract of the stems and leaves of plant was prepared by using ASE 150 system (automated extraction system). The solution was evaporated to dryness under vacuum and weighed. The lyophilized extract was then dissolved in DMSO to produce a final concentration of 100 mg/ml and stored at -20°C until it was used later.

2.2 Cell Culturing

MCF-7 (Breast Adenocarcinoma) cell line was purchased from the American Type Cell Culture Collection (ATCC, Manassas, VA). Cells were grown in DMEM (Dulbecco's Modified Eagle's Medium), containing 10% FCS (Fetal calf serum), and antibiotics streptomycin (100 µg/ml) and penicillin (100U/ml) at 37°C and 5% CO₂.

2.3 MTT Assay

The MTT assay was used to determine cytotoxicity of AM [20]. Cells (2.5 x 10³ per well) grown in 384-well plates were treated for 48 and 72 h (hours) with 100 and 200 µg/ml of AM extract. At the end of the experiment, 5 µl of MTT (5 mg/ml) solution was added per well for 4 h (hour) followed by 30 µl of solubilisation solution (10% SDS, 10 mM HCl). The following day OD (optical density) at 595 nm was measured with a microtiter plate reader (BMG Labtech PHERAstar FS, Germany).

2.4 APO Percentage Assay

MCF-7 cells (5 x 10³ per well) were cultured in 45 µl of media in 96-well plates. The next day cells were treated with AM as described previously (MTT assay) for 24, 48 and 72 h. 10 mM H₂O₂ for 30 min (minutes) was used as a positive control. Cells were stained with APOPercentage dye as per manufacturer's instructions (Biocolor, UK). Percentage of apoptotic cells was measured by flow cytometry (IntelliCyt Corporation, Albuquerque, NM) [21].

2.5 Mitochondrial Assay

MCF-7 cells at a density of 5 x 10³ cells per well was cultured in 45 µl of media in 96 well plates. The cells were treated with AM extract for 8, 16, 24 and 48 h and stained with 2

µM cyanine dye JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide) (Life Technologies, UK) for 1 h. 10 mM H₂O₂ was used as a positive control. Cells were analyzed by using flow cytometry by plotting FL2-H vs. FL-1H and applying a quadrant gate to determine JC-1 aggregates (red) and monomers (green) [22]. The cells were also incubated with 1 µg/ml Hoechst (Pierce) dye at room temperature for 15 min and visualized using a EVOS® FLoid® Cell Imaging Station (Life Technologies, UK).

2.6 RNA Extraction

Cells were grown in 6-well plates for 24 h and treated with 100 or 200 µg/ml of AM extract. After desired incubation time, cells were washed twice with 2 ml cold 1 x PBS followed by addition of 0.6 ml of Trizol. Cell lysis was performed by incubating the cells with Trizol for 5 min at room temperature and samples were transferred to 1.5 ml eppendorf tubes and 0.12 ml of chloroform was added. Tubes were then inverted for 15 seconds and incubated on ice for 10 min followed by centrifugation at 8000 rpm for 15 min at 4°C. To the aqueous phase, 0.3 ml of isopropanol was added and RNA was precipitated overnight at -20°C. Samples were centrifuged at 8000 rpm for 30 min at 4°C. The supernatant was removed and the resulting pellet was washed with 0.6 ml of 75% ethanol. Samples were then subjected to centrifugation at 8000 rpm for 20 min at 4°C, the supernatant was discarded and the pellet was air-dried and then resuspended in 30 µl of DEPC water. RNA concentration was determined by using a Nanodrop (Thermo Scientific, USA) [21].

2.7 cDNA Synthesis

cDNA reaction was set up by taking 2 µg of RNA along with 250 ng of Oligo-dT primer (Promega) and 500 µM of dNTPs. The reaction tube was incubated at 65°C for 5 min followed by a pulse spin and then shifted to ice. Each cDNA reaction was made up to 20 µl containing 1 x first strand reaction Buffer (Invitrogen), 5 mM DTT, 200 units SuperScript III RT (Invitrogen) and 40 units of RNase (Invitrogen). The PCR cycles used were: 25°C for 10 min followed by 37°C for 2 h, 85°C for 10 min and finally 4°C hold [21].

2.8 Quantitative Real Time PCR (qRT-PCR)

Each 20 µl PCR reaction in PCR fast reaction tubes (Applied Biosystems), contained 6 µl of DI

water, 10 µl of TaqMan Universal Master Mix (Applied Biosystems), 0.6 µl (300 nM) of each forward and reverse primer (Eurofins, Germany), 0.8 µl (200 nM) probe and 2 µl of cDNA. In a StepOnePlus Real-Time PCR machine (Applied Biosystems), the PCR cycles used were: 1 cycle of 95°C for 3 min to activate the enzyme, followed by 40 cycles of 95°C for 1 second (denaturation) and 95°C for 20 seconds (annealing and extension). Fold change in gene expression was calculated using the $\Delta\Delta CT$ method [21]. The sequences of the human genome based primers, probes and details of TaqMan assays used for RT-PCR are tabulated in supplementary file 1.

2.9 Caspase-3/7 Activity

MCF-7 cells were cultured in 384 well plates at a density of 2.5×10^3 cells per well overnight. Next cells were incubated with AM extract for 8, 16, 24 and 48 h. Caspase-3/7 activity was determined using the ApoTox-Glo kit (Promega). The luminescence was measured with a luminescence plate reader (BMG Labtech PHERAstar FS, Germany). Luminescence values were normalized to cell viability (measured using MTT assay) [21].

2.10 Western Blotting

MCF-7 cells, cultured in 6-well plates at 3×10^5 cells per well, were treated with AM extract for 8, 24 and 48 h. After cell lysis with RIPA buffer (150 mM NaCl, 1% Triton X 100, 0.1% SDS, 10 mM Tris pH 7.5, 1% sodium deoxycholate) protein was harvested and quantitated with a BCA protein determination kit (Pierce Thermo Scientific, USA). Protein lysate (20 µg) underwent electrophoresis on 10% SDS page gels, was transferred to nitrocellulose membranes and probed with antibodies specific to PARP-1 (Trevigen), Caspase-7 (Sigma) and p53 (Santa Cruz Biotechnology). For a loading control β -Tubulin (Santa Cruz Biotechnology) was used [22].

2.11 Cell Cycle Analysis

MCF-7 cells were seeded at a density of 1×10^5 cells per well in a 12-well plate and left overnight to settle. Cells were treated with 100 or 200 µg/ml of AM extract for 24 h. Cells were then trypsinized and collected into 1.5 ml eppendorf tubes. Samples were centrifuged for 5 min at 1000 rpm and the supernatant was removed. The resultant pellet was fixed in 800 µl of absolute ethanol and stored at -80°C overnight.

Following fixation, samples were centrifuged at 1000 rpm for 5 min, the supernatant was removed and the pellets were resuspended in 500 µl of 1 x PBS. Samples were again centrifuged (1000 rpm for 5 min), the supernatant was removed and the pellets were resuspended in 50 µl RNaseA (Roche) (50 µg/ml) for 15 min at 37°C or 30 min at room temperature. 200 µl of PI Staining solution (0.1% Triton X-100, 2 mM $MgCl_2$, 100 mM NaCl, 10 mM PIPES pH 6.8 and 10 µg/ml PI) was added per sample and incubated for 10 - 15 min prior to analyzing on HTFC Screening System (IntelliCyt Corporation, Albuquerque, NM) [21].

2.12 Autophagy Assay

The previously reported method [23] was modified for flow cytometry. MCF-7 cells were seeded at a density of 2.5×10^3 cells per well in 45 µl of media in 96-well plates. After 24 h, AM extract was added and incubated for 8, 16, 24, 48 and 72 h. Autophagy in cells was determined by staining cells with 50 µM of MDC (monodansylcadaverine) (Sigma) for 15 min at 37°C, where 10 µM Z36 (Sigma) was used as a positive control. Pretreatment with 50 µM of Chloroquine (Sigma) for 4 h was used to inhibit autophagy, where needed. The percentage autophagy positive cells were determined by flow cytometry (IntelliCyt Corporation, Albuquerque, NM) recording a minimum of 1000 events per well.

2.13 Reactive Oxygen Species (ROS) Assay

MCF-7 cells were seeded at a density of 2.5×10^3 cells per well in 45 µl of media in 96-well plates. The following day AM extract was added and incubated for 1, 2, 3 and 4 h. ROS activity was determined by staining the cells with 10 µM DCFDA (2',7'-dichlorofluorescein diacetate) (Sigma) and incubating for 1 h. 10 mM H_2O_2 was used as a positive control. ROS activity was measured by flow cytometry (IntelliCyt Corporation, Albuquerque, NM). and a minimum of 1000 events per well was acquired [21].

2.14 Statistical Analysis

Z-factor was determined for each assay and a Z-factor of ≥ 0.6 was recorded indicating good to excellent robustness for assays [24]. Student's *t*-test was used to compare the samples (treated vs. untreated) and were found to be statistically significantly different with $P = .05$. All statistics

including mean and SD calculations were performed using Microsoft Office Excel®.

3. RESULTS

3.1 Cell Growth Inhibition and Apoptosis Induction

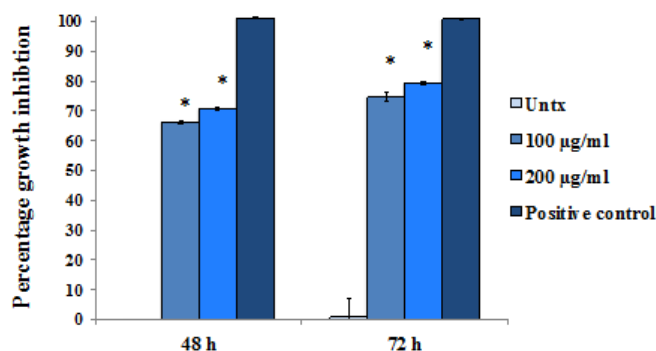
The effect of AM extract on growth inhibition of MCF-7 cell line was determined by using MTT assay. Significant cell growth inhibition i.e. 65% and 75% was observed at 100 µg/ml and 200 µg/ml of AM extracts, respectively at 48 h (Fig. 1A). The ability of AM extract to induce apoptosis was determined by using APOPercentage kit. Cells treated with 100 µg/ml of AM extract showed 10% apoptosis at 24 h and no further increase in apoptosis was detected at 48 h and 72 h time points (Fig. 1B). However, a significant increase in apoptosis was observed for 200

µg/ml of AM extract in a time dependent manner, i.e. percentage of cells undergoing apoptosis were 25% at 24 h, which further increased to 55% and 75% at 48 h and 72 h, respectively.

3.2 Disruption of Mitochondrial Membrane Potential (MMP)

Mitochondria is referred to as a powerhouse of the cell and is responsible for most of the ATP production while it maintains the proapoptotic factors like cytochrome c confined within the mitochondria. MMP disruption causes a drop in energy, which further leads to the release of proapoptotic factors and cell death. Disruption in MMP is generally measured by using JC-1 dye. In cells with disrupted MMP, JC-1 cannot aggregate and remains in a monomeric state emitting green fluorescence at ~529 nm.

A



B

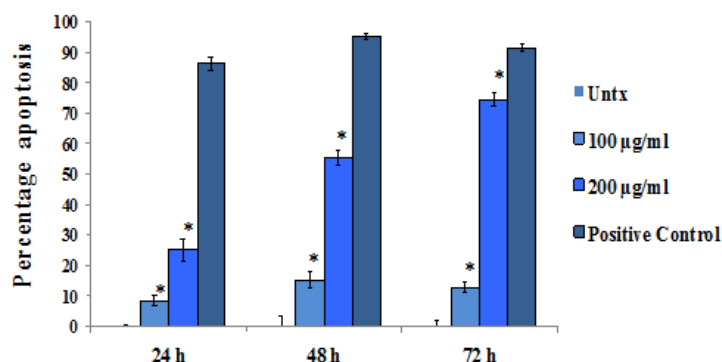


Fig. 1. Growth Inhibition and apoptosis in MCF-7 cells treated with AM extract. MCF-7 cells were seeded in quadruplicate at 5×10^3 cells per well in 96-well plate and treated with 100 or 200 µg/ml AM extract for the indicated time. (A) Growth inhibition was measured in MCF-7 cells by using MTT assay and 100 mM H₂O₂ used as a positive control. (B) The percentage cells undergoing apoptosis was assessed using the APOPercentage assay and 10 mM H₂O₂ was used as a positive control. 'Untx' represents untreated control

AM extract: significant from untreated control, * $P < 0.0001$; Z factor (> 0.7); Mean \pm SD = Mean values \pm Standard deviation of points in quadruplicate

In our study, 95% disruption of MMP was observed after 8 h of treatment with 100 µg/ml of AM extract; however, recovery in MMP with time was noticed (36% at 48 h) (Fig. 2A). For 200 µg/ml of AM extract more than 90% disruption in MMP was detected for the time course of the experiment (Fig. 2B).

3.3 Expression Analysis of Apoptosis Related Genes

qRT-PCR was used to investigate the changes in expression levels of apoptosis related genes in MCF-7 cells after 24 h of treatment with 100 and 200 µg/ml of AM extract (Fig. 3). Although, both the concentrations of AM extract downregulated the expression of most of apoptosis related genes, yet the expression of BAX and BAD (proapoptotic genes) was observed to be higher than BCL2 (antiapoptotic). Another anti-apoptotic gene, NF-kB was found to be downregulated only in response to 200 µg/ml of AM extract. No significant change in expression of CASP7 was noticed.

3.4 Caspase-independent Apoptosis

Caspase-3/7 activity was determined in MCF-7 cells treated with 100 and 200 µg/ml of AM extract for 8, 16, 24 and 48 h (Fig. 4A). Relative to untreated cells, no significant increase in caspase-3/7 activity was observed at both concentrations. Since MCF-7 cells are caspase-3 deficient, protein level of total caspase-7 was determined to further confirm the results of caspase-3/7 activity assay. Caspase-7 protein levels were also found to be reduced in cells at 24 and 48 h for both 100 and 200 µg/ml of AM extract (Fig. 4B and C). The cleavage of PARP-1 (a substrate of caspases) was also not detected, which further confirmed caspase-independent apoptosis (Fig. 4B and C). Furthermore, protein levels of PARP-1 and p53 (a regulator of cell cycle) were significantly reduced at 48 h in cells treated with 200 µg/ml of AM extract. Cell cycle distribution was also found unaltered after 24 h of treatment with AM.

3.5 Autophagy and ROS Production

Autophagy was detected as early as 8 h in AM extract treated MCF-7 cells, which continued to increase with time i.e. 66% and 84% for 100 and 200 µg/ml of AM extract respectively at 48 h (Fig. 5). Since ROS is a known inducer of autophagy, we also measured the ROS production in MCF-7 cells in response to AM

extract. In our experiments, an increase in ROS activity was observed as early as 1 h which maximized at 2 h, i.e. 52.5% and 37.4% for 100 and 200 µg/ml of AM extract, respectively (Table 1); however, ROS levels decreased with time and returned to normal levels at 8 h and 16 h (data not shown).

3.6 Effect of Autophagy Inhibition on Apoptosis

Autophagy has dual role in determining the cell fate and has been linked to both cell survival and death. In our experiments, we checked whether under stressful conditions autophagy is inhibiting the apoptosis by providing an alternative survival pathway to the cells. Cells were pretreated with the autophagy inhibitor 'chloroquine' for 1 h followed by 200 µg/ml of AM extract for 24 h. A significant increase in apoptosis i.e. from 20% to 45% was observed, which confirmed the anti-apoptotic role of autophagy in our study (Fig. 6). However, no difference in apoptosis was observed for 100 µg/ml of AM extract even after inhibiting autophagy with chloroquine.

4. DISCUSSION

A. marina has been described in the literature for its antibacterial, antifungal, anti-inflammatory and anticancer properties [7,10,18]. The present study was conducted to investigate the anticancer potential of ethyl acetate extract of leaves and stems of AM. In our initial screening for cytotoxicity, AM extract was able to inhibit metabolic growth of MCF-7 cells at 100 and 200 µg/ml concentrations. Both concentrations were able to induce the following effects: an increase in intracellular ROS production, MMP disruption, no PARP-1 cleavage, autophagy and no increase in caspase-3/7 activity. A significant increase in apoptosis from 20% to 45% was observed for 200 µg/ml of AM extract after inhibiting autophagy with chloroquine; however, no difference in apoptosis was observed for 100 µg/ml of AM extract in the same assay.

Multiple assays performed during this study such as gene expression, caspase-3/7 activity assay, caspase-7 and PARP-1 cleavage provided conclusive evidences of caspase-independent mechanism of apoptosis induction in MCF-7 cells after treatment with AM extract. We did not detect DNA damage or DNA fragmentation in our experiments. Similar observations have been reported in the published literature where MCF-7 cells treated with Resveratrol displayed loss of

MMP and underwent apoptosis; however no release of cytochrome C, caspase activation, PARP cleavage and DNA damage was recorded [25]. Shrivastava et al. [26] also observed that iodine reduced the MMP and ROS with no

concomitant activation of caspases in MCF-7 cells. Plant derived lectin 'Concanavalin A' has been shown to induce autophagic cell death via mitochondrial pathway in the absence of caspase-dependent apoptosis [27].

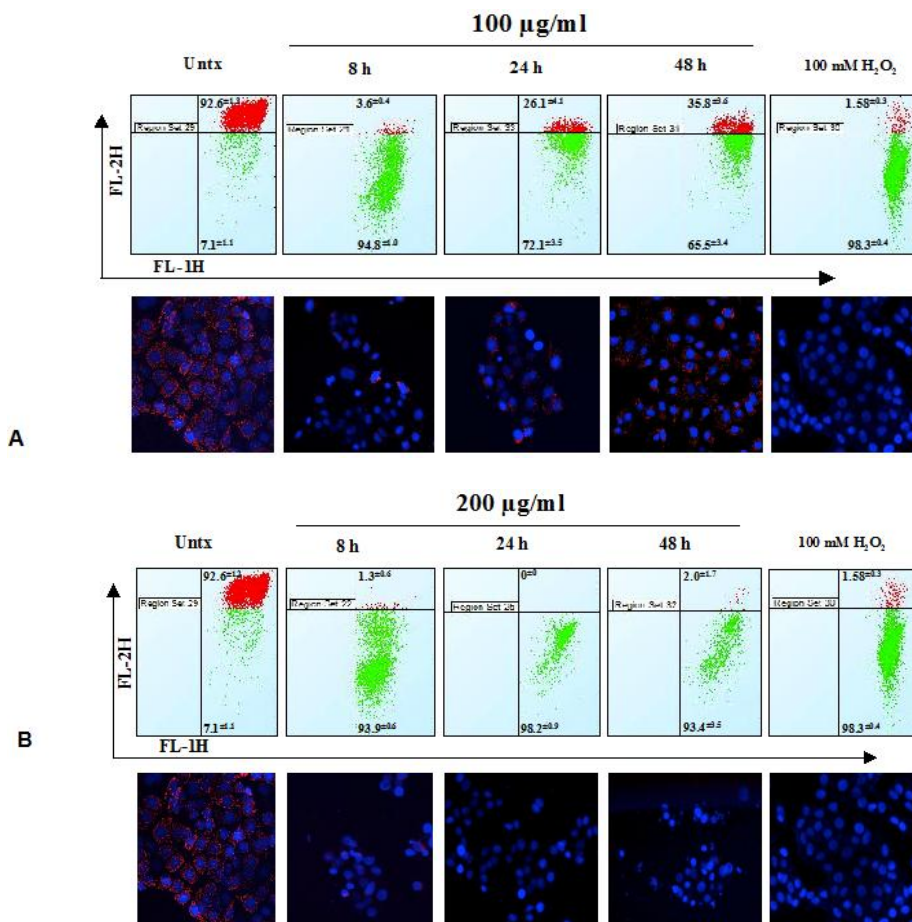


Fig. 2. MMP in MCF-7 cells treated with AM extract. MCF-7 cells were seeded in quadruplicate at 5×10^3 cells per well in 96-well plate and treated with (A) 100 µg/ml or (B) 200 µg/ml AM extract for the indicated time. Cells were stained with 2 µM JC-1 dye (red/green) and 1 µg/ml Hoechst to stain the nucleus (blue). Cells were visualized by fluorescence microscopy and analyzed by flow cytometry measuring fluorescence in the FL-2H vs. FL-1H channels. 'Untx' represents untreated control, 100 mM H₂O₂ was used as a positive control
Mean ± SD = Mean values ± Standard deviation of points in quadruplicate

Table 1. ROS generation in MCF-7 cells treated with 100 or 200 µg/ml of AM extract. Cells treated with 5 mM H₂O₂ for 1 h were used as a positive control

Sample	1 h	2 h	3 h	4 h
Untreated	2.49 ^{+/-0.70}	1.31 ^{+/-0.89}	1.65 ^{+/-1.56}	1.76 ^{+/-0.62}
100 µg/ml AM extract	32.21 ^{+/-2.34}	52.50 ^{+/-1.06}	36.64 ^{+/-1.23}	13.98 ^{+/-2.38}
200 µg/ml AM extract	22.64 ^{+/-4.84}	37.37 ^{+/-2.81}	19.88 ^{+/-2.42}	6.01 ^{+/-0.84}
5 mM H ₂ O ₂	99.71 ^{+/-0.08}	99.37 ^{+/-0.45}	99.38 ^{+/-0.42}	98.68 ^{+/-0.80}

Mean ± SD = Mean values ± Standard deviation of points in quadruplicate

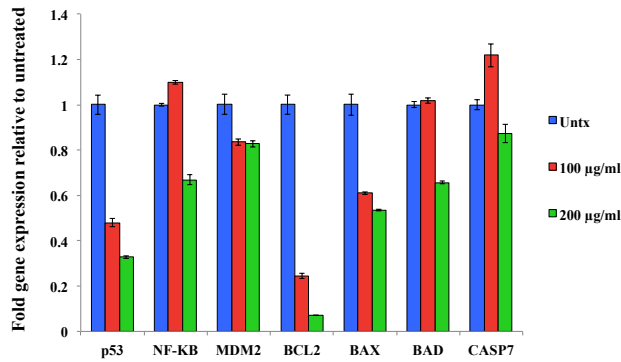


Fig. 3. Gene expression analysis of key apoptosis related genes in MCF-7 cells treated with AM extract. MCF-7 cells were seeded at 3×10^5 cells in a 6-well plate and treated with 100 or 200 µg/ml AM extract for 24 h. RNA was harvested from MCF-7 cells, converted to cDNA and gene expression for apoptosis related genes was determined by using RT-PCR
Mean ± SD = Mean values ± Standard deviation of points in quadruplicate

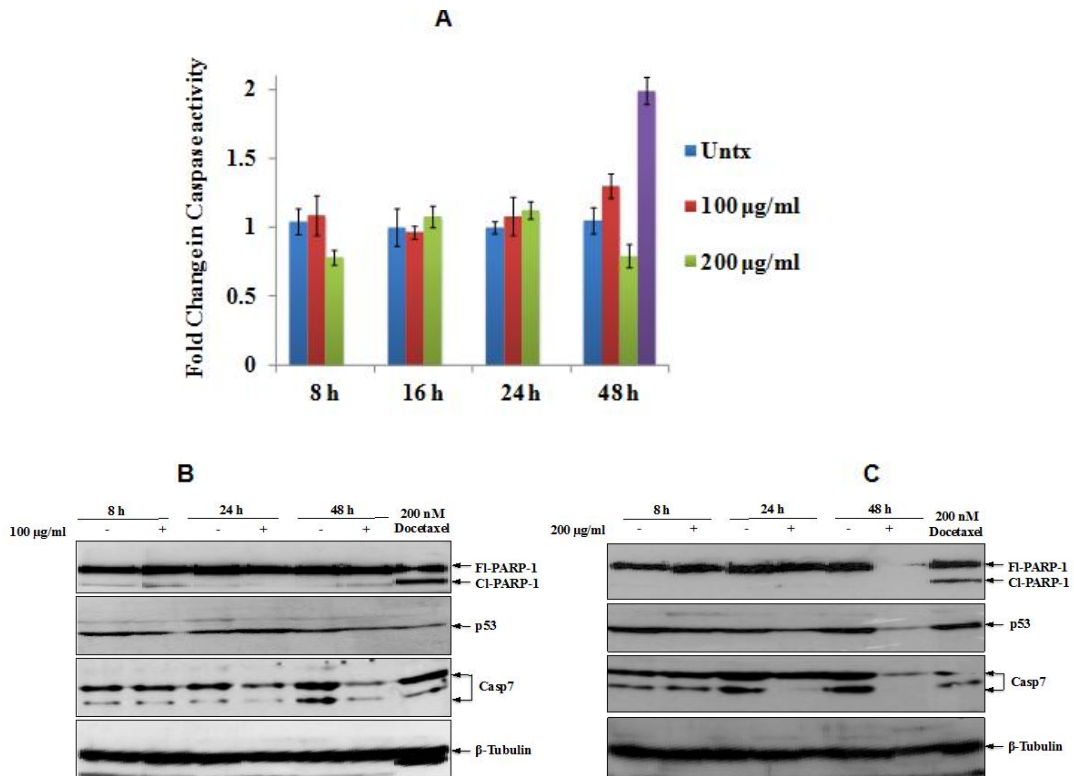


Fig. 4. Caspase-3/7 activity and protein expression in MCF-7 cells treated with AM extract. Legend: MCF-7 cells were seeded in quadruplicate at 2.5×10^3 cells per well in 384-well plates and treated with 100 or 200 µg/ml AM extract for the indicated time. (A) Caspase-3/7 activity was determined using the ApoTox kit where 200 nM Docetaxel was used as a positive control. The caspase-3/7 activity is represented as fold-change in activity when compared to untreated (Untx) cells. (B and C) Proteins were resolved on SDS-page gels probing for PARP-1, p53 and Caspase-7. Cells treated with 400 nM docetaxel for 24 h were used as a positive control, Untx represent untreated control and β-tubulin was used as a loading control. Where PARP-1 is 'Fl-' (full length) and 'Cl-' is (Cleaved), '-' represent untreated sample and '+' represent sample treated with AM extract

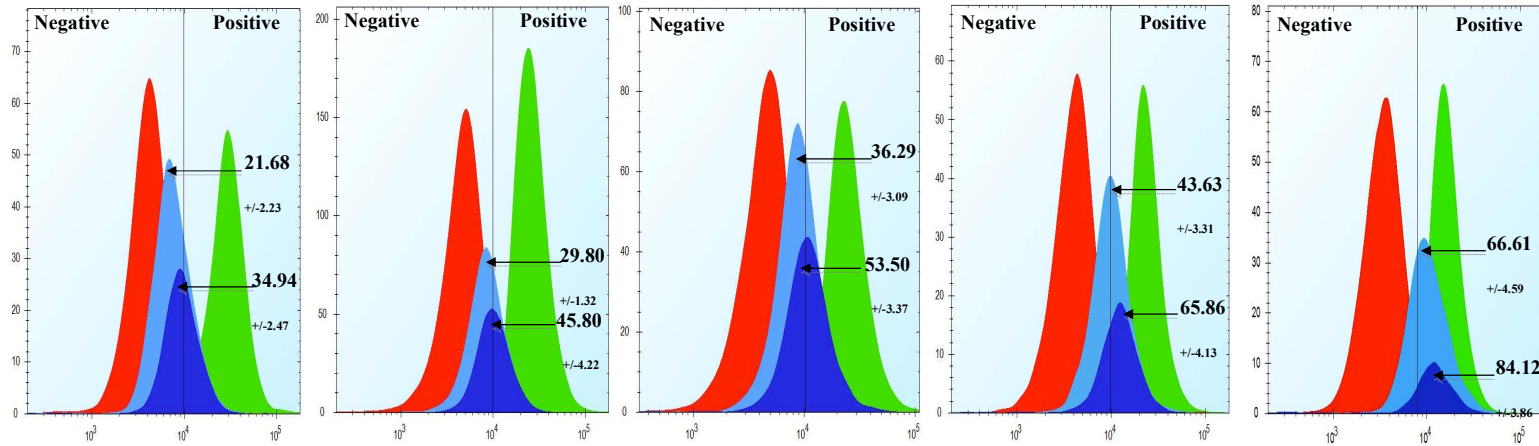


Fig. 5. Autophagy in MCF-7 cells treated with AM extract. MCF-7 cells were seeded in quadruplicate at 5×10^3 cells per well in 96-well plate and treated with 100 or 200 $\mu\text{g/ml}$ AM extract for the indicated time. (A) Cells were stained with 50 μM MDC dye and autophagy was determined by flow cytometry. Cells treated with 10 μM Z36 for 1 h were used as a positive control (green peak) while untreated cells (red peak) were used as a reference for setting gates

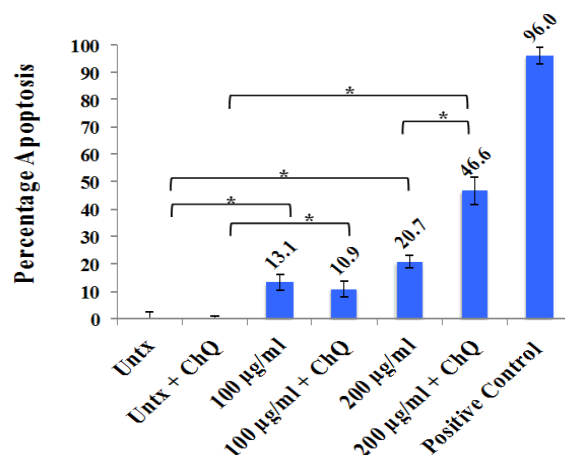


Fig. 6. Apoptosis in MCF-7 cells treated with AM extract and Chloroquine. MCF-7 cells were seeded in quadruplicate at 5×10^3 cells per well in 96-well plate and pretreated with $50 \mu\text{M}$ Chloroquine (ChQ) for 4 h followed by treatment with 100 or 200 $\mu\text{g/ml}$ of AM extract for 24 h. Percentage apoptosis was determined with the APOPercentage assay and cells treated with 10 mM H_2O_2 were used as a positive control. 'Untx' represents untreated control and bars represent standard deviation of points in quadruplicate

AM extract: significant from untreated control, * $P < 0.006$

Mean \pm SD = Mean values \pm Standard deviation of points in quadruplicate

Apoptotic cell death is triggered by altered cellular redox potential and disrupted energy metabolism in mitochondria [28]. Mitochondria are also the main source of ROS production in a cell [29; 30] and excessive production of ROS can activate cell death pathways [31]. Increases in ROS levels were shown to be an early event in apoptotic cell death and precedes MMP disruption [32,33,34,35]. We performed various assays to assess the chronology of events leading to apoptotic cell death in MCF-7 cells after AM extract exposure. Our data shows that ROS generation was initiated within 1 h of AM extract treatment and peaked at 2 h, further causing the depolarization of MMP. A significant increase in autophagy was also observed for both the concentrations. Autophagy is generally associated with cell survival by maintaining ATP and amino acids and removing damaged proteins and organelles [32,36].

In our experiments, an increase in percentage of apoptosis from 20% to 70% was observed in MCF-7 cells treated with 200 $\mu\text{g/ml}$ of AM extract during the course of the experiment. However, in cells treated with 100 $\mu\text{g/ml}$ of AM extract, only about 10% of apoptosis was observed at 24 h and no increase in apoptosis was seen over time. It was also observed that MMP damage was recovered over time in cells treated with 100 $\mu\text{g/ml}$ of AM extract. A possible explanation for this observation could be that the energy derived from autophagy and clearance of damaged

mitochondria along with MMP recovery, may have been sufficient to inhibit further cell death in case of 100 $\mu\text{g/ml}$ treatment (Fig. 7).

Restoration of lost MMP has been shown to prevent further release of cytochrome c (proapoptotic factor) in the case of crotoxin treatment to MCF-7 cells [37]. Our data suggests that autophagy acted as a survival mechanism in our experiments and once the threshold of energy compensation provided by autophagy was overwhelmed by using higher dose (as seen for 200 $\mu\text{g/ml}$ AM extract), cells underwent apoptosis. Similar observations were reported for paraquat (PQ) (1, 1'-dimethyl-4,4'-bipyridinium dichloride), a widely used herbicide, which was found to induce autophagy in the beginning while cells eventually died through apoptosis [36]. Several compounds have also been reported for simultaneous induction of autophagy and apoptosis in cancer cells [38,39,40,41]. In the past decade, it has become increasingly evident that massive ROS generation modulates autophagic pathways [42] contributing to cancer initiation and progression [30,43,44,45]. It has also been reported that a mannose-binding lectin (*Polygonatum cyrtonea* lectin) induced apoptosis and autophagy in A375 cells and was proposed that ROS may be connecting the autophagy and apoptosis by switching the availability of various proteins, and hence influencing the cell death fate [28].

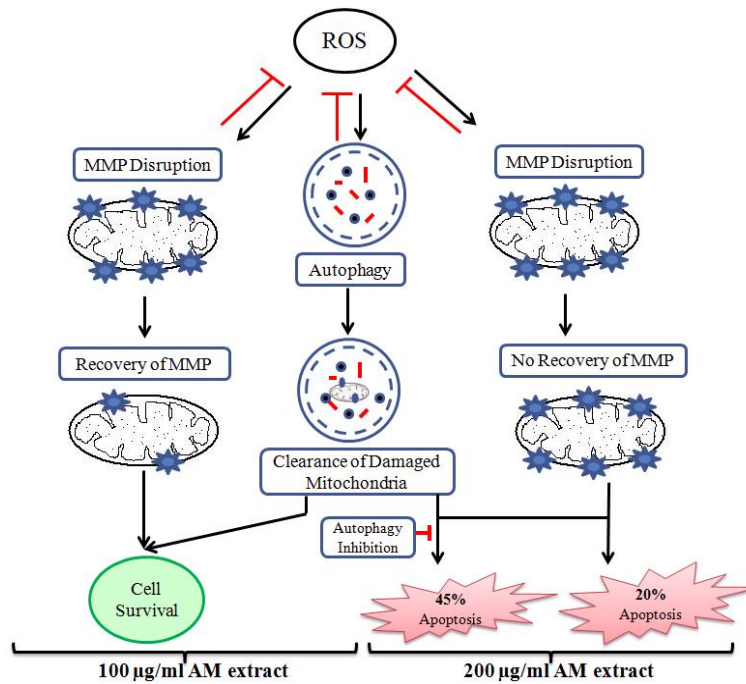


Fig. 7. Diagrammatic representation of the dose-dependent cell death mechanisms induced by AM extract in MCF-7 cells and their manipulation by autophagy inhibition. Legend: Black arrows represent the activation, red bars represent the inhibition and blue stars represent the MMP disruption

Studies have also demonstrated that inhibition of autophagy triggers apoptosis [46,47]. The suppression or inhibition of autophagy in cancer cells has been shown to increase the efficacy of several classes of anticancer agents including vorinostat, cyclophosphamide, and imatinib [48,49,50,51]. Therefore, the combination of autophagy inhibitor and apoptosis inducers has been proposed to be an effective strategy to find new treatment options for cancer. Several clinical trials have been initiated in this direction [48,52]. In the present study, we demonstrated that pretreatment of MCF-7 cells with autophagy inhibitor 'chloroquine' for 1 h followed by 200 µg/ml of AM extract treatment for 24 h significantly increased the apoptosis from 20% to 45%.

5. CONCLUSION

The present study describes the interplay of various mechanisms involved in MCF-7 breast cancer cell death in response to treatment with AM extract. Various assays performed during the study confirmed the caspase-independent apoptosis in the cells. AM extract induced ROS in the cancer cells leading to the changes in

MMP and autophagy induction. We further showed that inhibiting autophagy increased apoptosis-inducing capability of the AM extract by more than two-folds. Since cancer cells are known to be more vulnerable to oxidative stress caused by ROS-generating agents [43], such agents coupled with autophagy inhibitors present unique opportunities to exploit cross-signaling between cell death pathways to enhance the effectiveness of chemotherapy. This study further supports the idea that the pharmacological modulation of autophagy can be a valuable tool for anticancer therapy.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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