Ind. J. Pharm. Edu. Res., 2024; 58(3):890-898.
https://www.ijper.org

Original Article

An Umbelliferone-induced Caspase-Mediated Apoptosis in MDA-MB-231 Breast Cancer Cells

Mohammed Albratty[1,*], Hafiz A Makeen[2]

[1]Department of Pharmaceutical Chemistry and Pharmacognosy, College of Pharmacy, Jazan University, Jazan, SAUDI ARABIA.
[2]Department of Clinical Pharmacy, Pharmacy Practice Research Unit, College of Pharmacy, Jazan University, Jazan, SAUDI ARABIA.

ABSTRACT

Background: A highly malignant tumor, Breast Cancer (BC) has a poor prognosis for patients with the highest mortality rates in women. BC is currently treatable with a limited number of therapeutic strategies. **Aim:** Natural compounds with anti-proliferative capabilities are gaining popularity as a way to mitigate the toxicity of radiation and synthetic anti-tumor drugs. Umbelliferone has been reported to possess diverse pharmacological activities, like anti-cancer, anti-inflammation and anti-oxidation properties. **Materials and Methods:** In the current investigation, the potential of umbelliferone as an anti-cancer agent for breast cancer was evaluated in MDA-MB-231 cell lines. The influence of umbelliferone on cell viability AO/EtBr, apoptotic induction, MMP levels, ROS generation, cell adhesion assays, apoptotic marker levels and anti-inflammatory has been examined. **Results:** The impact of umbelliferone on the cell viability revealed that its cytotoxic potential increased in a dose-dependent pattern and IC_{50} concentrations were chosen for further experiments. The results showed that apoptosis was induced by umbelliferone in MDA-MB-231 cells by staining with AO/EtBr, triggering ROS pathway and DNA damage level of MMP. The caspase and inflammatory marker levels were also increased upon umbelliferone treatment. **Conclusion:** Umbelliferone cytotoxic potential in cells is mediated by inducing apoptosis, which is supported by significant levels of ROS and MMP level as well as the findings of the staining. All these results suggest that umbelliferone can be used as a potent anti-cancer drug for breast cancer cells.

Keywords: Breast cancer, Umbelliferone, MDA-MB-231 cells, Apoptosis, Reactive oxygen species.

Correspondence:
Dr. Hafiz A. Makeen
Department of Clinical Pharmacy, Pharmacy Practice Research Unit, College of Pharmacy, Jazan University, Jazan, SAUDI ARABIA.
Email: hafiz@jazanu.edu.sa

Received: 03-01-2024;
Revised: 11-03-2024;
Accepted: 26-05-2024.

INTRODUCTION

Breast cancer is the leading type of cancer in women globally, contributing significantly to cancer-related deaths. In Saudi Arabia, it ranks as the second most common cancer among women. Studies have highlighted a lack of awareness about breast cancer, negatively impacting breast self-examination practices. This is a cross-sectional study conducted in Saudi Arabia's Eastern Province to assess breast cancer awareness among adult females.[1,2] Many factors are responsible for causing breast cancer, but diet, hormones, pregnancy, obesity and abnormal breast development are among the most prevalent. There are no known risk factors for approximately half of breast cancers and surgery is the first line form of therapy, along with adjuvants like chemotherapy and radiotherapies.[3] Several multimodal therapies are available to treat breast cancer, but death from cancer-related causes among women worldwide remains the leading cause. This emphasizes the requirement for new treatment modalities.[4] To address these issues, it is imperative to develop innovative therapeutic approaches that can enhance the efficacy of treatment for this deadly disease.

The body has developed a way of eliminating excess, damaged, or harmful cells by using apoptosis or programmed cell death. The pathways of apoptosis involve intrinsic (mitochondria-regulated) and extrinsic (death receptor-regulated) cascades.[5] An extrinsic pathway is triggered when related ligands bind to the cell surface-bound death receptors, which subsequently activates caspase-8 and/or -10.[6] Caspase-9 is activated when mitochondrial proteins, Apaf-1 and apoptogenic proteins are released as a result of an intrinsic apoptotic pathway.[7] Caspases 8, 9 and 10 are the initiators of apoptosis since they activate the effector caspases 3-7, initiating the caspase cascade. However, for caspase-8 to enhance apoptotic stimuli, the intrinsic apoptotic pathway must be activated.[8] The Bcl-2 is activated by caspase-8 which releases its proteolytic inhibitor, causing the mitochondrial outer membrane to permeabilize which induces the cytochrome c release, an apoptogenic protein.[9] Several cancer therapeutic agents have been shown to induce apoptosis during the past decade. Though apoptosis-induced death of cancer cells has been

DOI: 10.5530/ijper.58.3.97

controversial, chemopreventive as well as therapeutic agents have been identified utilizing inducing apoptosis.

In an investigation of 1,2-dimethylhydrazine-challenged colon cancer, umbelliferone alone or in combination with 5-fluorouracil showed significant anti-carcinogenic effects and 5-fluorouracil was controlled by umbelliferone.[10] Kielbus and co-workers were able to decrease the growth of the laryngeal cancer RK33 cells by using this compound.[11] A cell line derived from Ferula showed anti-cancer effects against liver cancer cells at all dosages (0-50 mM). Cell cycle arrest, apoptosis and nuclear damage were identified as mechanisms through which the activity was conducted.[12] Hepatocellular Carcinomas (HCCs) are deadly cancers connected to poor patient prognoses and with high morbidity and mortality rates as well as limited treatment options. Combined with a lipopolysaccharide, coumarin stopped the growth of Sarcoma 180 tumors and increased the survival of the tumor-bearing mice.[13] Umbelliferone isolated from *Coriandrum sativum* L. showed cytotoxicity against HepG2 (Human Liver Carcinoma), RPMI (Human Nasal Septum Carcinoma), HT-29 (Colon Carcinoma), A-549 (Small Lung Carcinoma) and HeLa (Cervical Carcinoma) cells. Comparative to controls, the viability of the cells was reduced by up to 62.5% when placed in concentrations of 1000 and 500 Lg/mL, whereas the dosage of the drug required to hinder 50% of cell viability was >1000 Lg/mL.[14]

MATERIALS AND METHODS

Chemicals

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), Acridine Orange and ethidium Bromide (AO/EtBr), paraformaldehyde, trypan blue, Rhodamine 123 (RH-123) stain and N-acetyl L-cysteine (NAC) were purchased from Sigma-Aldrich (USA). Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), Anti-mitotic solution 100X and trypsin-Ethylene Diamine Tetraacetic Acid (EDTA) were purchased from standard suppliers.

Cell culture

DMEM enriched with 10% FBS, 1% L-glutamine and the antibiotic cocktail was utilized to maintain the MDA-MB-231 human breast carcinoma cells acquired from the laboratory. To cultivate the cells, a 5% CO_2-supplemented incubator was used in this work.

Cell viability assay

The MTT assay was employed to assess control and treated cell growth. Specifically, cells (2×10^4 cells/well) were loaded into 96-well plates after 24 hr under variations in umbelliferone concentration (2.5-15 µg). Then cells were incubated at 37°C for 3 hr after treatment with a 5 mg/mL MTT solution. The microplate reader used in this study (Molecular Devices, USA) to observe the absorbance at 540 nm.

Apoptotic staining with AO/EtBr

MDA-MB-231 cells were cultivated and administered with the 10 and 12.5 µg of umbelliferone for 24 hr. Later, cells were rinsed with PBS and fixed for 10 min with paraformaldehyde. Using 0.1% Triton X-100 for 10 min, the cells were permeabilized; the supernatant was discarded and rinsed thrice with PBS. At 37°C and in the dark, cells were incubated for 10 min with AO/EtBr at a 1:1 ratio. A fluorescent microscope (Olympus) was employed to note the stained cells under PBS twice and with AO/EtBr-stained cells (apoptotic cells).

Staining with the level of MMP

This Rhodamine 123 (RH-123) probe was used to measure the values of MMP. We collected cells and incubated them (10 and 12.5 µg of umbelliferone) for 30 min at 37°C with 10 µM Rhodamine 123 (RH-123). In addition, the cells were rinsed with saline to discard the excess stain and then examined under a fluorescent microscope (Olympus).[15]

ROS (reactive oxygen species) measurement

DCFDA, a stable nonpolar dye, was used to monitor ROS production. In this study, cells were cultivated at a population of 1×10^5 cells/mL, permitted to adhere for 24 hr and then administered with 10 and 12.5 µg of Umbelliferone. During 20 min of incubation at 37°C in the dark, the cells were treated with 10 mM DCFDA. A fluorescent microscope (Olympus) was used to monitor ROS production in the cells.

Assay for cellular adhesion

To determine the cell adhesion characteristic feature of MDA-MB-231 cells administered with 10 and 12.5 µg Umbelliferone, cell adhesion assays were performed. After seeding cells in serum-free DMEM medium on coated plates, the cells were incubated for 2 hr. The cells were rinsed thrice with PBS using PBS after 2 hr to remove unattached cells. Methanol fixation was applied for 10 min to the remaining adherent cells. The fixed cells were stained for 15 min with methylene blue dye and assessed using a microscope.

Estimation of caspases activity

Determination of caspase 3 and 9 activity in MDA-MB-231 cells administered with 10 and 12.5 µg was performed by colorimetric-based caspase 3 and 9 assay kits (Caspase Colorimetric Protease Assay Sampler Kit, Thermo Fischer Scientific and the USA). The experiment was executed as per the guidelines of the manufacturer. Analyzing this product with microplates at 405 nm allowed the optical density to be determined. Based on the graphed curve with OD values the experiments were performed in triplicates.

Cytokine assay

A method for expressing IL-1β, IL-6 and IL-8 has been described elsewhere, using the ELISA method.[16] As described above, we grew MDA-MB-231 cells according to the protocol and then treated them with 10 µg or 12.5µg Umbelliferone after 24 hr of incubation. After exposed cells had been removed by centrifugation, we examined their IL-1β, IL-6 and IL-8 levels using kits as per the guidelines of the manufacturer (Sigma-Aldrich, USA).

Statistical analysis

The results of triplicate experiments were given as mean±SD and the variations between them are determined by one-way ANOVA. The $p<0.05$ was fixed to represent the significant levels.

RESULTS

Anti-proliferative activity Umbelliferone against MDA-MB-231 cells

To examine the influence of Umbelliferone on MDA-MB-231 cell growth was assessed by the MTT assay. As exhibited in Figure 1, umbelliferone considerably inhibited 37°C and 24 hr the cell growth of MDA-MB-231 cells. The cell growth was inhibited by >50 or 65% in cells administered with 10 or 12.5 µg/mL of the umbelliferone, respectively. Therefore, it was clear that the umbelliferone exhibited potent cytotoxicity hence; we employed the umbelliferone for additional experiments.

Analysis of apoptosis in MDA-MB-231 cells by umbelliferone treatment

The evaluation of cell morphology was conducted through the utilization of AO/EtBr staining. The outcomes of the study revealed that the nucleus structure of the control cells remained undamaged. However, in the cells treated with umbelliferone, distinct characteristics of apoptosis such as nuclear chromatin condensation were found. These changes were accompanied by other alterations in cellular morphology, as depicted in Figure 2. Following the administration of umbelliferone, it was seen that cell shrinkage and cytoplasm condensation occurred in a manner that was dependent on the dosage. According to the data presented in Figure 2, the administration of umbelliferone led to a notable augmentation in the accumulation of apoptotic cells as compared to the control cells that were not subjected to any treatment.

Impact of the umbelliferone on MMP level

In this work, we scrutinized the influence of umbelliferone on the integrity of mitochondrial membranes, which is considered an initial step in the process of apoptosis. To assess this, we employed the Rhodamine 123 (RH-123) fluorescent probe. The findings depicted in Figure 3 demonstrate that the administration of umbelliferone resulted in a diminution in the MMP status in comparison to the control cells.

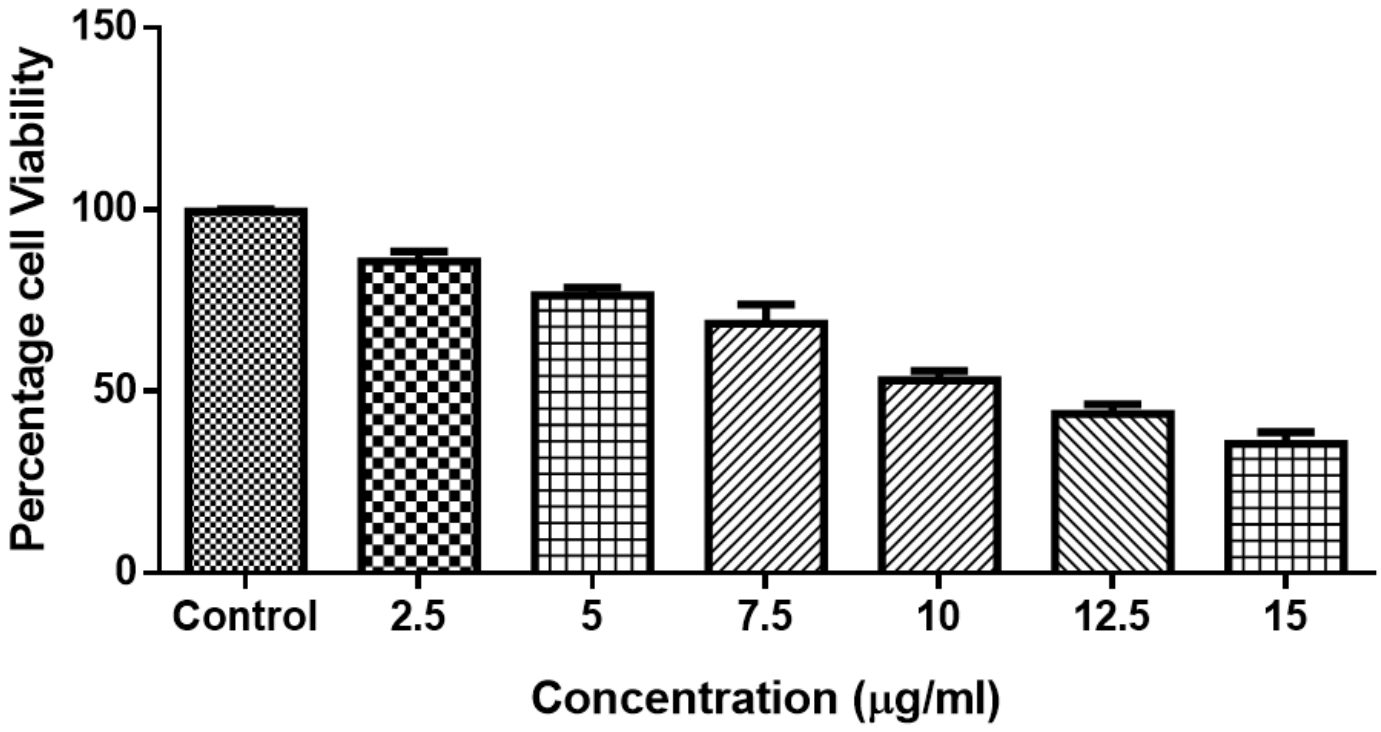

Figure 1: Umbelliferone triggers cytotoxicity in MDA-MB-231 cells at 2.5-15µg/mL dosages treatment for 24 hr. Both control and treated cells were assessed by the MTT assay and the outcomes are revealed as a mean±SD of triplicates.

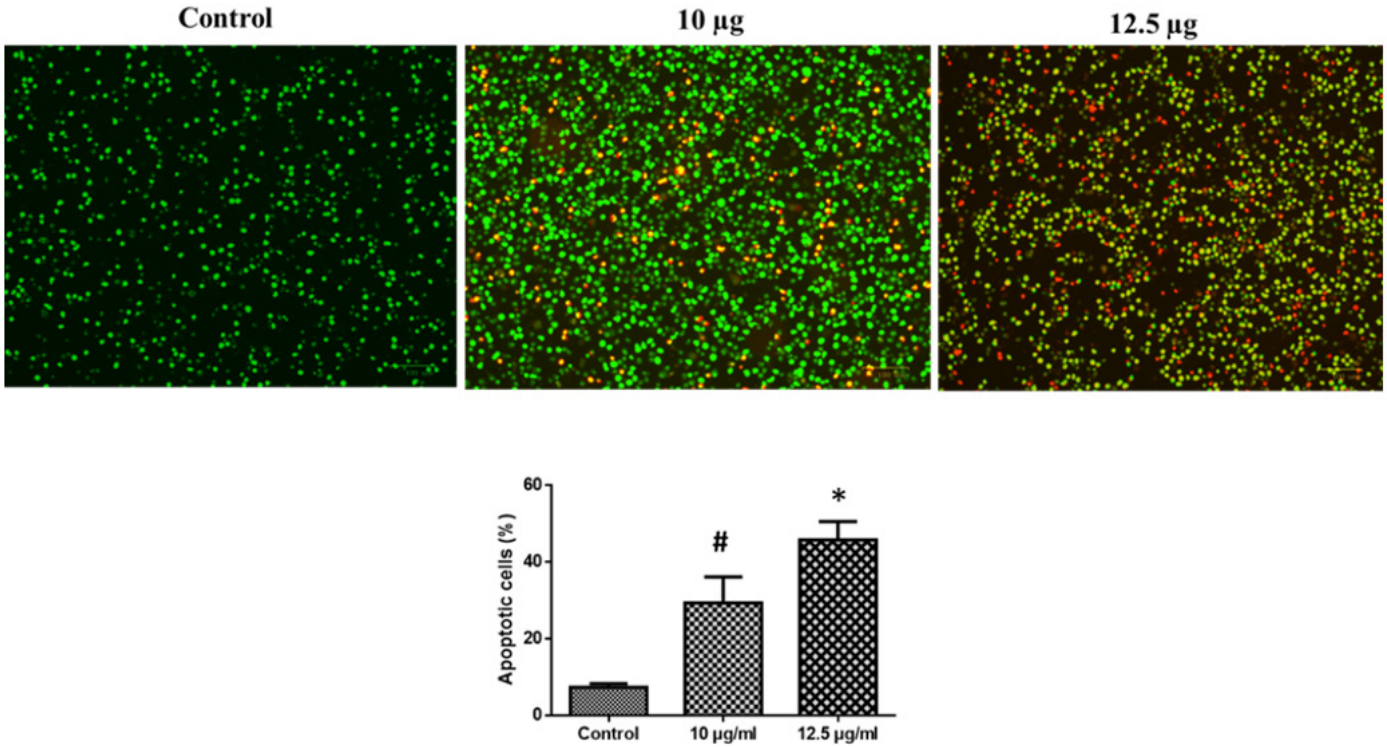

Figure 2: MDA-MB-231 cells are apoptotic when Umbelliferone is applied at 10 and 12.5 µg dosages treatment for 24 hr. AO and EtBr (1:1), were employed to stain the cells.

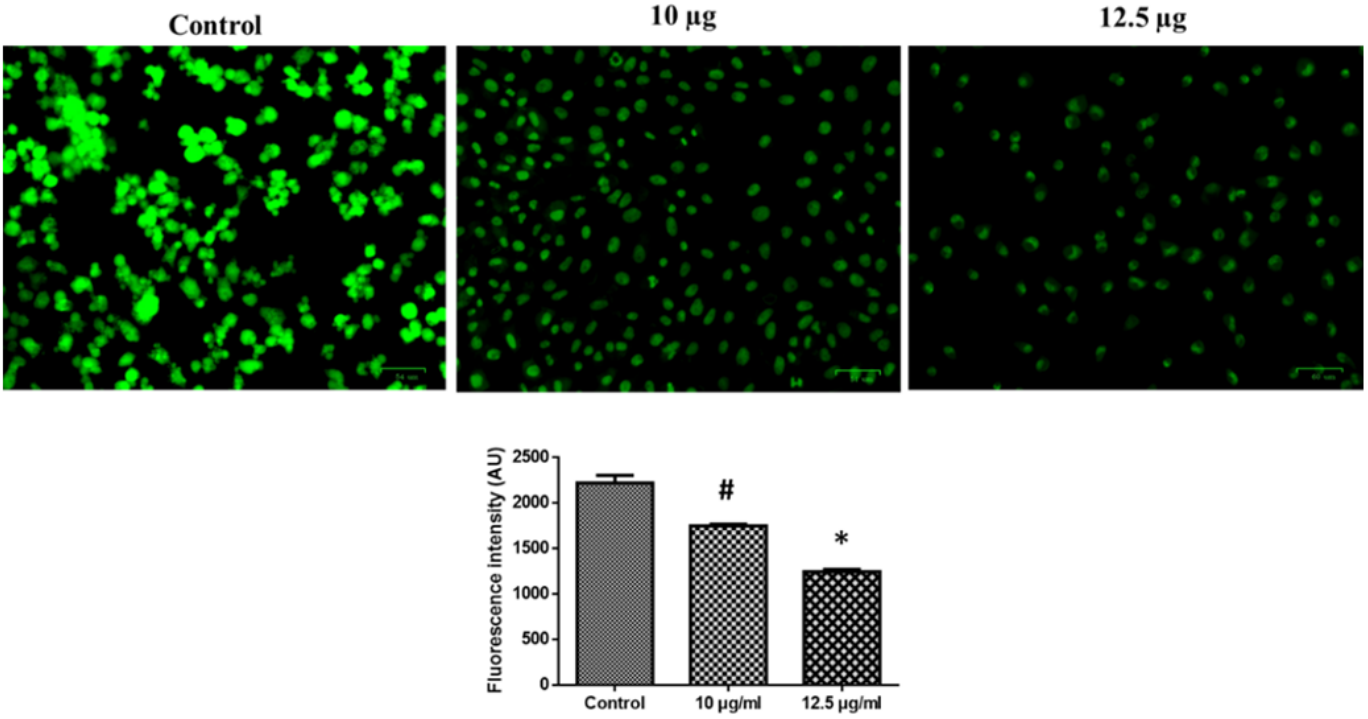

Figure 3: MDA-MB-231 cells with Umbelliferone demonstrate the diminished MMP levels.

Umbelliferone-induced ROS accumulation in MDA-MB-231 cells

Oxidative stress arises as a consequence of an inequilibrium between the concentrations of pro-oxidants and anti-oxidants inside cellular systems. The overproduction of ROS due to a disproportion between pro-oxidants and anti-oxidants can cause damage to cellular macromolecules such as DNA, proteins and lipids. This can ultimately result in the physical and chemical deterioration of tissues, potentially leading to cellular demise.

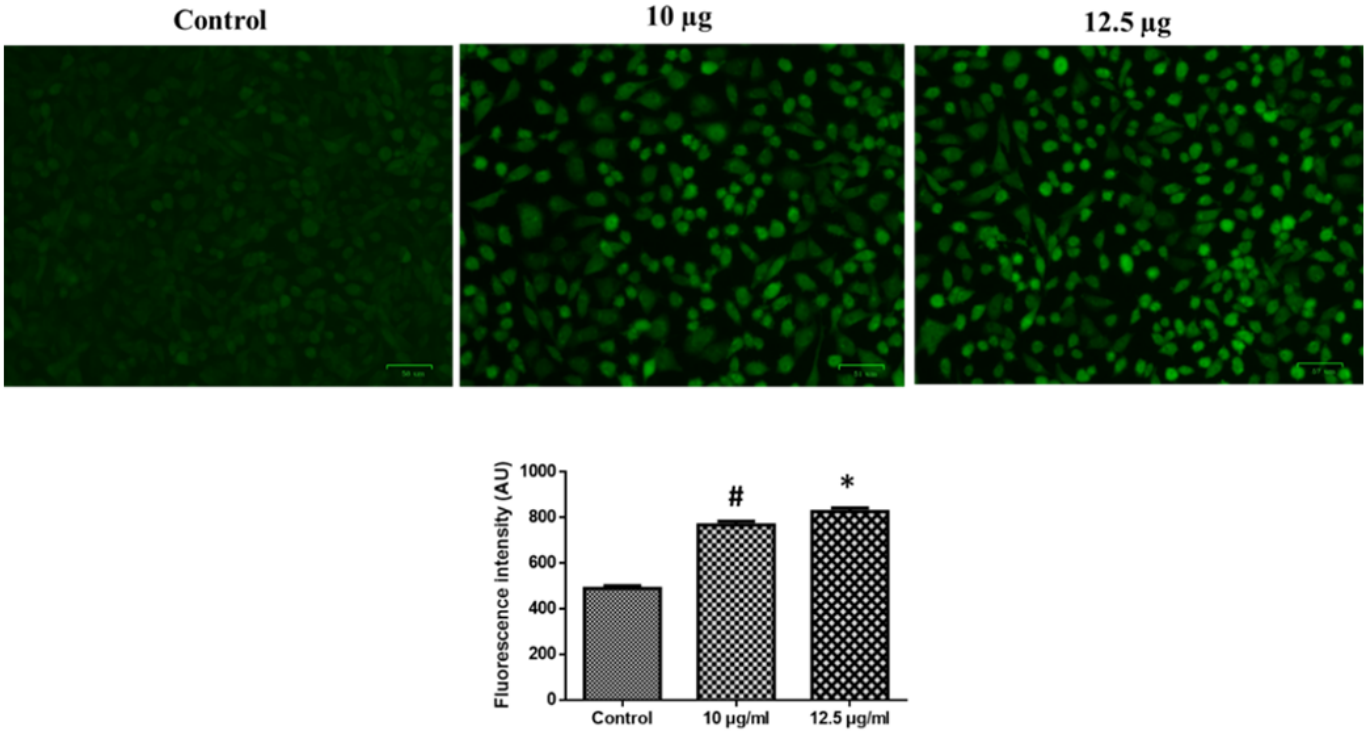

Figure 4: The MDA-MB-231 cells were treated with Umbelliferone for 24 hr and stained using the DCFH-DA dye. Control and different concentrations on 10 and 12.5 µg of Umbelliferone treated cells.

Hence, an investigation was conducted to determine the potential involvement of ROS formation in the induction of apoptosis by umbelliferone. As depicted in Figure 4, the compound umbelliferone exhibited a significant increase in intracellular levels of ROS within a time frame of 15 min. The findings of this study suggest that ROS has a role in inducing apoptosis in MDA-MB-231 cells treated with umbelliferone.

MDA-MB-231 cells are ineffective at adhesion when Umbelliferone is added

Using MDA-MB-231 cells treated with Umbelliferone for gel cell adhesion assays. Compared to Umbelliferone-treated cells, the control cells revealed higher adherent cells. 10 and 12.5 µg of Umbelliferone administration reduced the adherent cells equally with 12.5 µg of Umbelliferone (Figure 5A).

Caspase activity Umbelliferone in MDA-MB-231 cells

We aimed to assess the activity levels of caspase-3 and caspase-9 in Umbelliferone-treated MDA-MB-231 cells that undergo apoptosis. There is a considerable augmentation in caspase-3 and 9 activities in Umbelliferone-treated cells over control cells. Expressions of caspase-3 and 9 are based in a dosage-dependent manner in MDA-MB-231 cells (Figure 5B).

Umbelliferone increases the inflammatory response in MDA-MB-231 cells

Figure 6 as shown below, Umbelliferon's effects on inflammation imply that produces more inflammatory cytokines. Umbelliferone-treated cell lines expressed significantly higher status of IL-1β, IL-6 and IL-8, than control cells and then showed a dose-dependent increase after Umbelliferone 10 and 12.5 µg administration.

DISCUSSION

7-hydroxycoumarin, also known as umbelliferone, is a common coumarin derivative. In addition to carrots, coriander, angelica, Hydrangea macrophylla and Ferula communis, umbelliferone has been found in numerous plants across nature.[17] In sunscreens, umbelliferone is used because it can absorb ultraviolet light effectively at multiple wavelengths.[18] A few studies have reported its analgesic, anti-inflammatory and anti-inflammatory properties, along with its anti-nociceptive properties. An animal study has demonstrated that umbelliferone inhibits cancer progression and enhances survival period in tumor-bearing animals undergoing sarcoma 180.[19,20] A dose-dependent effect of umbelliferone on RK33 laryngeal cancer cells was found by the authors. A study on umbelliferone has also shown that it inhibits the growth of rat mammary cancer stimulated by 7,12-dimethylbenz(a) anthracene.[21]

There are a variety of biological properties of this component that may contribute to health benefits but the mechanisms that prevent malignant cells from proliferating have not been fully determined. While we examined traditional medicinal plants for their ability to induce apoptosis from natural sources, we studied the compound's efficacy. Concentration-dependent inhibition of the MDA-MB-231 cell growth induced by umbelliferone

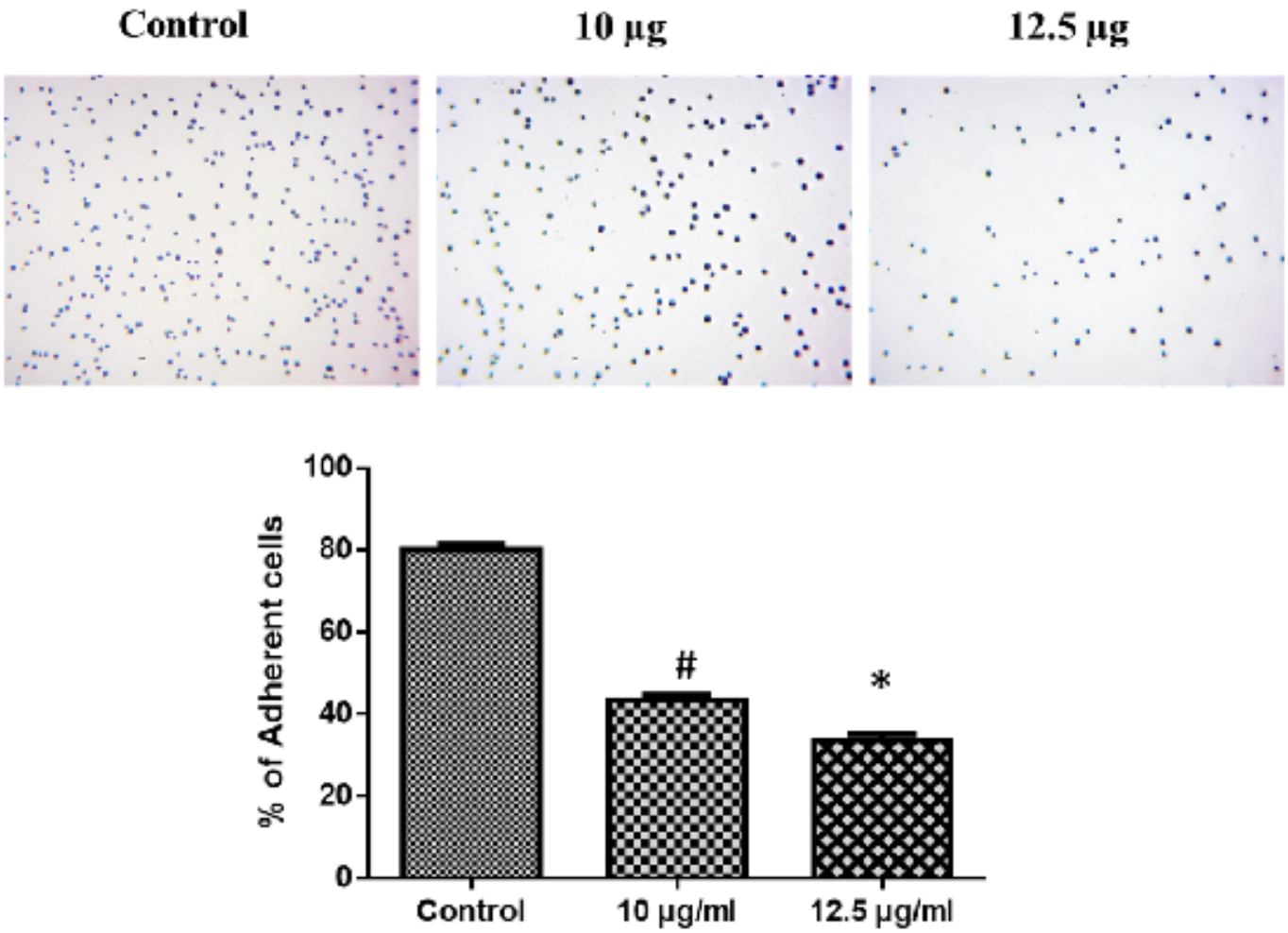

Figure 5A: Cell adhesion is disrupted in the MDA-MB-231 cells after the treatment with the Umbelliferone for 24 hr. Control, 10 µg and 12.5 µg of Umbelliferone-treated cells.

was demonstrated in the current study (Figure 1). By assessing the nuclear damages and apoptotic cells (Figure 2), we further confirmed that the umbelliferone-promoted anti-cancer properties due to apoptosis.

Multiple pathological conditions can be caused by dysregulated apoptosis, including cancer and when apoptosis fails, an inequity in cell number develops, resulting in tumorigenesis. Consequently, cancer therapy has developed around the stimulation of apoptosis in tumor cells.[22] According to our results, umbelliferone-induced apoptosis due to the stimulation of the caspase-3 and inactivation of survivin (Figures 5B). Additionally, the outcomes showed that the umbelliferone exposure resulted in caspase-3 and caspase-9 activation and the destruction of PARP which leads to diminution of the MMP (Figure 3). Apoptosis occurs via the extrinsic pathway where caspase-8/-10 is activated, resulting in the stimulation of the downstream caspases and consequently, death receptors are recruited.[23]

When mitochondria become dysfunctional, stressors, such as chemotherapeutic agents, trigger the intrinsic pathway. As a result of mitochondrial dysfunction, caspase-9 is activated and caspases subsequently become active. Caspases cleave a variety of specific substrates including PARP following caspase-3 activation. This results in apoptosis. Occasionally, caspase-8 regulates the intrinsic cascade through the cleavage of Bad, pro-apoptotic genes. Several molecules, such as those in the Bcl-2 and IAP families, control caspases.[23] Apoptosis is mediated by Bcl-2 proteins by interacting with pro-apoptotic members (like Bax and Bad) and antiapoptotic members (like Bcl-2 and Bcl-xL), especially the intrinsic signaling members with dysfunction of mitochondria. Despite inhibiting caspase-3 and 9, IAP family proteins are not capable of inhibiting caspase-8 use.[24]

We also observed a significant apoptosis-induced response to umbelliferone treatment (Figure 5A). According to these data, caspases are the pivotal players in causing umbelliferone-mediated apoptosis in MDA-MB-231 cells, denoting that umbelliferone-stimulated apoptosis involves caspase-dependent pathways. According to growing evidence, the redox state affects cell fate and may contribute to apoptosis via oxidative DNA injury and diminution of the MMP status.[25] We found that umbelliferone treatment promoted the endogenous

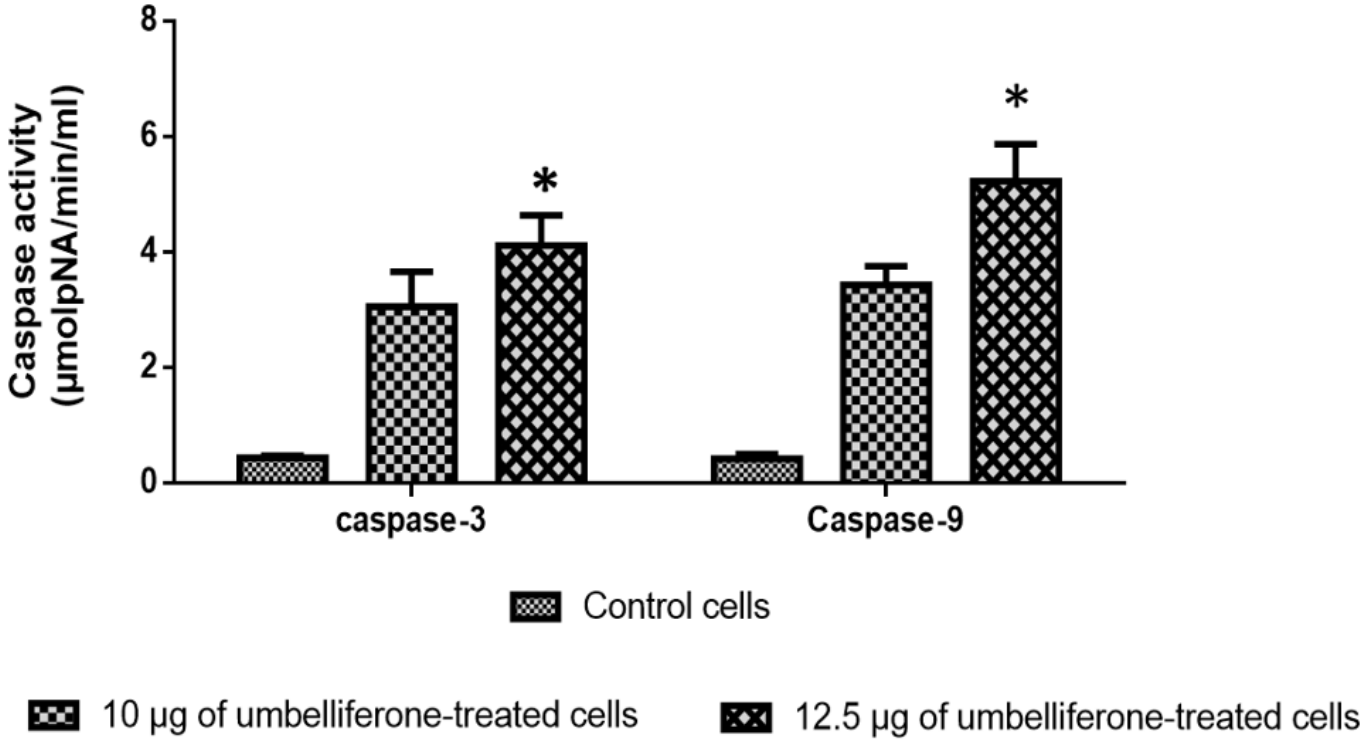

Figure 5B: An assay kit based on colorimetry was used to assess caspase-3 and 9 activities in cell lysates. The data are revealed as a mean±SD of triplicates. *n*=3, **p*<0.005 compared to the control.

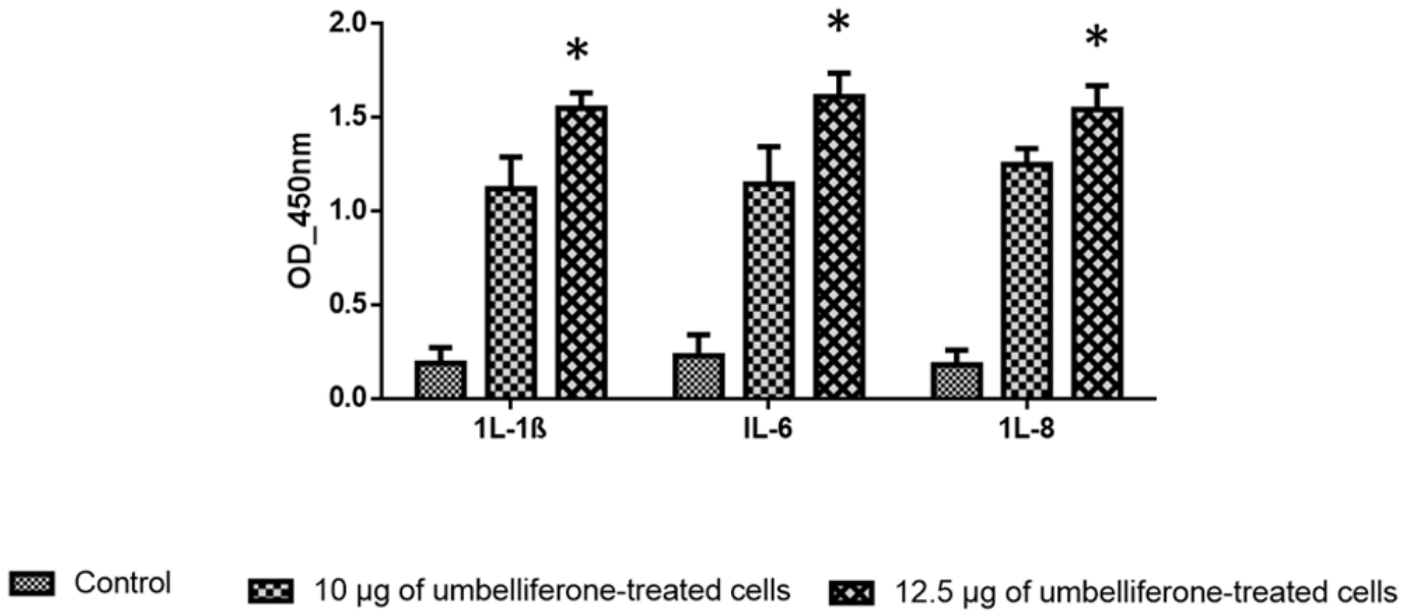

Figure 6: MDA-MB-231 cells treated with Umbelliferone have elevated IL-1β, IL-6 and IL-8 levels. An assay kit based on colorimetric was employed to examine inflammatory marker levels in the cell lysates. The data are revealed as a mean±SD of triplicates. *n*=3, **p*<0.005 compared to the control.

ROS accumulation in MDA-MB-231 cells, which demonstrated that ROS are generated for apoptosis caused by umbelliferone treatment. IL-1β, IL-8 and IL-6 status are remarkably elevated in umbelliferone-treated MDA-MB-231 cells than in control cells, confirming that inflammation leads to apoptosis (Figure 6).

The present study suggests that death receptors initiate this pathway by tying up Death Receptors to stimulate proximal caspases (caspases 3 and 8) which in turn stimulate the effector caspases like caspase-3. A disrupted mitochondrial membrane initiates the caspase-9 cascade, hence initiating the apoptotic signaling through the mitochondrial pathway. Furthermore, truncated Bid seems to be a potential mechanism to increase umbelliferone-induced caspase-dependent apoptosis through crosstalk between the two pathways.[26] The active compounds of Umbelliferone need to be identified; nonetheless, this is a pioneering study that offers a pivotal novel understanding of the anticancer properties of Umbelliferone.

CONCLUSION

To our knowledge, umbelliferone hasn't previously been shown to exhibit any anti-cancer activity against MDA-MB-231 cells. Hence, this study explores the mechanism of umbelliferone cytotoxicity for the first time. MDA-MB-231 cells were found to be apoptosis by umbelliferone. The role of umbelliferone to treat and manage breast cancer might therefore be able to be determined with additional research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest

ABBREVIATIONS

AO/EtBr: Acridine Orange and Ethidium Bromide; **DCFH-DA:** Dichloro-dihydro-fluorescein diacetate; **HDI:** Human Development Index; **HCCs:** Hepatocellular carcinomas; **RPMI:** Human Nasal Septum Carcinoma; **HT-29:** Colon Carcinoma; **A-549:** Small Lung Carcinoma; **DMEM:** Dulbecco's modified Eagle's medium; **FBS:** Fetal bovine serum.

SUMMARY

Studies have highlighted a lack of awareness about breast cancer, negatively impacting breast self-examination practices. This is a cross-sectional study conducted in Saudi Arabia's Eastern Province to assess breast cancer awareness among adult females. Many factors are responsible for causing breast cancer, but diet, hormones, pregnancy, obesity and abnormal breast development are among the most prevalent. Natural compounds with anti-proliferative capabilities are gaining popularity as a way to mitigate the toxicity of radiation and synthetic anti-tumor drugs. Umbelliferone has been reported to possess diverse pharmacological activities, like anti-cancer, anti-inflammation and anti-oxidation properties. We estimated the cell viability of MDA-MB-231 cells by MTT, AO/EtBr, Cell adhesion assays, MMP, ROS, levels of caspase-3, -9 for apoptosis and inflammatory markers IL-1β, IL-6 and IL-8. The results showed that apoptosis was induced by umbelliferone in MDA-MB-231 cells by staining with AO/EtBr; triggering ROS pathway and DNA damage level of MMP. The caspase and inflammatory marker levels were also increased upon umbelliferone treatment. All these results suggest that umbelliferone can be used as a potent anti-cancer drug for breast cancer cells

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Cite this article: Albratty M, Makeen HA. An Umbelliferone-Induced Caspase-Mediated Apoptosis in Mda-Mb-231 Breast Cancer Cells. Indian J of Pharmaceutical Education and Research. 2024;58(3):890-8.