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Cycloastragenol suppresses the abnormal proliferation of breast cancer cells *in vitro*

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ABSTRACT

Breast cancer remains the most prevalent malignancy among women. This form of cancer contributes to approximately 16% of cancer related mortality worldwide. In this study, antiproliferative effect of cycloastragenol was investigated in MCF-7 mammary cancer cells. The cytotoxic efficacy of cycloastragenol was assessed using the MTT assay. Intracellular ROS levels, mitochondrial membrane potential and nuclear morphological alterations were examined to explore the apoptotic efficacy of cycloastragenol. Cycloastragenol induced DNA damage was evaluated through comet assay. Flow cytometry was used to study the cell cycle analysis. The present study noticed the IC_{50} concentration of cycloastragenol at 60 µg/mL in MCF-7 cells. Cycloastragenol treated MCF-7 cells showed elevated ROS accumulation, loss of mitochondrial membrane potential, DNA fragmentation and cell-cycle arrest at the G0/G1 phase. Present findings indicate that cycloastragenol exerted significant *in vitro* anticancer effects against MCF-7 cells..

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INTRODUCTION

Cancer is the second leading cause of death worldwide, accounting for about one sixth of all global deaths and disproportionately affecting low and middle income countries, where nearly 70% of cancer related mortality occurs (Harakeh *et al.*, 2023). In 2022 alone, approximately 20 million new cases and nearly 10 million deaths were reported globally (Mfotie Njoya *et al.*, 2025). Cancer develops through uncontrolled cellular proliferation driven by accumulated genetic mutations, disrupted signaling pathways, resistance to growth inhibitory signals and evasion of programmed cell death. Additional hallmarks include limitless replicative potential, sustained angiogenesis, invasiveness and the ability to produce autonomous growth signals (Vahrmeijer *et al.*, 2013; Nardin *et al.*, 2020).

Breast cancer accounts for nearly 25% of all cancer cases and over 16.7% of cancer related deaths among women worldwide. In 2022, it was the second most commonly diagnosed cancer globally, with approximately 2.3 million new cases and 666,000 deaths (Bray *et al.*, 2024).

Standard treatment options include surgery, radiation therapy and chemotherapy. However, resistance to these therapies and adverse effects remain major clinical challenges. These drawbacks have heightened interest in phytocompounds as potentially safer therapeutic alternatives (Wali *et al.*, 2025). Traditional medicinal plants have long served as sources for identifying natural compounds with therapeutic potential (Chaachouay *et al.*, 2024). Herbal medicines are increasingly explored as promising agents in cancer therapy, providing a rich source of novel bioactive compounds for the development of effective chemotherapeutic drugs (Siddiqui *et al.*, 2025). MCF-7 (Michigan Cancer Foundation-7) cells are derived from a 69-year-old Caucasian woman with metastatic adenocarcinoma of the breast. These cells exhibit an epithelial morphology and are classified as a luminal epithelial phenotype. They express estrogen receptors, enabling them to respond to estrogen with increased proliferation (Surya *et al.*, 2023).

Oxidative stress plays a significant role in cancer initiation and progression as well as in diabetes, metabolic disorders and atherosclerosis (Ju *et al.,* 2024). ROS exert dual functions depending on their concentration. At physiological concentrations, ROS act as important signaling molecules that regulate cell proliferation, survival, migration, gene expression and angiogenesis. In cancer cells, these normal redox-regulated processes become dysregulated, allowing physiological ROS levels to support tumor growth, invasion and therapeutic resistance (Nakamura and Takada, 2021). Apoptosis, or programmed cell death, is a genetically controlled mechanism that removes damaged or unwanted cells during development and in response to cellular injury (Fuchs and Steller, 2011). It can be initiated through intrinsic or extrinsic pathways involving caspase activation, both of which converge to activate effectors caspases that produce the characteristic morphological and biochemical features of apoptosis (Pistritto *et al.*, 2016).

Phytonutrients have gained considerable attention for their therapeutic safety and efficacy. Cycloastragenol is a major phytochemical derived from the dried roots of Astragalus membranaceus (He *et al.*, 2022). It is a cycloartane-type triterpenoid known for its anti-inflammatory and neuroprotective properties (Zhu *et al.*, 2021; Ikram *et al.*, 2021). Recent research has demonstrated its anticancer potential in colorectal, lung and gastric cancers (Park *et al.*, 2022; Zhu *et al.*, 2024; Hwang *et al.*, 2019). Cycloastragenol attenuates liver fibrosis in CCl_4-treated mice (Luangmonkong *et al.*, 2023). However, no studies have investigated the anticancer activity of cycloastragenol against breast cancer using *in vitro* models. Therefore, the present study aims to evaluate the *in vitro* antitumor efficacy of cycloastragenol in MCF-7 cells.

MATERIALS AND METHODS

Cell culture

MCF-7 cancer cell line was acquired from the National Centre for Cell Science, India. Cells were cultured in DMEM containing FBS and penicillin–streptomycin. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO_2.

MTT assay

The MTT assay was used to determine the IC_{50} value of cycloastragenol (Mosmann, 1983). MCF-7 cells were seeded into 96-well plates and incubated overnight. The medium was replaced with fresh medium after 24 hrs containing various concentrations of cycloastragenol. The culture is then incubated for 24 hrs. MTT solution was added to each well and kept for 4 hrs. The absorbance of the resulting formazan crystals was read at 595 nm. Cell viability was determined using the formula.

Cell viability = (OD of treated cells / OD of control cells) × 100

Evaluation of reactive oxygen species generation

ROS levels were measured using the method of Rastogi *et al.* (2010). MCF-7 cells were treated with IC_{50} value of cycloastragenol and kept for 24 hrs. The cells were then harvested and resuspended in PBS. 1ml of this suspension was treated with 100 µL of DCFH-DA and kept at 37°C for 30 minutes. Fluorescence had been visualized with excitation at 480 nm and emission at 530 nm under microscope.

Assessment of mitochondrial membrane potential (MMP)

MMP was evaluated using the JC-1 fluorescent probe (Huang *et al.*, 2024). MCF-7 cells were seeded in six well plates and incubated overnight. The cells were then treated with the IC_{50} concentration of cycloastragenol and kept for 24 hours. Cells were stained with JC-1 for 30 minutes at 37°C and examined using an inverted fluorescence microscope. The green (485nm) and red (525nm) fluorescence were measured.

AO/EtBr staining

The effect of cycloastragenol on cell viability was examined using AO/EtBr dual staining (Alasmari, 2024). MCF-7 cells were added in six well plates and kept overnight. This is followed by treatment with the IC_{50} concentration of cycloastragenol and kept for 24 hours. Cells were treated with AO/EtBr staining solution in the dark for 10 minutes and observed under a fluorescence microscope and images were captured.

DAPI staining

DAPI staining was used to examine nuclear morphological changes associated with apoptosis (Moon *et al.*, 2025). MCF-7 cells were added in six well plates and kept overnight. Cells were then treated with the IC_{50} concentration of cycloastragenol and kept at further 24 hours. Cells were washed with PBS, fixed with 4% formaldehyde for 15 minutes at room temperature, washed again with PBS, stained with DAPI and examined using a fluorescence microscope.

Comet assay

The alkaline comet assay was performed to evaluate DNA damage in MCF-7 cells treated with cycloastragenol (Ping *et al.*, 2013). Cells were seeded in six well plates and incubated for 24 hours. The cells were then treated with the IC_{50} concentration of cycloastragenol for further 24 hours. Cells were then trypsinized and resuspended in ice cold PBS. A known volume of aliquot of the suspension was mixed with low melting agarose at 37°C and layered onto slides. Slides were allowed to solidify at 4°C in the dark, and then immersed in pre-chilled lysis buffer for 1 hour. DNA unwinding was performed in alkaline buffer for 30 minutes at 4°C. Electrophoresis was carried out at 35 V and 300 mA for 20 minutes. Slides were washed, fixed in 70% ethanol, air-dried, stained with SYBR Gold in TE buffer and visualized under a fluorescence microscope.

Cell cycle analysis

MCF-7 cells were treated with the IC_{50} value of cycloastragenol and kept for 24 hrs. The cells were then fixed in 75% ethanol kept overnight. Fixed cells were stained with (PI)/RNase solution. Samples were incubated at 37°C in the dark for 15 minutes and cell cycle distribution was analyzed using flow cytometry (Li *et al.*, 2025).

RESULTS

The effect of cycloastragenol and cisplatin on breast cancer cells viability is presented in Fig. 1 and 2 respectively. Cycloastragenol reduced cell viability in a dose dependent mode with an IC_{50} value of 60.60 µg/mL. However, the effect of cycloastragenol was found to be lesser than that of cisplatin (IC_{50} value). Morphological changes in untreated, cisplatin treated and cycloastragenol treated cells are shown in Fig. 3.

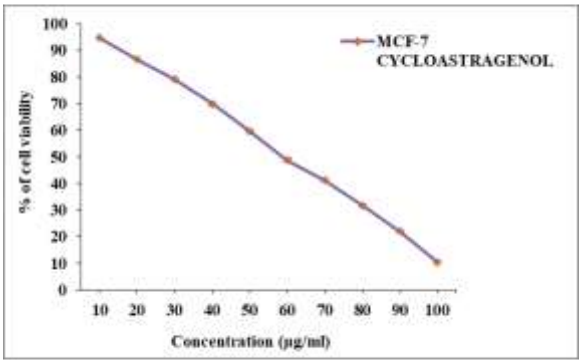

Fig. 1. Efficacy of cycloastragenol on the cell viability of MCF-7 cells

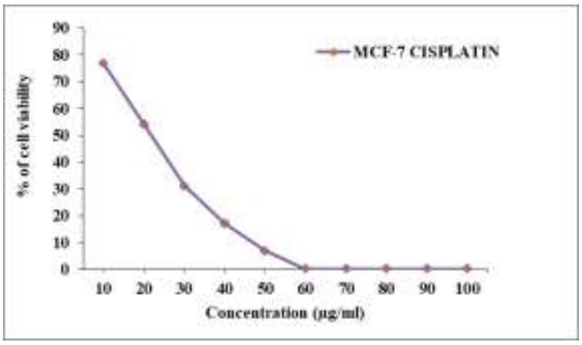

Fig. 2. Efficacy of cisplatin on the cell viability of MCF-7 cells

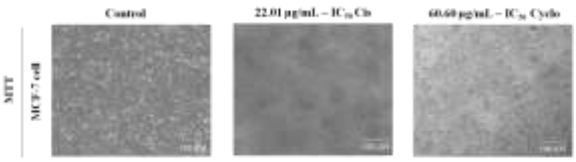

Fig. 3. Morphology of untreated MCF-7, cisplatin and cycloastragenol treated MCF-7 cells. The IC_{50} dose of cycloastragenol and standard cisplatin against MCF-7 cells was recorded at 60 µg/mL and 22 µg/mL

The effect of cycloastragenol on intracellular accumulation of ROS in breast cancer cells and corresponding fluorescence intensity are presented in Fig. 4a and 4b respectively. Minimal ROS generation was observed in the untreated cells while treatment with the IC_{50} dose of cycloastragenol markedly elevated ROS levels. These findings suggest that cycloastragenol may trigger apoptosis in MCF-7 cells through ROS mediated mechanisms.

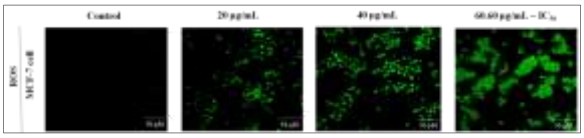

Fig. 4a. Effects of cycloastragenol on ROS generation of low, medium and IC_{50} dosage in MCF-7 cells

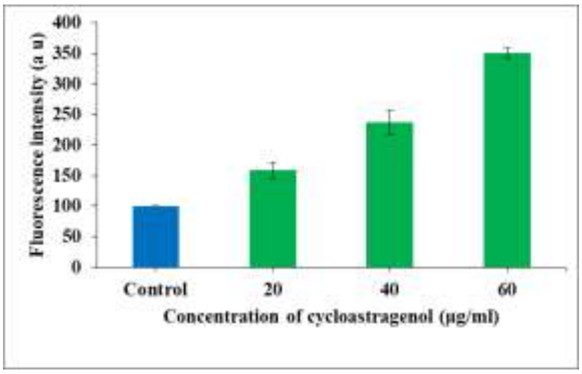

Fig. 4b. % of fluorescence intensity for ROS generation in control and cycloastragenol-treated MCF-7 cells

The effect of cycloastragenol on MMP alterations in breast cancer cells and corresponding fluorescence intensity are presented in Fig. 5a and 5b respectively. Control cells exhibited red fluorescence indicating intact mitochondrial membrane potential. However, cells treated with cycloastragenol showed a shift from red to dominant green fluorescence reflecting a significant loss of mitochondrial membrane potential.

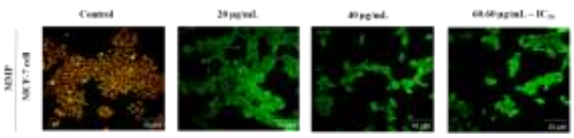

Fig. 5a. Efficacy of cycloastragenol in MCF-7 cells on mitochondrial membrane potential by JC-1 staining

The effect of cycloastragenol on apoptosis induced morphological alterations in breast cancer cells and corresponding fluorescence intensity are presented in Fig. 6a and 6b respectively. AO/EB staining revealed clear morphological evidence of apoptosis in breast cancer treated with cycloastragenol. Control cells appeared uniformly green indicating viable cells. Treated cells showed orange/red fluorescence with chromatin condensation and nuclear fragmentation, characteristic features of late apoptotic or necrotic cells. This confirms cycloastragenol mediated apoptotic cell death in breast cancer cells.

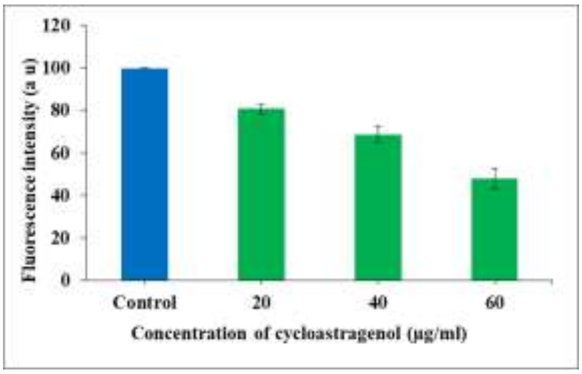

Fig. 5b. Fluorescence intensity of JC-1 staining in control and cycloastragenol-treated MCF-7 cells

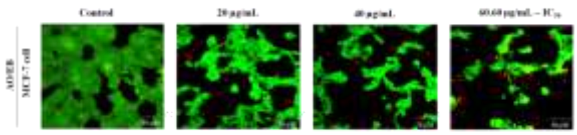

Fig. 6a. Cycloastragenol elicited apoptosis in human breast cancer cell line (MCF-7) demonstrated by staining with acridine orange/ethidium bromide (AO/EB) (Scale bar = 50 µm). The MCF-7 cells were treated with low, medium and IC_{50} dose of cycloastragenol for 24 h

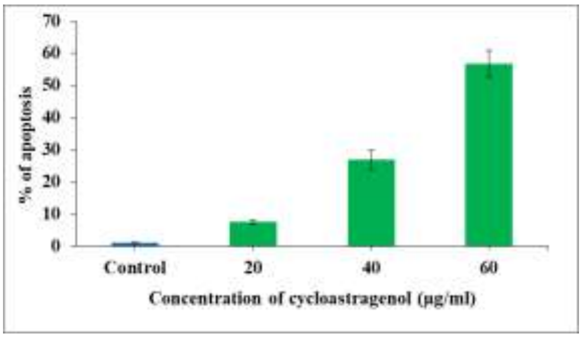

Fig. 6b. Bar diagram shows % of apoptotic cells were calculated by AO/EtBr staining in control and cycloastragenol-treated MCF-7 cells

The effect of cycloastragenol on nuclear membrane damage in breast cancer cells and corresponding fluorescence intensity are presented in Fig. 7a and 7b respectively. Control cells displayed intact, uniformly stained nuclei, whereas treatment with cycloastragenol resulted in pronounced nuclear condensation and fragmentation. These observations confirm that cycloastragenol induces apoptosis associated nuclear damage.

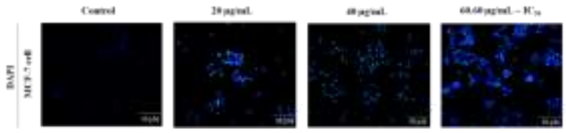

Fig. 7a. Cycloastragenol stimulates apoptosis in MCF-7 cells with low, medium and IC_{50} dose of cycloastragenol

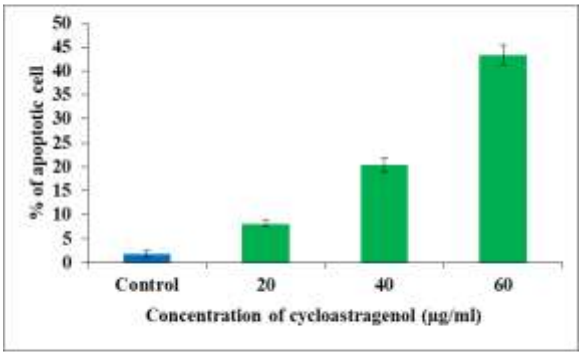

Fig. 7b. Bar diagram shows % of apoptotic cell by DAPI staining in control and cycloastragenol-treated MCF-7 cells

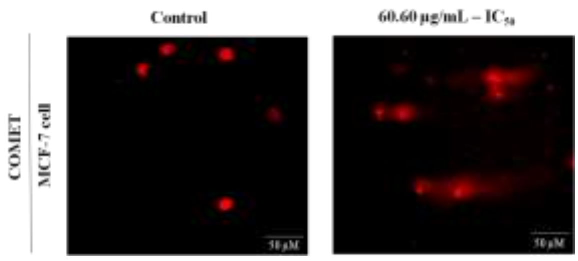

Fig. 8a. Efficacy of cycloastragenol on DNA damage in MCF-7 cells

The effect of cycloastragenol on nuclear DNA damage in breast cancer cells are presented in Fig. 8a and 8b (Graphical representation). Control cells showed intact nuclei without comet formation. Cycloastragenol treated MCF-7 cells exhibited pronounced comet tails indicating significant DNA damage. These results confirm the genotoxic potential of cycloastragenol in MCF-7 cells.

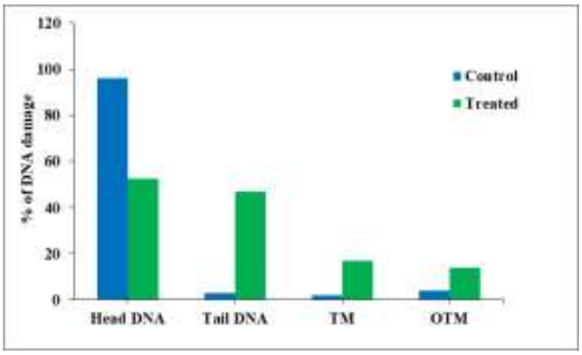

Fig. 8b. Graphical representation of DNA damage by comet assay in control and cycloastragenol-treated MCF-7 cells

Flow cytometry revealed that cycloastragenol induced cell cycle arrest predominantly at G0/G1 phase in MCF-7 cells as compared to untreated cells (Fig. 9a and 9b). This demonstrates that cycloastragenol inhibits cell proliferation by inducing G0/G1 arrest.

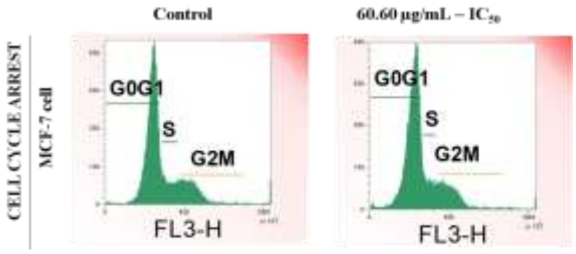

Fig. 9a. Cell cycle analysis of untreated MCF-7 cells and cells treated with IC_{50} dose of cycloastragenol

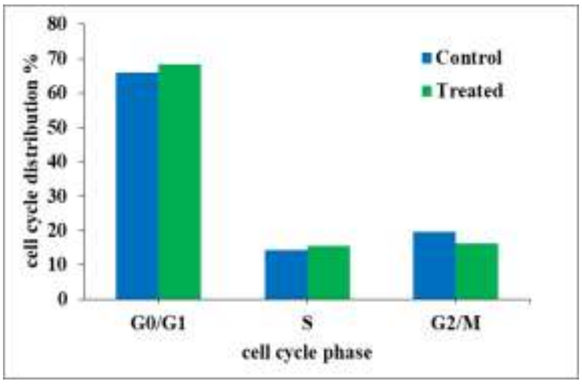

Fig. 9b. This graph shows cell cycle arrest by using flow cytometry in control and treated MCF-7 cells

DISCUSSION

Cancer is the important cause of mortality worldwide and is the most critical issue in global public health as well. While the primary treatment options for cancer are surgery, chemotherapy, immunotherapy and their combinations, chemotherapy is recognized as an effective treatment for cancers that have metastasized. However, chemotherapy often fails to eliminate all cancer cells and thus may reappear (Giaquinto *et al.*, 2022; Behranvand *et al.*, 2022). The development of safe and effective anticancer agents remains a significant challenge due to toxic side effects and drug resistance. Plant based phytochemicals have gained considerable attention for their potential to inhibit tumor progression with fewer side effects (Ali *et al.*, 2012). Cell line based research offers a valuable strategy for preliminary screening of anticancer compounds from medicinal plants and herbs (Niu and Wang, 2015).

The MTT assay is widely used to evaluate cell viability and cytotoxicity. It relies on the enzymatic reduction of tetrazolium salt to formazan crystals by metabolically active cells (Stockert *et al.*, 2018). Breast cancer cells treated with cycloastragenol showed a progressive reduction in viability in dose dependent manner.

ROS promotes cancer related signaling pathways at low to moderate concentration. However, excessive ROS can induce oxidative damage to DNA, proteins and lipids, thereby causing cell dysfunction or death (Shah and Rogoff, 2021; Yang *et al.*, 2018). Thus, measuring intracellular ROS provides important insight into the anticancer potential of new compounds. ROS generation was found to be enhanced in cycloastragenol treated MCF-7 cells. The present result thus suggests that cycloastragenol suppress the MCF-7 cell proliferation by inducing extensive oxidative DNA damage.

Mitochondria play a central role in ROS mediated apoptosis. Loss of MMP is a critical early event that facilitates mitochondrial outer membrane permeabilization and the release of cytochrome c, which activates downstream apoptotic pathways

(Wang and Youle, 2009). In the present study, cycloastragenol markedly disrupted MMP in MCF-7 cells as indicated by reduced fluorescence intensity compared to untreated cells. This suggests that cycloastragenol induces mitochondrial dysfunction as part of its proapoptotic action.

AO/EtBr dual staining is a reliable method for assessing apoptosis related morphological changes. Acridine orange stains both live and dead cells, whereas ethidium bromide penetrates only nonviable cells with damaged membranes (Elumalai *et al.*, 2012; Varadarajan *et al.*, 2023).

Cycloastragenol treated MCF-7 cells showed distinct apoptotic features, including nuclear condensation and fragmentation. The present results thus confirming the induction of cell death by cycloastragenol. Apoptotic nuclear morphology was further examined using DAPI staining that highlights chromatin condensation and the formation of apoptotic bodies (Han *et al.*, 2022). Cycloastragenol treated MCF-7 cells showed marked increase in apoptotic bodies compared with controls, supporting the cycloastragenol's apoptotic potential.

Apoptosis serves as a critical mechanism for eliminating severely damaged cells and maintaining tissue homeostasis (Diao *et al.*, 2021). This study showed cycloastragenol has the ability to induce DNA damage and impairs DNA repair mechanisms in MCF-7 cells as evidenced by increase in tail length and olive tail moment. The present results further supporting the cycloastragenol's apoptotic potential.

Cell cycle regulation is essential for maintaining controlled cell proliferation. Thus, targeting cell cycle checkpoints represents an important therapeutic approach in breast cancer treatment (Aziz *et al.*, 2019). Cycloastragenol arrested the proliferation of MCF-7 cells in the G0/G1 phase. It also reduced the proportion of cells in the S phase indicating that cycloastragenol effectively inhibits the proliferation of breast cancer cells.

CONCLUSION

This research has examined on cycloastragenol activity in cancer treatment. This study has pointed out the cytotoxic properties of cycloastragenol in MCF-7 breast cancer cells. It induced cell death by generating ROS, depolarizing mitochondrial membranes and eventually leading to apoptosis and necrosis. The IC_{50} value of cycloastragenol significantly enhanced its antitumor effects by inhibiting cell growth. Comet assay further confirm its anticancer activity as evidenced by increased DNA damage. Cycloastragenol induced G0/G1 phase arrest showing its potential to reduce tumor cell growth. These findings revealed the strong antiproliferative effect of cycloastragenol and explores as a promising candidature for breast cancer treatment. However, further *in vivo* studies are needed to further confirm its safety and therapeutic efficacy.

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