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MORPHOLOGICAL AND FLUORESCENCE-BASED EVALUATION OF EPIGALLOCATECHIN GALLATE-INDUCED APOPTOSIS IN MDA-MB-231 TRIPLE-NEGATIVE BREAST CANCER CELLS

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ABSTRACT

Background: Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer with limited targeted therapeutic options owing to the absence of estrogen receptor, progesterone receptor, and HER2 expression. Epigallocatechin-3-gallate (EGCG), the major polyphenolic catechin of green tea, has demonstrated promising anticancer activity by inducing oxidative stress and apoptosis. Morphological and fluorescence-based techniques provide valuable insights into the cellular events associated with EGCG-induced apoptotic cell death.

Objective: To evaluate the morphological and fluorescence-based changes associated with EGCG-induced apoptosis in MDA-MB-231 triple-negative breast cancer cells.

Materials and Methods: MDA-MB-231 cells were treated with increasing concentrations of EGCG (0–50 μM) for 24 h. Morphological alterations were assessed using phase-contrast microscopy. Intracellular reactive oxygen species (ROS) generation was qualitatively evaluated using DCFH-DA fluorescence staining. Apoptotic changes were further examined by Acridine Orange/Ethidium Bromide (AO/EB) dual staining and Propidium Iodide (PI) staining. Representative microscopic images were analyzed to characterize cellular and nuclear changes associated with apoptosis.

Results: EGCG treatment induced marked morphological alterations in MDA-MB-231 cells, including cell shrinkage, rounding, membrane blebbing, reduced cellular adherence, and loss of normal spindle-shaped morphology. DCFH-DA fluorescence imaging demonstrated enhanced intracellular green fluorescence in EGCG-treated cells, indicating increased ROS generation. AO/EB dual staining revealed a progressive increase in early and late apoptotic cells characterized by chromatin condensation, nuclear fragmentation, and orange-red fluorescence. PI staining demonstrated increased membrane permeability with a higher proportion of PI-positive cells following EGCG treatment, indicating late apoptotic cell death. Collectively, the microscopic findings consistently demonstrated concentration-dependent apoptotic changes following EGCG exposure.

Conclusion: EGCG induces characteristic morphological and fluorescence-based features of apoptosis in MDA-MB-231 triple-negative breast cancer cells. The observed cellular and nuclear alterations, together with enhanced ROS fluorescence and increased PI uptake, provide qualitative evidence supporting oxidative stress-mediated apoptosis. These findings further support the potential of EGCG as a promising naturally derived therapeutic agent for the treatment of triple-negative breast cancer.

KEYWORDS: Epigallocatechin-3-gallate; EGCG; Triple-negative breast cancer; MDA-MB-231; Apoptosis; Phase-contrast microscopy; Acridine Orange/Ethidium Bromide staining; Propidium Iodide staining; DCFH-DA.

INTRODUCTION

Triple-negative breast cancer (TNBC) is one of the most aggressive molecular subtypes of breast cancer and accounts for approximately 15–20% of all breast cancer cases. The absence of estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor-2 (HER2) limits the effectiveness of targeted therapies, making chemotherapy the primary treatment option. However, poor therapeutic response, frequent recurrence, and the development of chemoresistance continue to contribute to unfavorable clinical outcomes. Consequently, there is growing interest in identifying novel therapeutic agents capable of selectively inducing apoptosis in TNBC cells

while minimizing toxicity to normal tissues (1–3).

Natural bioactive compounds have emerged as attractive candidates for cancer therapy because of their ability to regulate multiple cellular signaling pathways involved in proliferation, survival, invasion, and apoptosis. Among these compounds, epigallocatechin-3-gallate (EGCG), the principal polyphenolic catechin present in green tea (*Camellia sinensis*), has been extensively investigated for its anticancer potential. Previous studies have demonstrated that EGCG suppresses tumour cell growth by modulating oxidative stress, inhibiting cell-cycle progression, reducing metastatic potential, and activating intrinsic apoptotic pathways. These diverse biological properties have positioned EGCG as a promising naturally derived anticancer molecule (4–7).

Apoptosis is a highly regulated form of programmed cell death that plays a critical role in maintaining tissue homeostasis and eliminating damaged or malignant cells. Unlike necrosis, apoptosis is characterized by distinct biochemical and morphological alterations, including cell shrinkage, chromatin condensation, nuclear fragmentation, membrane blebbing, and formation of apoptotic bodies. These structural changes occur in a sequential manner and provide important evidence for evaluating the mechanism of action of potential anticancer agents.

Therefore, detailed morphological assessment has become an integral component of preclinical studies investigating apoptosis-inducing compounds (8–10).

Microscopy-based techniques are widely employed to identify and characterize apoptotic cell death. Phase-contrast microscopy enables direct visualization of cellular morphology, allowing observation of characteristic apoptotic features such as loss of adherence, cytoplasmic shrinkage, and membrane blebbing. In addition, fluorescence-based staining techniques provide greater specificity for detecting different stages of apoptosis. Acridine Orange/Ethidium Bromide (AO/EB) dual staining differentiates viable, early apoptotic, late apoptotic, and necrotic cells based on membrane integrity and nuclear morphology, whereas Propidium Iodide (PI) selectively stains cells with compromised plasma membranes, indicating late apoptosis or secondary necrosis. Together, these methods provide reliable qualitative evidence supporting apoptosis induced by anticancer compounds (11–14).

Oxidative stress is closely associated with apoptotic cell death in cancer cells. Excessive intracellular reactive oxygen species (ROS) disrupt mitochondrial function, promote cytochrome *c* release, activate executioner caspases, and initiate the morphological changes that characterize apoptosis. Fluorescence imaging using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) has become a widely accepted technique for visualizing intracellular ROS generation and correlating oxidative stress with apoptotic progression. When combined with phase-contrast and fluorescence microscopy, DCFH-DA staining provides valuable mechanistic insights into the sequence of cellular events leading to programmed cell death (15–17).

The human MDA-MB-231 cell line is an established in vitro model of triple-negative breast cancer because of its highly invasive phenotype, mesenchymal characteristics, and resistance to conventional therapy. Although several studies have reported the antiproliferative effects of EGCG in breast cancer cells, relatively few have comprehensively evaluated the accompanying morphological and fluorescence-based changes associated with apoptosis using complementary microscopic techniques. Such characterization is essential for confirming apoptotic cell death and strengthening the mechanistic evidence supporting the anticancer activity of EGCG (6,18–20).

Therefore, the present study aimed to evaluate the morphological and fluorescence-based changes associated with EGCG-induced apoptosis in MDA-MB-231 triple-negative breast cancer cells. Phase-contrast microscopy, DCFH-DAfluorescence imaging, Acridine Orange/Ethidium Bromide dual staining, and Propidium Iodide staining were employed to characterize cellular and nuclear alterations following EGCG treatment. The findings provide qualitative evidence supporting oxidative stress-mediated apoptosis and further clarify the cellular mechanisms underlying the anticancer activity of EGCG.

MATERIALS AND METHODS:

Cell Culture

The human triple-negative breast cancer cell line (MDA-MB-231) was obtained from a certified cell repository. Cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cell cultures were maintained at 37°C in a humidified incubator with 5% CO_2. Cells were subcultured upon reaching approximately 80–90% confluence, and exponentially growing cells were used for all experiments.

Experimental Design

MDA-MB-231 cells were seeded in appropriate culture plates and allowed to attach overnight. Cells were treated with increasing concentrations of EGCG (0, 10, 20, 30, 40, and 50 µM) for 24 h, while untreated cells served as the control group. Following treatment, morphological alterations, intracellular ROS generation, and apoptosis-associated cellular changes were evaluated using phase-contrast microscopy and fluorescence-based staining techniques.

Phase-Contrast Microscopy

Morphological alterations induced by EGCG treatment were examined using an inverted phase-contrast microscope. Following 24 h of treatment, cells were observed directly without staining for characteristic features of apoptosis, including cell shrinkage, rounding, membrane blebbing, loss of cell-to-cell contact, and detachment from the culture surface. Representative images were captured at ×20 magnification under identical microscopic settings.

DCFH-DA Fluorescence Imaging
Intracellular reactive oxygen species (ROS) generation was qualitatively assessed using the DCFH-DA fluorescence staining method. After treatment with EGCG, cells were washed twice with phosphate-buffered saline and incubated with DCFH-DA solution under dark conditions at 37°C. Excess dye was removed by washing with PBS, and fluorescence images were immediately captured using a fluorescence microscope with appropriate excitation and emission filters. Increased green fluorescence intensity was considered indicative of enhanced intracellular ROS generation.

Acridine Orange/Ethidium Bromide (AO/EB) Dual Fluorescence Staining
Apoptotic cells were identified using Acridine Orange/Ethidium Bromide (AO/EB) dual staining. Following EGCG treatment, cells were washed with PBS and incubated with freshly prepared AO/EB staining solution. Stained cells were examined immediately under a fluorescence microscope. Viable cells exhibited uniform green fluorescence with intact nuclei, whereas early apoptotic cells showed bright green nuclei with chromatin condensation. Late apoptotic cells displayed orange to reddish fluorescence with condensed or fragmented nuclei, while necrotic cells exhibited intense red fluorescence due to loss of membrane integrity.

Propidium Iodide (PI) Staining
Membrane permeability associated with late apoptosis was evaluated using Propidium Iodide staining. Following treatment, cells were washed with PBS and incubated with PI solution for the recommended duration under dark conditions. After washing, cells were observed using a fluorescence microscope. Cells exhibiting red fluorescence were considered PI-positive, indicating compromised plasma membrane integrity and late apoptotic or necrotic cell death.

Image Acquisition and Interpretation
All microscopic observations were performed using identical imaging parameters for both untreated and treated groups. Representative images from at least three independent experiments were selected for qualitative comparison. Morphological changes and fluorescence staining patterns were interpreted independently by two investigators to minimize observer bias.

Statistical Analysis
Data are presented as **mean ± standard deviation (SD)** of three independent experiments. Graphical representation and descriptive statistical analysis were performed using **GraphPad Prism version 9.0** (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

Morphological assessment
Phase-contrast microscopic examination revealed marked morphological differences between untreated and EGCG-treated MDA-MB-231 cells. Control cells exhibited normal spindle-shaped morphology with intact cellular architecture and firm adherence to the culture surface. In contrast, EGCG-treated cells displayed characteristic apoptotic changes, including cell shrinkage, rounding, membrane blebbing, reduced cell-to-cell contact, and detachment from the culture surface. These morphological alterations became more pronounced following EGCG treatment, indicating progressive cellular damage and apoptosis (Figure 1).

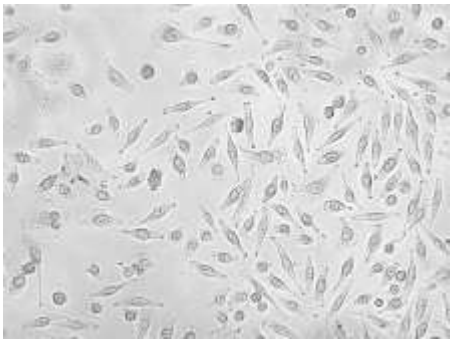
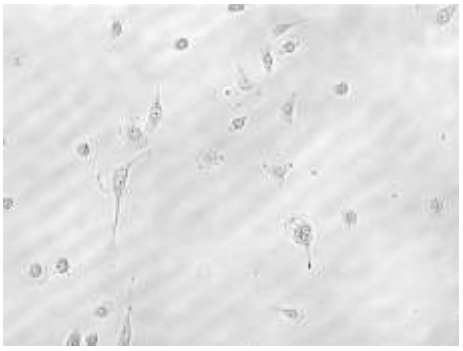


FIG.1: Morphological Changes in MDA-MB-231 Cells Following EGCG Treatment

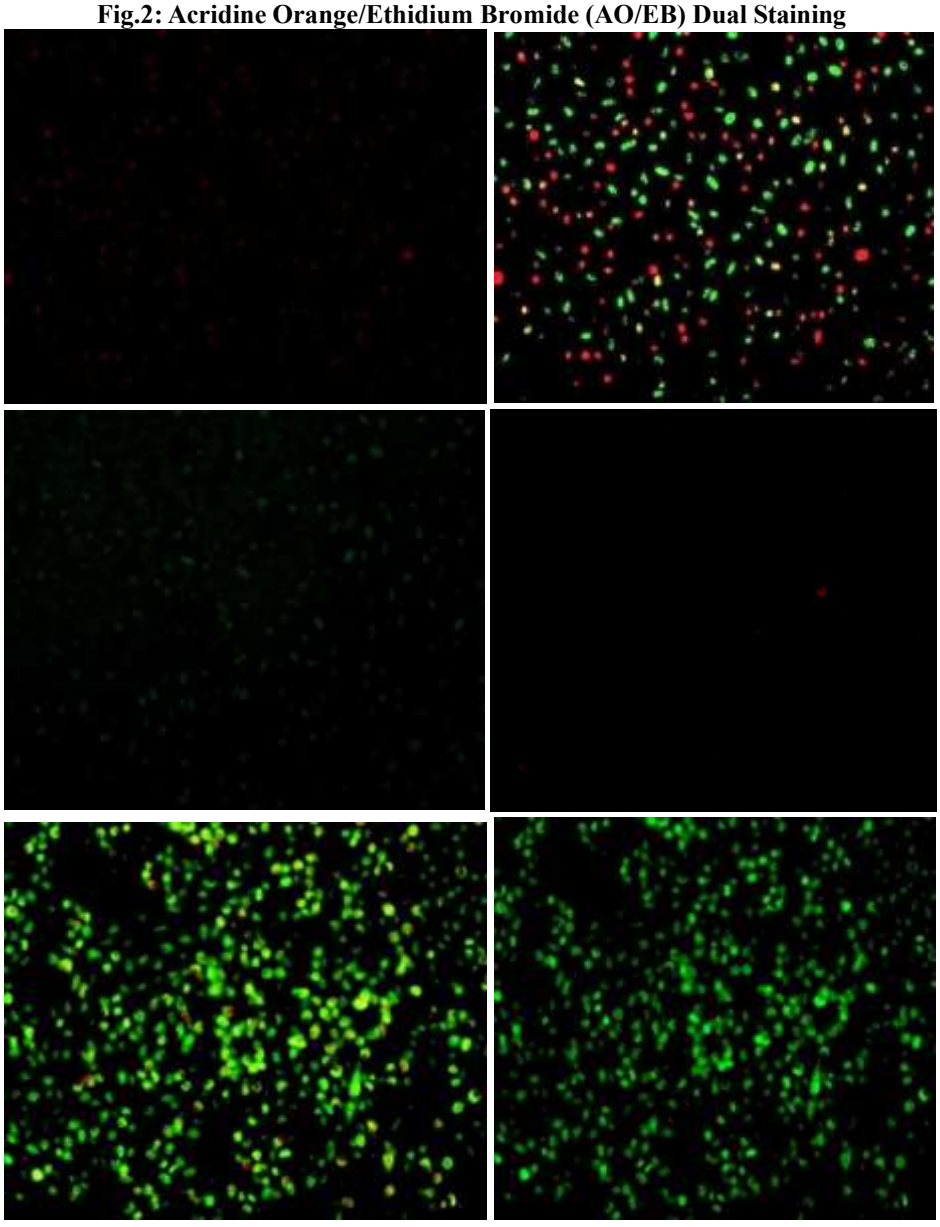


Control MD MB 231 CELLS-EGCG Treated MD MB 231 CELLS- IC5

Acridine Orange/Ethidium Bromide (AO/EB) dual staining

AO/EB dual fluorescence staining demonstrated distinct differences between untreated and EGCG-treated cells. Control cells predominantly exhibited uniform green fluorescence with intact nuclear morphology, indicating viable cells. In contrast, EGCG-treated cells showed an increased number of cells with chromatin condensation, nuclear fragmentation, and orange-red fluorescence, representing early and late apoptotic cells. The increased proportion of apoptotic cells following EGCG treatment confirmed apoptosis as an important mechanism of EGCG-induced cytotoxicity (Figure 2).

Fig.2: Acridine Orange/Ethidium Bromide (AO/EB) Dual Staining



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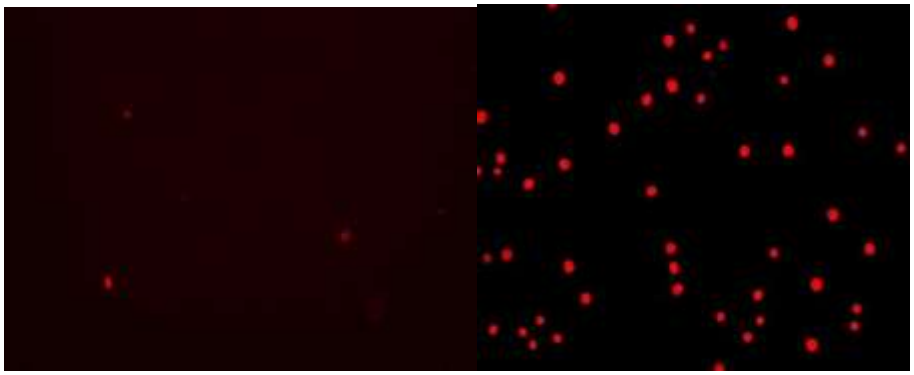
Propidium iodide (PI) staining

Propidium iodide staining revealed minimal PI-positive cells in the untreated control group, indicating intact plasma membrane integrity. EGCG-treated cells exhibited a marked increase in PI-positive staining, reflecting increased membrane permeability and loss of membrane integrity. The enhanced PI fluorescence observed in treated cells indicated increased late apoptotic cell death following EGCG exposure (Figure 3).

FIG.3: PROPODIUM IODINE STAINING



Control MD MB 231 CELLS- PI-2 EGCG Treated MD MB 231 CELLS-PI.3

DISCUSSION

The present study evaluated the morphological and fluorescence-based alterations associated with EGCG-induced apoptosis in MDA-MB-231 triple-negative breast cancer cells. Using complementary microscopic techniques, including phase-contrast microscopy, DCFH-DA fluorescence imaging, Acridine Orange/Ethidium Bromide (AO/EB) dual staining, and Propidium Iodide (PI) staining, the study demonstrated characteristic apoptotic changes following EGCG treatment. These observations provide qualitative evidence supporting apoptosis as the predominant mechanism of EGCG-induced cell death in MDA-MB-231 cells.

Phase-contrast microscopy revealed marked morphological alterations in EGCG-treated cells compared with untreated controls. Control cells maintained their characteristic spindle-shaped morphology with intact cellular architecture and firm adherence to the culture surface, whereas treated cells exhibited progressive cell shrinkage, rounding, membrane blebbing, reduced cell-to-cell contact, and detachment. These morphological alterations are classical features of apoptosis and reflect cytoskeletal reorganization and progressive cellular condensation during programmed cell death. Rodríguez-Teja et al. demonstrated similar apoptotic morphological changes following EGCG treatment in breast cancer cells (21). Likewise, Bimonte et al. highlighted apoptosis as one of the principal mechanisms responsible for the anticancer activity of EGCG in triple-negative breast cancer (22). Fujiki et al. also reported that green tea catechins induce characteristic apoptotic morphological changes in several human cancer cell lines (23).

Fluorescence microscopy using DCFH-DA staining demonstrated enhanced intracellular green fluorescence in EGCG-treated cells, indicating increased reactive oxygen species (ROS) generation. Although quantitative ROS estimation was presented separately in the first manuscript, the fluorescence images obtained in the present study visually confirmed oxidative stress induction following EGCG exposure. Schumacker described excessive ROS generation as a critical mediator of mitochondrial dysfunction and apoptosis in cancer cells (24). Similarly, Moloney and Cotter emphasized that disruption of redox homeostasis activates multiple apoptotic signalling pathways (25). Negri et al. further reported that EGCG selectively exhibits pro-oxidant activity in malignant cells by increasing intracellular ROS beyond their antioxidant capacity, thereby promoting apoptosis (26).

AO/EB dual staining clearly demonstrated progressive apoptosis following EGCG treatment. Untreated cells exhibited uniform green fluorescence with intact nuclei, whereas treated cells showed chromatin condensation, nuclear fragmentation, and orange-red fluorescence corresponding to early and late apoptotic stages. Elmore described these nuclear alterations as characteristic features of programmed cell death (27). Crowley et al. reported that AO/EB dual staining reliably differentiates viable, early apoptotic, late apoptotic, and necrotic cells based on fluorescence characteristics (28). Kerr et al. first established chromatin condensation and apoptotic body formation as defining morphological features of apoptosis (29). The present observations closely resemble these well-established apoptotic characteristics.

Propidium Iodide staining provided additional confirmation of apoptosis by demonstrating increased membrane permeability in EGCG-treated cells. Only a few PI-positive cells were observed in untreated controls, whereas treated cells exhibited intense red fluorescence, indicating compromised plasma membrane integrity during late apoptosis. Chan et al. demonstrated that PI staining is a reliable indicator of late apoptotic and necrotic cell death resulting from membrane disruption (30). The increased PI uptake observed in the present study therefore provides additional qualitative evidence supporting EGCG-induced apoptotic cell death.

The combined use of phase-contrast microscopy and fluorescence-based staining techniques represents an important strength of the present investigation. While biochemical assays provide quantitative evidence of apoptosis, microscopic techniques directly visualize the sequential structural and nuclear changes occurring during programmed cell death. The concordance among morphological alterations, enhanced DCFH-DA fluorescence,

AO/EB staining patterns, and increased PI positivity strongly supports oxidative stress-mediated apoptosis as the predominant mechanism underlying EGCG-induced cytotoxicity.

The findings of the present study are consistent with previous reports demonstrating that EGCG suppresses breast cancer progression by modulating oxidative stress and apoptotic signalling pathways. Rodríguez-Teja et al. reported that EGCG promotes mitochondrial dysfunction and apoptosis in breast cancer cells (21). Bimonte et al. summarized the multitargeted anticancer effects of EGCG in triple-negative breast cancer (22). Singh et al. further demonstrated that EGCG regulates multiple molecular pathways involved in oxidative stress, apoptosis, angiogenesis, and tumour progression, highlighting its potential as a naturally derived anticancer agent (31).

CONCLUSION

The present study demonstrated that EGCG induces distinct morphological and fluorescence-based features of apoptosis in MDA-MB-231 triple-negative breast cancer cells. Phase-contrast microscopy revealed characteristic apoptotic changes, including cell shrinkage, membrane blebbing, and loss of cellular adherence, while DCFH-DA fluorescence imaging confirmed increased intracellular ROS generation. AO/EB dual staining and PI staining further verified apoptotic progression through chromatin condensation, nuclear fragmentation, and increased membrane permeability. The consistent findings obtained using multiple complementary microscopic techniques provide strong qualitative evidence that EGCG promotes oxidative stress-mediated apoptotic cell death. These results reinforce the potential of EGCG as a promising natural anticancer agent for triple-negative breast cancer and warrant further molecular and in vivo studies to establish its therapeutic applicability.

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