

Ziziphus mauritiana Leaf Extract Induces ROS-dependent Apoptosis by Downregulation of CD81 in MDA- MB 231 Triple Negative Breast Cancer Cells

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Research Article

Keywords: Triple Negative Breast Cancer, Ziziphus mauritiana, CD81, ROS, Apoptosis

Posted Date: January 1st, 2025

DOI: <https://doi.org/10.21203/rs.3.rs-5734006/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background. Breast cancer (BC) is the most prevalent cancer among women, with triple-negative breast cancer (TNBC) representing a particularly aggressive subtype. Current treatments often have limited efficacy and significant toxicity, highlighting the need for alternative therapeutic strategies. *Ziziphus mauritiana* (ZM) leaf extract contains triterpenoids which have been proven to be used as anticancer agents, but have not been studied in TNBC cells.

Aim. To examine the triterpenoid content in ZM, especially BA and OA, ZM cytotoxicity, intracellular ROS levels, the amount of apoptosis, and CD81 expression on MDA-MB 231 cells after being given ZM.

Material and Methods. The extract was prepared using a sequential extraction method. The presence of triterpenoids was confirmed via HPLC and LC-MS analysis. The cytotoxicity of the extract was assessed using the CCK-8 assay, while flow cytometry was employed to analyze CD81 expression and ROS levels. Apoptosis was quantified using an Annexin V-FITC/PI detection kit.

Results. Based on HPLC, the BA content in ZM is 28.3 µg/ml, and the OA content is 13.4 µg/ml. ZM significantly reduced MDA-MB 231 cell viability by 28.28% at 400 µg/ml after 48 hours. Flow cytometry revealed a notable downregulation of CD81 expression ($p < 0.0001$) and an increase in intracellular ROS levels ($p < 0.0001$). Furthermore, ZM induced early and late apoptosis in treated cells, with increases of 2.34-fold and 2.66-fold, respectively.

Conclusion. ZM leaf extract exhibits potent cytotoxic effects against MDA-MB 231 through ROS-dependent apoptosis and CD81 downregulation, suggesting its potential as a novel therapeutic agent for TNBC treatment.

Introduction

Breast cancer (BC) is the most frequently diagnosed cancer in women and is the leading cause of cancer death, followed by lung and colorectal cancer. The International Agency for Research on Cancer (IARC) reported 2,295,686 new cases worldwide in 2022, resulting in a mortality rate of 665,684 [1]. Biomarker expression divides breast cancer into four subtypes: estrogen receptor, progesterone receptor, human epidermal growth factor receptor 2 (HER2), and Ki67, which correspond to luminal A, luminal B, HER-2 overexpression, and triple-negative breast cancer (TNBC) [2–4]. TNBC is a subtype of BC characterized by the absence of estrogen, progesterone, or HER2 receptors. When compared with other BC subtypes, TNBC accounts for 15–20% of BC incidents and usually occurs in young women under 40 years of age, and is associated with increased recurrency and mortality [3, 5]. The Food and Drug Administration (FDA) has approved anti-metabolites, paclitaxel, and anthracyclines as adjuvant and neoadjuvant chemotherapy regimens for patients with TNBC. However, the toxicity of chemotherapy poses a significant risk to patients, and some patients continue to experience no clinical benefit. Therefore, finding effective targets for accurate TNBC therapy is a challenging and important clinical problem to be solved [4].

Plants serve as a source of therapeutic chemicals due to their capacity to generate bioactive substances, particularly secondary metabolites. (6) *Ziziphus mauritiana* (ZM), referred to as chinee apple, indian jujube or ber [7], is a species of plant in the Rhamnaceae family. This plant species is commonly found in South Asia (China, India, Pakistan) and the eastern Africa [7], flourishing at an elevation of 400 meters under arid conditions [8, 9]. Traditional herbal medicine employs many plant components of ZM, including leaves, stems, and roots, to treat conditions such as infections, diabetes, hypertension, leishmaniasis, and insomnia. The pharmacological action of ZM is attributed to its abundant phytochemical content, which includes alkaloids, flavonoids, phenolic compounds, tannins, and triterpenoids. These compounds exhibit a wide array of pharmacological properties, encompassing cardiovascular, antioxidant, antimicrobial, antiviral, antidiabetic, anti-inflammatory, anti-ulcerogenic, anti-obesity, anti-aging, analgesic, immunomodulatory, hypolipidemic, neuroprotective, hepatoprotective, and anticancer activities [10–12]. Previous research has documented the cytotoxic activity of ZM against various cancers, including the root extract's effect on MCF7 breast cancer cells [13], the seed extract's impact on Ehrlich ascites carcinoma [14], the aerial parts' efficacy against hepatic carcinoma [15], and the methanol extract of the leaves' activity against A549 lung cancer cells [16]. However, no study has reported the cytotoxic activity of ZM leaf extract against TNBC.

Triterpenoids in ZM are known to promote the apoptosis of cancer cells in different types of cancers, including TNBC [17]. Natural derivatives like betulinic acid (BA) or oleanolic acid (OA) are two chemically distinct triterpenoids [18]. Previous research has shown that BA can induce apoptosis in TNBC cells by damaging mitochondrial membrane potential [19]. BA also has the ability to enhance the generation of reactive oxygen species (ROS) in cancer cells, leading to excessive oxidative stress [20–22]. The elevation of ROS might lead to oxidative damage to the DNA, proteins, and lipids of cancer cells, ultimately initiating pathways that result in cell death [23]. Studies have shown that OA exerts anti-cancer attributes against TNBC [24].

CD81, or target antiproliferative 1 (TAPA1), is a member of the tetraspanins, which are transmembrane proteins [25]. Normally, CD81 plays a role in the immune system, plays an important role in the activation of B lymphocyte cells [26, 27]. In tumorigenesis, tetraspanins play a role in tumor growth, invasion and angiogenesis. CD81 has been identified to promote tumor cell cluster formation and metastasis in TNBC through an interaction with other cell surface proteins such as CD44. CD81 expression in TNBC also correlated with poor prognosis and an increased metastatic capability [28]. CD81 downregulation can inhibit cancer cell communication by exosomes, essential for tumor growth and metastasis [29].

The purpose of this study was to examine the amount of triterpenoids in ZM, primarily BA and OA, and to demonstrate cytotoxicity of ZM through MDA-MB 231 cells. This study aims to examine the amount of triterpenoids in ZM, especially BA and OA, and to demonstrate the cytotoxic effect of ZM through MDA-MB 231 cells. In addition, this study also analyzed the relationship between CD81, increased reactive oxygen species (ROS), and apoptosis. The results of this study are expected to prove the potential of ZM in the treatment of TNBC. This has the potential to result in a new sort of target treatment for TNBC.

Methods

Collection and Authentication of Plant

Fresh ZM leaves collected from Malang, East Java, Indonesia. Authentication by the Technical Executives Unit of Herbal Materia Medica Laboratory Batu, Malang, East Java, Indonesia (Ref No. 000.9.3/3189/102.0/2023).

Preparation of Extract

The dried leaves were then crushed and sieved with a mesh size of 80#. The obtained powder (500 g) was extracted successively with nonpolar to polar menstruum with increasing polarity, starting with petroleum ether (60°C-70°C) and then MeOH (65°C-75°C), followed by water (80°C-100°C) using a continuous hot extraction method for 6 hours. In MeOH extract, the menstruum is distilled and concentrated at low pressure to form a dark green mass. The concentrated ZM mass was stored at 2°C-4°C for further use.

Standard stock solutions, leaf extract ZM, and chromatographic conditions.

BA (GLPBio, USA) and OA (GLPBio, USA) each in the amount of 10 mL dissolved in 10 mL Acetonitrile (1000 ppm). Both stock solutions were then combined and diluted serially to several concentrations: 2; 4; 6; 8; 10 ppm, and analyzed using Shimadzu High-Performance Liquid Chromatography (HPLC) UV LC-20AD with manual injection and UV detector at 210 nm wavelength. BA and OA were analyzed using SHIMPACK® VP ODS-C18 (4.6 x 250 mm, 5 µm). Mobile phase analysis was run at a flow rate of 1.0 mL/minute with an isocratic pump mode of ddH₂O:acetonitrile (80:20, v/v) ratio with an injection volume of 20 µL. The column was set at 30 °C. ZM extract samples were filtered through a 0.22 µm PTFE filter and analyzed in an HPLC system for the determination of recoverable BA and OA from the mixture.

LC-MS analysis of BA and OA standards and ZM leaf extract.

LC-MS analyzed the standard solutions of BA and OA to confirm the peaks and their positions. LC-MS also analyzed the ZM leaf extract to identify the presence of BA and OA. The analysis used UHPLC from a Thermo Vanquish Autosampler with a Hypersil Gold C18 column (2.1 × 100 mm, 1.9 µm). The mass spectrometry used was Thermo TSQ Fortis QqQ (Triple Quadrupole) with Heated-Electron Spray Ionization (H-ESI) method.

Cell Line and Culture

The MDA-MB 231 cells were obtained from the American Type Culture Collection (ATCC; RRID: CVCL_0062; Rockville, USA), and cultured in the culture division of the biomedical laboratory, Faculty of Medicine, Pajajaran University, Indonesia. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM), containing 10% FBS (fetal bovine serum) and 1% antibiotic solution (10,000 U/ml penicillin and 10 mg/ml streptomycin). Cells were maintained in 5% CO₂ and humidified air at 37°C.

Cell Viability Assay

The cytotoxic activity of ZM against MDA-MB 231 cells was determined using the CCK-8 assay (GLPBio, USA). Viable cancer cells were grown in 96-well plates at a density of 10^3 cells/well in 100 μ l DMEM. The cells were incubated for 24 hours at 37°C in an environment containing 95% air humidity and 5% CO₂. ZM was utilized at the following concentrations: 300 μ g/ml, and 400 μ g/ml. The negative control of this study used 0.05% dimethyl sulfoxide (DMSO). After adding 10 μ L of CCK-8 solution to each well of the plate using a repeating pipettor, then incubate the plate for 1-4 hours. The absorbance was determined at 450 nm by a microplate reader, and the percentage of viable cells was computed employing the following formula:

$$\text{Cell viability (\%)} = [(As-Ab)/(Ac-Ab)] \times 100$$

As = absorbance of the experimental well

Ab = blank well absorbance

Ac = control well absorbance

Analysis of CD81 expression by flow cytometry

MDA MB 231 cells were grown in 96-well plates at a density of 10^5 cells/well in 100 μ l of DMEM 0.05%. The suspension (50 μ L) was removed and placed into a clean 1.5 mL centrifuge tube and then diluted by adding 1 μ L of fluorescent antibody CD81 (GLPBio, USA) to each 50 μ L of cell staining buffer. Cell surface antigen staining was carried out and incubated in the dark for 20 minutes at room temperature. Then 300-500 μ L of cell staining buffer was added and stirred by pipetting gently. Finally, it was transferred into a 5 cm round-bottom tube and analyzed using flow cytometry.

Intracellular ROS detection assay

The production of ROS was detected by 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) staining. After the culture cells were treated, they were stained with DCFH-DA (GLPBio, USA). The cells, which density 10^5 cells/well, were harvested and incubated with 10 μ M DCFH-DA dye for 30 min and incubated at 37°C, and then rinsed 2-3 times with PBS. ROS measurement using flow cytometry with a 525 nm wavelength.

Cell Apoptosis

Cell apoptosis was analyzed by flow cytometry. The cells, which density 10^5 cells/well, were treated with BA and OA for 24 h in a humidified incubator with 5% CO₂ at 37 °C. The cells were harvested, washed twice, and then resuspended. The cell suspension was fixed with 400 μ L of Annexin binding buffer after incubation with 5 μ L of FITC-Annexin V (BioLegend, USA) and 10 μ L of PI for 15 minutes at room

temperature (25 °C) in the dark room. Apoptotic cells were detected with a FACSCalibur flow cytometer (BD).

Statistical Analysis

In vitro research data is presented in the form of mean ± standard deviation (SD) with four repetitions. SPSS 23.0 (SPSS,Chicago, IL) software was applied to perform statistical analysis. Statistically significant differences was one-way analysis of variance. P values were labeled as follows: *P < 0.05; **P < 0.01; ***P < 0.001.

Result

HPLC and LCMS/MS analysis of BA and OA standard and extract of ZM leaves

The retention times for standard BA and OA were 10.864 min (± 0.23 min) and 12.058 min (± 0.15 min), respectively, according to HPLC analysis at various concentrations. LCMS/MS analysis at various concentrations revealed that the retention times for standard BA and OA were 4.586 min (± 0.19 min) and 5.020 min (± 0.22 min), respectively (table 1). Fig. 1a displays the chromatogram for the ZM extract of leaves by HPLC. A distinct peak appearing at 10.662 min indicates BA, while the peak at 12.208 min matches the OA retention time. Fig. 1d displays the chromatogram for the ZM extract of leaves by LCMS/MS. A distinct peak appearing at 4.622 min indicates BA, while the peak at 5.042 min matches the OA retention time. These data clearly demonstrated the validity of the HPLC method for BA and OA analysis. The results from the 210 nm UV detector matched the results from the MS detector for the analysis of BA and OA from the ZM leaf extract. The area under the peak of the BA vs. concentration plot shows a linear fit with a correlation coefficient of 0.9986 (Fig. 1b). The plot of the injected OA standard's area against its concentration displays a linear fit with a correlation coefficient of 0.9981 (Fig. 1c). According to the calibration results, the BA content in ZM was 28.3 µg/ml, and the OA content was 13.4 µg/ml.

Table 1. HPLC and LCMS results of standard solutions and ZM leaf extract

standar (ppm)		HPLC				LCMS			
		Ret. Time	Area	Height	Conc	Ret. Time	Area	Height	amount ppm
BA	2	10.895	18600	1259	1.951	4.552	10.942.008	10.596.323	20.528
	4	10.883	37990	2555	4.119	4.592	18.951.669	17.979.810	40.445
	6	10.855	53634	3824	5.868	4.592	26.876.492	24.849.639	60.150
	8	10.847	73903	5362	8.133	4.592	33.351.399	29.320.048	76.251
	10	10.841	89970	6404	9.829	4.602	43.958.338	38.553.171	102.626
OA	2	12.079	19975	1317	1.903	4.982	20.014.205	17.773.136	22.238
	4	12.068	39616	2683	4.073	5.022	35.280.884	30.905.056	41.310
	6	12.054	57157	3830	6.010	5.022	47.629.971	41.891.266	56.738
	8	12.042	76880	5255	8.189	5.032	61.159.961	55.882.018	73.640
	10	12.046	91687	6242	9.596	5.042	87.121.389	84.161.935	106.073
ZM									
BA		10.662	26526	928		4.622	6.655.475	6.317.725	1
OA		12.208	14960	1224		5.042	161.717	334.547	n.a

Ret. Time : retention time

Conc. : concentration

n.a: not applicable

ZM inhibited proliferation in MDA-MB 231 cells

The CCK-8 assay determined the impact of ZM on MDA-MB 231 cells proliferation and compared it with controls. The CCK-8 assay results showed that administering ZM at concentrations of 300 g/ml and 400 g/ml can reduce the viability of MDA-MB 231 cells. It could be seen that a concentration of 400 µg/ml can reduce the viability of MDA-MB 231 cells by 28.28% at 48 hours of observation.

ZM leaf extract downregulates CD81 expression

Flow cytometry analyzed the effect of ZM leaf extract on CD81 expression. MDA-MB-231 cells treated with ZM leaf extract showed a decrease in CD81 expression (Fig. 3e) compared with untreated group. These results indicate that ZM leaf extract affects CD81 levels in TNBC cell types.

ZM Increased ROS intracellular

The enzymatic cleavage of DCFH-DA to DCF was measured in TNBC cells to evaluate intracellular ROS levels and characterize the mechanism of ZM-induced cytotoxicity. We found that ZM significantly increased the ROS level in the MDA-MB-231 cell line compared with other groups. These results indicate that ZM leaf extract triggers the formation of ROS in TNBC cells.

ZM induced intrinsic apoptosis in MDA-MB 231 cells

We measured the apoptotic TNBC cells using Annexin-V/FITC. In this study, ZM with a concentration of 300 µg/ml increased early apoptosis 2.34-fold and late apoptosis 2.66-fold in MDA-MB 231 cells compared to the control. Meanwhile, ZM with a concentration of 400 µg/ml increased early apoptosis 2.26-fold and late apoptosis 2.98-fold in MDA-MB 231 cells compared to the control.

Discussion

The importance of herbal medications in chemoprevention has been increasingly acknowledged, especially for complementary and adjunctive therapy. A bioassay research is crucial for assessing herbal medications and their phytoconstituents, which are responsible for many interesting pharmacological actions [16]. Phytochemicals are a broad term for secondary metabolites found in plants that have a wide range of structures and functions, both in plants and in traditional medicine for humans. Naturally widespread plant triterpenes and triterpenoids, comprising more than twenty thousand described compounds, are the most important group of phytochemicals [30].

The plants of the genus *Ziziphus* widely distribute terpenoids. Researchers have extracted approximately 43 triterpenes from the fruits, bark, flowers, aerial, leaves, and seeds of the genus *Ziziphus* to date, and several of these triterpenes have demonstrated promising biological activities [31]. It is known that ZM contains many triterpenoids. Researchers isolated three new medicinal agents, zizimauritic acids A, B, and C, from the roots of ZM [32]. It was also found that BA, ceanothenic acid, and ceanothic acid were isolated from the roots of ZM [11]. Another study also mentioned that ZM leaf extract contains triterpenoids, specifically BA, OA, and ursolic acid [33]. Using HPLC-LCMS/MS, this study showed that BA and OA were present in ZM leaf extract. The levels of BA were found to be 28.3 µg/ml and those of OA were 13.4 µg/ml.

Recognized as a promising anti-tumor compound, BA demonstrates effectiveness against various types of cancer and is regarded as a potential drug candidate. Recent studies have confirmed that BA has the potential to combat breast cancer [34], specifically TNBC [35]. One mechanism of BA as an anticancer is the activation of the mitochondrial apoptotic pathway, which is achieved by damaging the potential membrane of the mitochondria. BA isolated from *Ziziphus jujuba* fruit was shown to inhibit MCF-7 cells by causing cell cycle arrest in the G2/M phase, inhibited Bcl2 expression, and triggered Bax expression [21]. In MDA-MB 231, BA inhibited Bcl2 expression and mediated the inhibition of topoisomeration I and II, which in turn triggers cell cycle arrest and apoptosis [35]. BA can also increased intracellular ROS thus triggering apoptosis in various cancers, such as multiple myeloma [22], leukemia [36], hepatocellular

carcinoma [37], melanoma [38], squamous cell carcinoma [20], bladder carcinoma [39], cervical carcinoma [40], and breast cancer [41]

This study found that ZM leaf extract can increase intracellular ROS and trigger apoptosis in MDA-MB 231 cells. Om Prakash et al. conducted research that demonstrated an increase in ROS in A549 lung cancer cells using ZM leaf extract [16]. Kabir et al.'s research, on the other hand, demonstrated that ZM fruit can both increase ROS and trigger apoptosis in MCF7 breast cancer cells [42]. In cancer, excessive levels of cellular ROS cause damage to proteins, nucleic acids, lipids, membranes, and organelles, which can lead to the activation of cell death processes such as apoptosis. Mitochondria play an important role in initiating apoptosis and are considered both a source and target of ROS. High levels of mitochondrial ROS can initiate intrinsic apoptosis, leading to the release of cytochrome c into the cytosol from the mitochondrial intermembrane space. In the cytosol, cytochrome c engages apoptosis protease-activating factor-1 (APAF1), followed by apoptosome formation, which activates caspase-9. Caspase-9 is an important part of the intrinsic pathway. It then turns on effector caspases like caspase-3, -6, and -7. This cuts proteins inside cells and kills them through apoptosis. A link between ROS and the extrinsic pathway of apoptosis also exists. ROS can activate transmembrane death receptors, including Fas, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL-R1/2), and tumor necrosis factor receptor 1. When transmembrane death receptors are activated, they bring adaptor proteins, Fas-associated protein with death domain (FADD), and procaspase-8 and -10 to the surface of the cytoplasm to create death-inducing signaling complexes (DISCs). This sets off the activation of caspase-8 and -10, which can then directly activate effector caspases and start apoptosis [43, 44].

This study also demonstrated that ZM leaf extract can reduce CD81 expression in MDA-MB 231 cells. Similar research conducted by Marni et al. showed the results that DATS has cytotoxicity against MDA-MB 231 by reducing tetraspanin expression [45]. Previous studies have shown that BA can reduce CD81 expression, resulting in increased intracellular ROS, increased apoptosis, and decreased viability of MDA MB 231 cells [46]. CD81 is normally present on the cell surface and plays a role in several mechanisms of cell function, such as cell-to-cell contact, cellular fusion, protein tracking, and membrane organization [47]. Breast cancer exhibited overexpression of CD81 in comparison to normal breast tissue [48, 49]. Downregulation of CD81 in various malignancies can decrease tumor growth, migration, and cell invasion [50–52], while overexpression of CD81 in breast cancer and melanoma increases metastasis [29, 53]. Studies have reported that suppressing CD81 expression inhibits cell proliferation in parotid cancer [48], breast cancer [47], prostate cancer [50], bladder cancer [51], and osteosarcoma [52]. Sugiyama et al. demonstrated that CD81 contributes to cell proliferation due to its significant association with EGFR. In order to change EGFR signaling, CD81 either makes EGFR bind more directly to EGF or stops the formation of EGFR signaling oligomers. The study also showed that CD81 or other tetraspanins like CD82 and CD151 is an important part of controlling receptor signaling, both in terms of how strong and sensitive it is as well as how certain EGFR tyrosine residues are phosphorylated [54]. The author of this study hypothesizes that administering ZM to MDA-MB 231 cells might downregulate CD81 expression, which in turn may decrease the binding between CD81 and EGFR, leading to increased ROS and the induction of apoptosis.

Conclusion

This study present evidence for the first time that ZM leaf extract administration can induce ROS-dependent apoptosis by downregulating CD81 in MDA-MB 231 cells. Therefore, our findings suggest that ZM may possess anticancer properties for the treatment of TNBC.

Statements and Declaration

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Competing Interest

The authors have no relevant financial or non-financial interest to disclose

Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Dian Yuliartha Lestari], [Gondo Mastutim] and [Indri Safitri Mukono]. The first draft of manuscript was written by [Dian Yuliartha Lestari] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Figures

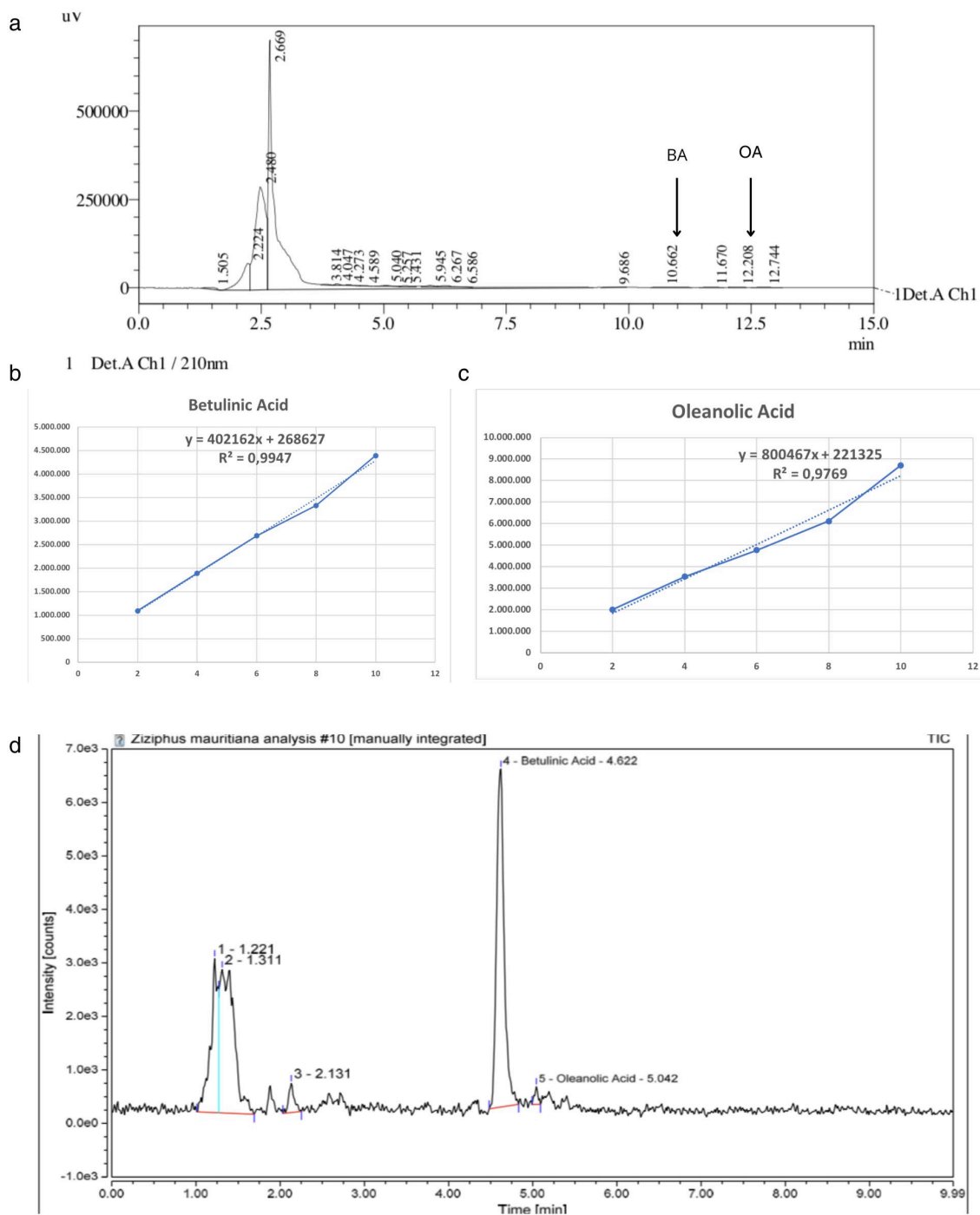


Figure 1

Chromatogram of ZM leaf extract. **a.** HPLC chromatogram. **b.** Concentration of BA (mg/L). **c.** Concentration of OA (mg/L). **d.** LCMS chromatogram

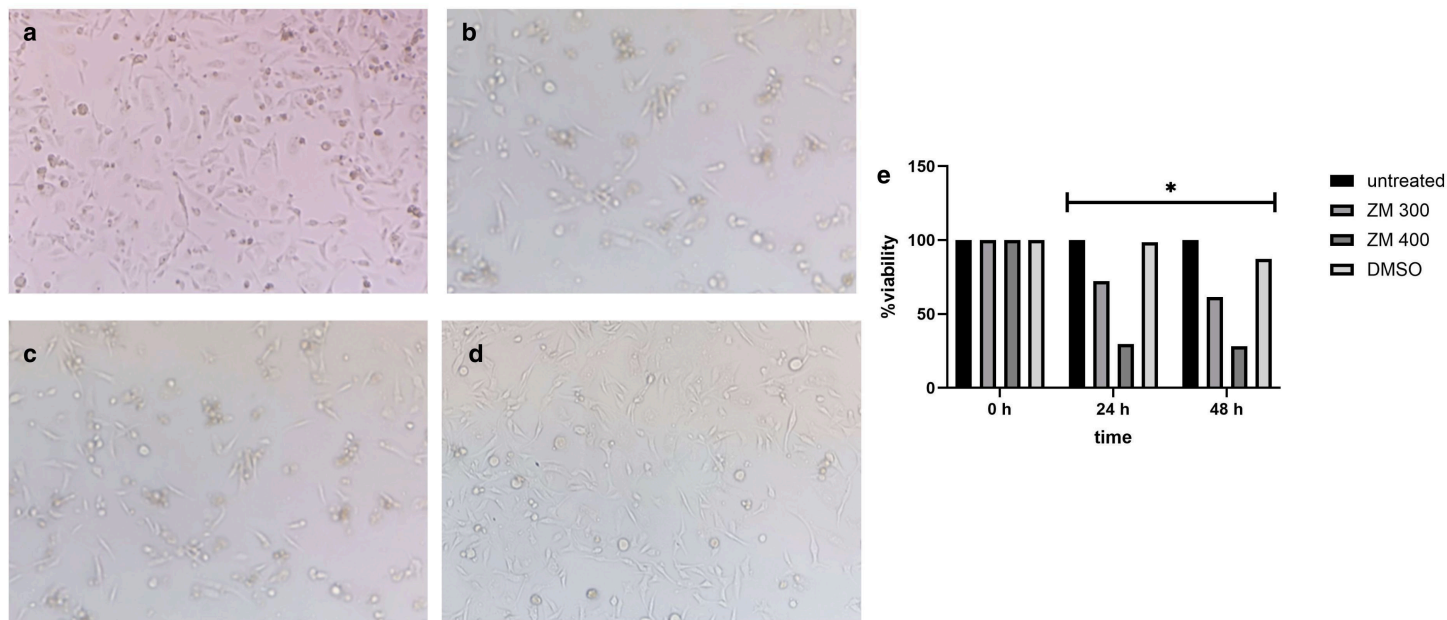


Figure 2

The viability of MDA-MB 231 cells was assessed after treatment with ZM at 24 and 48 hours. **a.** The photomicrograph of untreated group on 24 h. **b.** The photomicrograph of ZM 300 $\mu\text{g/ml}$ group on 24 h. **c.** The photomicrograph of ZM 400 $\mu\text{g/ml}$ group on 24 h. **d.** The photomicrograph of DMSO (negative control) group on 24 h. **e.** Statistical analysis suggests the percent of viability cells as compared to the untreated group in 24 and 48 hours. * $p < 0.05$

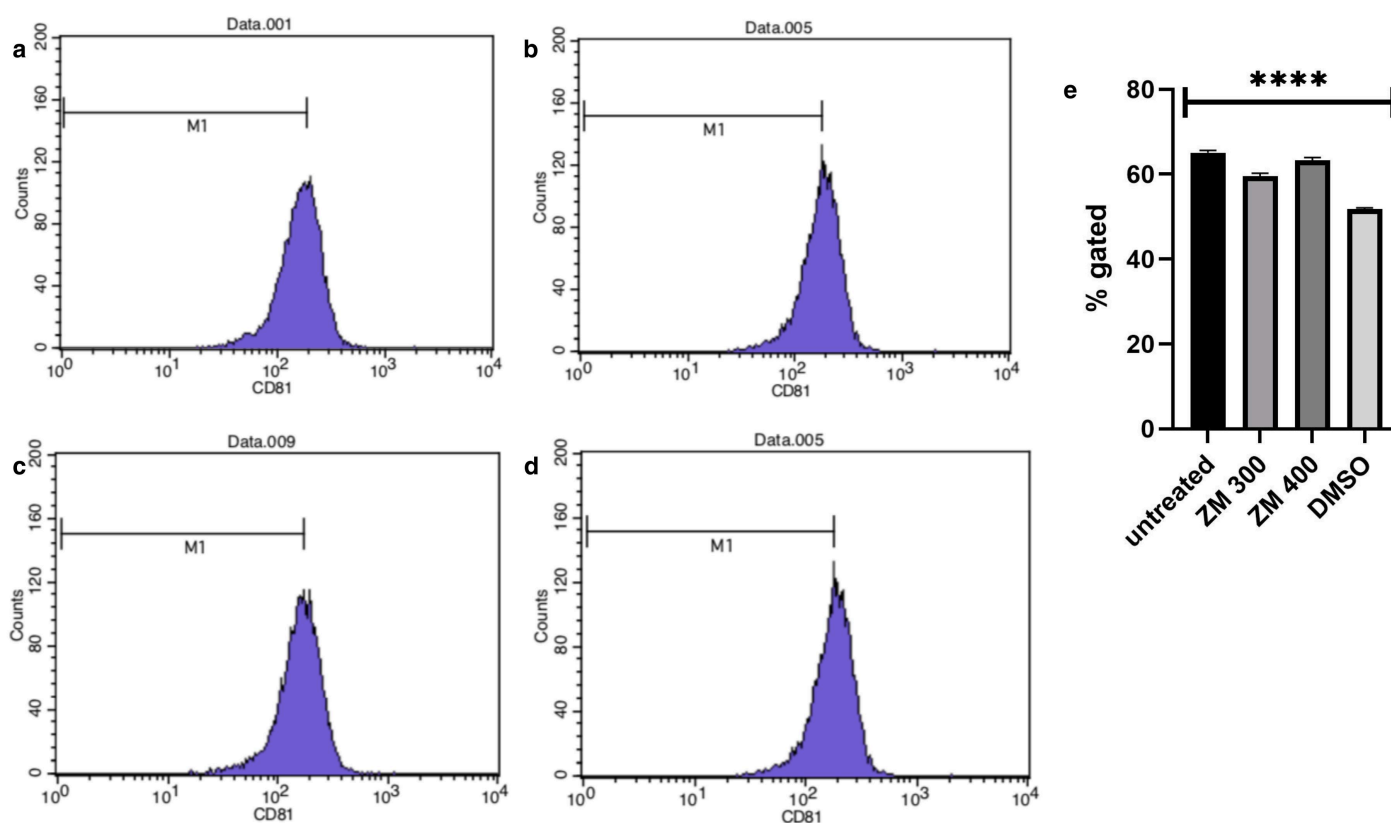


Figure 3

CD81 expression in MDA-MB 231 cells after ZM administration. **a.** Untreated group. **b.** ZM 300 µg/ml. **c.** ZM 400 µg/ml. **d.** DMSO (negative control). **e.** Statistical analysis suggests the percent of CD81 as compared to the untreated group. Minimum four independent experiments were carried out, and the values are expressed as means ± SEM, ****p < 0.0001 compared to the untreated group.

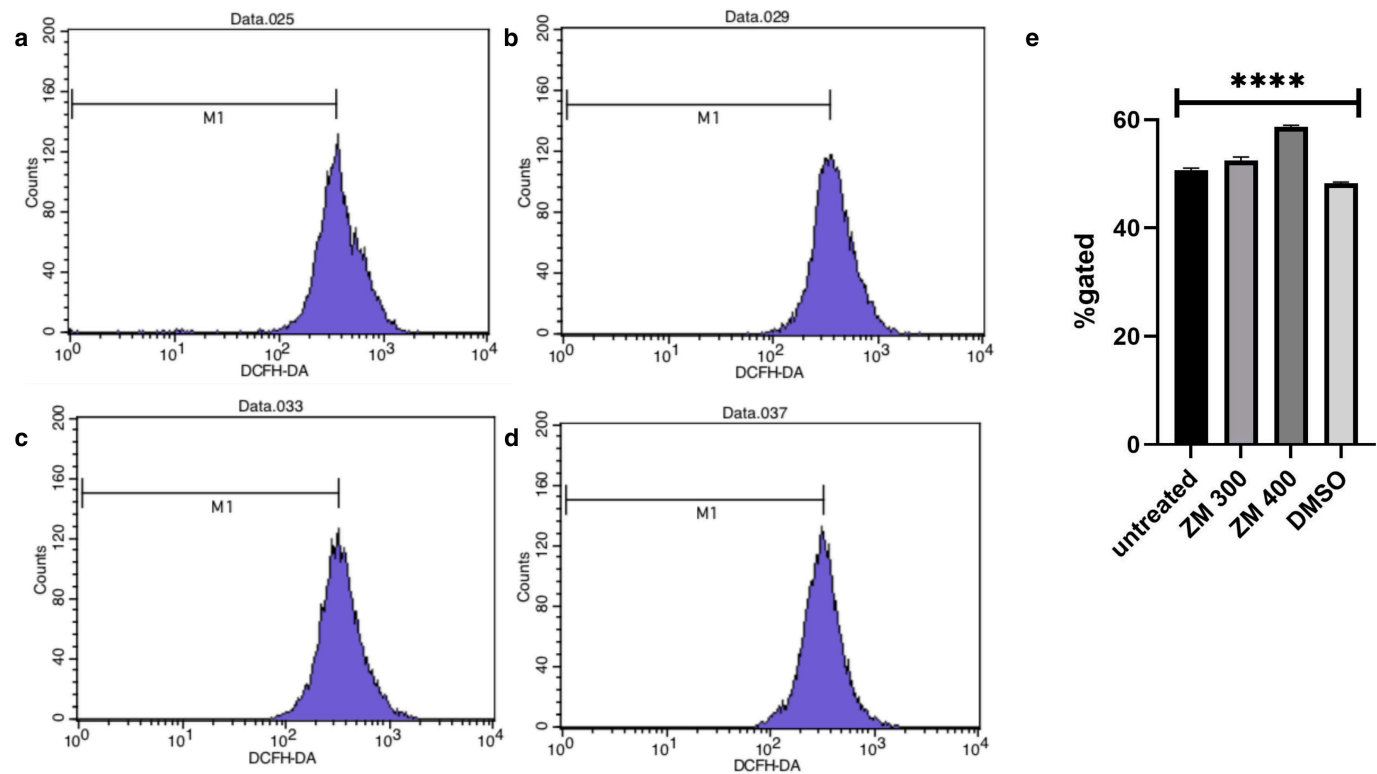


Figure 4

Intracellular ROS levels of MDA-MB 231 cells after ZM administration. **a.** Untreated group. **b.** ZM 300 µg/ml. **c.** ZM 400 µg/ml. **d.** DMSO. **e.** Statistical analysis suggests the percent of ROS as compared to the untreated group. Minimum four independent experiments were carried out, and the values are expressed as means ± SEM, ****p < 0.0001 compared to the untreated group.

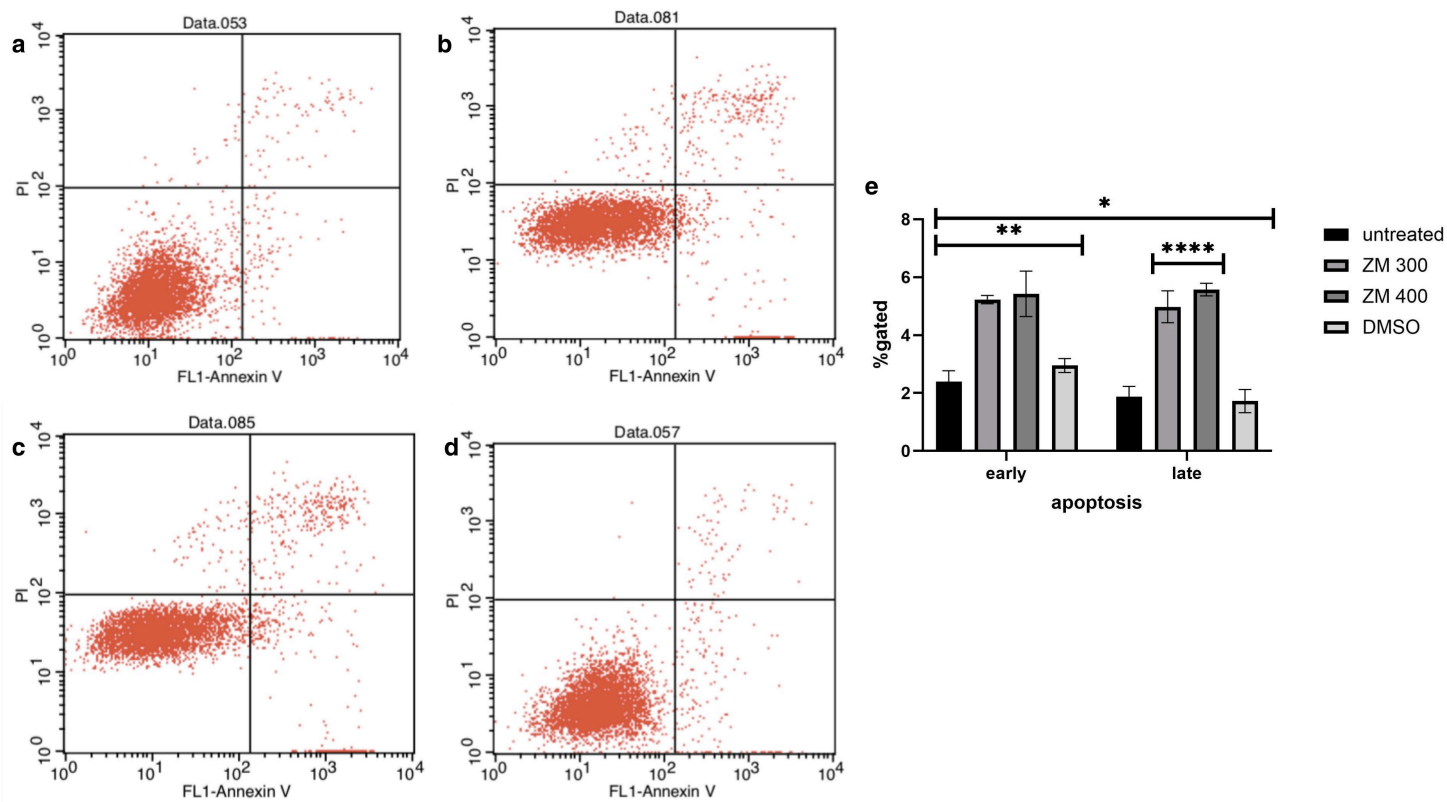


Figure 5

Early and late apoptosis of MDA-MB 231 cells after ZM administration. **a.** Untreated group. **b.** ZM 300 µg/ml. **c.** ZM 400 µg/ml. **d.** DMSO. **e.** Statistical analysis suggests the percent of apoptotic cells as compared to the untreated group. Minimum four independent experiments were carried out, and the values are expressed as means $\pm$ SEM, *p < 0.05, **p < 0.01, and ***p < 0.001 compared to the untreated group.