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Effect of Various Solvent Extracts of Annona Cherimola Mill Leaf Extract on Breast Cancer Cell Line MCF-7

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Abstract

Background: In the last few years, various research has focused on the potential of herbal medicines as an alternative therapy for cancer. This is due in large part to the fact that these medications have very few side effects on healthy cells. Cherimoya, or Annona cherimola mill, is a natural fruit that is edible and recognized to exhibit several biological functions due to its significant content of phytochemical components. The anti-cancer effects of A. cherimola ethanolic leaf extract on leukemia have been described in previous investigations. The purpose of this in-vitro research is to investigate the possible anti-cancer efficacy of several solvent extracts of Annona cherimola mill in breast cancer cell lines, namely MCF-7. Methods: Cell viability assays were used to assess the antiproliferative effects of different solvent extracts. The purpose of this study was to analyze the aqueous extract of A.cherimola mill for cell viability, cell cycle, apoptosis detection, intracellular reactive oxygen species measurement, and protein characterization using western blotting. Results: Cell viability was reduced by 47% treated with aqueous extract, demonstrating its selectivity for the line of breast cancer cells. MCF-7. The IC25 for the extract was 0.43 mg/ml (MCF-7). Conclusion: Our findings reveal that the aqueous extract of A. cherimola mill has anti-tumor properties by selectively inhibiting the breast cancer cells (MCF-7) and promoting their apoptosis.

1. Introduction

Researchers have paid close attention to functional foods in recent years owing to their physiological and biological activities as well as their nutritional advantages[1]. Major contributions from plant-derived compounds have been made to medicine, particularly in the identification of new drugs [2]. Over the past few decades, there has been a lot of research on medicinal plants that are beneficial in treating a variety of illnesses [3-6]. The tropical plant family Annonaceae, also known as the custard apple, is enormous and has undergone much research [7, 8]. The plant's phytochemical research revealed that it contains significant amounts of alkaloids, terpenoids, flavonoids, and acetogenins [9]. Among the most genus- or species-rich families due to its members' wide range of morphological diversity [10]. One of the 129 genera in the Annonaceae family, Annona contains 119 species [11] that differ in terms of their place of origin, climate, and geography [9]. Because of its antimalarial [4], antiparasitic [12], anti-inflammatory [13], and antiproliferative [11] characteristics, Annona species were widely utilized in traditional medicine. Flavonoids and acetogenins discovered in the leaves of A. muricata Linn, or Graviola, have been shown to decrease prostate cancer cell growth [14,15]. The oxidative stress and Bcl-2 downregulation mechanisms were responsible for the cytotoxic effects of A. squamosa seed extract on breast and leukemic cell lines in vitro [16]. Cherimoya, also known as Annona cherimola Mill, is a subtropical fruit that may be eaten and has a pleasant flavor as well as essential elements, including vitamins and minerals [17]. In contrast, A. cherimola was extensively employed for a variety of uses in skin problems and digestive disorders [18]. Cherimoya seeds contain annomolin and annocherimolin, which have been proven in studies to

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have a cytotoxic impact on human cancer cell lines [19,20]. Similar results were seen when A. cherimola's ethanolic leaf extract, which is rich in terpenes, was applied to AML cell lines [21]. The most often diagnosed cancer in women and the second-leading cause of mortality globally is breast cancer [22]. It is a diverse illness with numerous subtypes that differ in both clinical and histological characteristics. Breast cancer is classified as either noninvasive or invasive, depending on whether it starts in the ducts or lobes of the breast and doesn't spread to other healthy breast cell line[23]. Additionally, it is divided into many groups based on the expression of certain receptors, such as the human epidermal growth factor (HEGF-R2), progesterone receptor (PR), and oestrogen receptor (ER) [24]. However, these methods have significant negative side effects, necessitating the use of an alternative therapy that can effectively treat malignant cells without causing harm to healthy cells [25]. This work examines the aqueous extract of A. cherimola's potential anti-cancer effects on breast cancer cell lines in vitro.

2. Materials and Methods

GENERATION OF *ANNONA CHERIMOLA* MILL EXTRACTS

We used the Soxhlet reflux extraction technique to get the extracts. Dried plant material from the Annona cherimola Mill species was subjected to Soxhlet extraction using a variety of solvents selected for their polarity index. Water, methanol, dichloromethane, ethyl ether, toluene, diethyl ether, and toluene were among the solvents tested. Each solvent was used to extract components from fresh plants. Over many hours, 25 g of plant powder was soaked in solvent using 500ml. After the completion of the extractions, Solvents were evaporated in a rotating evaporator at temperatures no higher than 50 °C while the pressure was reduced, and the samples were then desiccated under a vacuum for a full 24 hours. The extraction powders were kept at 40 degrees Celsius. The plant materials used in the tests described in this study were extracted in water for 24 hours in the manner described above.

Breast Cancer Cell Culture

The MCF-7 cells were cultured in RPMI 1640 media supplemented with 10% foetal calf serum and 50 U/ml of penicillin and streptomycin. When the cell confluence reached 90%, trypsin-EDTA (PAA, UK) was used to pass them on. The cells were kept at 37 degrees Celsius and 5% CO_2. Specifically, 5 x 105 cells were plated out for every 75 cm^2. After they achieved 90% confluency, they were passed using trypsin-EDTA under experimental condition.

CELL VIABILITY ASSAY

Cells were seeded at a density of 2 x 105 cells per ml in 100 µl volume into 96-well plates and allowed to adhere for a whole night. A concentration of 160M hydrogen peroxide was used as a control sample, and 100 l of fresh medium was added even before cells received treatment in accordance with the appropriate chapters. After 4 hours incubation at 37°C, the plates were washed with 50 µl of MTT lysis solution to dissolve the formazan product. After allowing the formazan solubilization process to finish overnight in the culture plates, spectrophotometric absorbance measurements were taken at 570nm.

CELL CYCLE ANALYSIS

Cultured cells were seeded at a density of 2 x 105 cells per ml in 2 ml onto a six-well plate and allowed to adhere for an entire night. Then, anti-cyclin Flow cytometry A and propidium iodide were used to stain MCF-7 cells. 1 ml of fresh medium was used to collect cells, which were then counted using a hemocytometer. After centrifuging at 300 g for 5 minutes, the supernatant was discarded and 1 ml PBS was used to wash a total of 5x105 cells. Prior to analysis, 500 µl of permeabilization buffer was used to resuspend the pellet. To create histograms, we plotted log FL1 versus linear FL3. Data was represented as a percentage of cells relative to the control population, and cell cycle populations were gated based on their fluorescence patterns.

DETECTION OF APOTOSIS

Cell viability was used to figure out how many MCF-7 cells died after being exposed to a crude extracts for up to 72 hours. Cells were plated at a density of 2x105 cells per milliliter in a 6-well plate and allowed to adhere for an entire day. After staining, the cells were run through a flow cytometer, where 20,000 events were recorded to determine the relative amounts of FITC fluorescence (FL1) and PI fluorescence (FL3) in each sample. Using the fluorescence profile of

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untreated controls (Q3 > 95% cells), quadrant gating was utilized to calculate the proportion of cells undergoing apoptosis.

MEASUREMENT OF INTRACELLULAR REACTIVE OXYGEN SPECIES

Overnight, cells in a 24-well plate were cultured before being rinsed twice with sterile PBS and allowed to reacclimate for two hours. The cells were treated, and then probed with 100 µM DCFH-DA one hour before a treatment was finished. The cells were dyed and then rinsed in PBS after being incubated for 60 minutes.

PROTEIN CHARACTERISATION BY WESTERN BLOT

This experiment seeded 2x105 cells per ml of cell culture media into a 6-well plate. The cell pellet was heated to 90 °C for 10 minutes in 100 µl of cell lysis buffer after 15 minutes on ice. A bicinchoninic acid protein kit and 560nm absorbance measurement estimated the cell lysate's protein content (Sigma). Bovine serum albumin (BSA) in tris-buffered saline (TBS) containing Tween-20 (TBST) was used to block the membrane after transfer for one night at 4 degrees Celsius. After that, we washed the membrane six times for five minutes each time in TBST to get rid of the main antibody. After that, the sample was left at room temperature for 2 hours with horseradish peroxidase (HRP)-conjugated secondary antibody. In order to detect the HRP-conjugated secondary antibody, the membrane was washed six times for 5 minutes in TBST before the enhanced chemiluminescence (ECL) detection reagent was used (CalBioChem), followed by three 10-minute washes in TBST. After 60 minutes of blocking with 3% BSA in TBST, the membrane was probed with a 1/10,000 dilution of mouse anti-human-actin primary antibody. After removing the primary antibody and washing the membrane, the membrane was incubated with an HRP-conjugated secondary antibody. After this, the antibody washes were performed in TBST for 5 minutes each, and the membrane was finally ready for HRP detection using an ECL detection reagent.

Detection of the DNA damage lesion 8-oxo-guanine

Overnight, adhering culture was implanted onto such a 6-well plate at 2 x 105 cells per milliliter in 3 ml. After three hours, the medium was changed to 1 ml and the cells were treated with 0.25, 0.5, 1, or 2 mg/ml extract or vehicle control. During 60 minutes, 80 µM hydrogen peroxide caused strand breaking as a positive control.. After 10 minutes, slides were submerged in ice-cold lysis solution at 4°C in the dark. To detect 8-oxo-guanine, slides were rinsed in ice-cold FPG buffer and treated either with diluted FPG protein or FPG buffer alone. After 30 minutes, the slides were placed in a flatbed tank where they were exposed to alkaline unwinding and electrophoresis. COMETscore was used to generate images of the comets and evaluate their data. The results were shown as a proportion of total DNA found in the tail.

3. Results and Discussion

MTT assay

The MTT test was used to assess the extract's efficacy. The MTT test was used to assess cell viability after 72 hours of treatment with different extracts at dosages of up to 2 mg/ml. To test NAC's capacity for safeguarding from cells treated, we or before MCF-7 cells for 1 hour before extract treatment with 3 mM NAC.

DCF-DA assay

Compound profiles extracted from the Soxhlet extraction were scrutinized via TLC. Although not completely identical, the compound profiles of all extracts separated by TLC, except PET-Ether extraction, contained comparable bands, indicating some overlap in compound extraction. A consistent extraction was seen when comparing extractions done at various times. Uneven sample loading may account for observed concentration variations in the profiles of the individual compounds.

Quantification of Bax, p21 and p53expression in aqueous extract-treated cells

After treating MCF-7 cells with aqueous extract, we used SDS-PAGE and western blotting to analyse the levels of p53, p21, and Bax protein expression. First, membranes were probed with either a rabbit anti-human p53, mouse anti-human p21, or 1:1,000 dilution of rabbit anti-human bax.

Aqueous extract treatment indues G0/ G1 growth arrest

Flow cytometry was used to examine the cell cycle progression of MCF-7 cells after they were treated for

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24 hours with 2 mg/ml extract. After Five hours of treatment with the extract, the percentage of cells in the G0/G1 population rose from 44.6% to 62.5% (figure 1a), and it improved furthermore to 73.3percentage points after 24 hours of treatment, extract administration drastically decreased the proportion of cells in the G2 phase from 16.3% after 7 hours to 10.2% after 24 hours (figure 1b).

AQUEOUS EXTRACT TREATMENT INCREASES THE EXPRESSION OF P21 AND P53

Activation of the p21 transcriptional target p53 controls cell cycle arrest in the G0/G1 phase. p21, a CDK-inhibitor, blocks cell cycle progression by reducing CDK activity, which in turn leads to decreased pRB phosphorylation, E2F inactivation, and G1 arrest. As a result, western blotting was used to measure how much p53 and p21 were expressed as a result of being treated with an aqueous extract. Lysates were collected for protein expression analysis. When extract is applied to MCF-7 cells, the expression of p21 and p53 steadily increases over time.

After 5 hours of treatment, p53 expression increased by 48.4 percent, and after 24 hours, it increased by 129.2 percent, as measured by densitometry of protein expression (figure 1b). After 5 hours, p21 expression increased by 49.1%, and after 24 hours, it increased by 82%, mirroring the rise in p53 expression.

Aqueous extract treatment induces apoptosis

An essential therapeutic response to chemotherapy is the activation of apoptotic mechanisms. Cell death was determined by flow cytometry after being stained with annexin V binding assay to detect surface phosphatidylserine. Apoptosis persisted after 48 and 72 hours of treatment. The percentage of cells undergoing apoptosis was shown to grow from 3.9% after 24 hours of treatment to 28.5% after 72 hours, and then to 73.4% after 72 hours.

Simultaneously, the percentage of viable cells dropped from 93.2% after the first 24 hours to 60.2% after the second 24 hours, and then to 18.5% after 72 hours. A member of the Bcl-2 family, BAX promotes cell death. Simultaneously, the percentage of viable cells dropped from 93.2% after the first 24 hours to 60.2% after the second 24 hours, and then to 18.5% after 72 hours. Up-regulation of BAX, a pro-apoptotic member of the Bcl-2 family, occurs as a result of p53-mediated apoptotic signaling. To confirm that the uptick in p53 expression was due to the stimulation of apoptosis, Bax protein expression changes in response to extract administration were also investigated. After 48 hours, Bax protein levels were dramatically elevated following treatment with 2 mg/ml of extract.

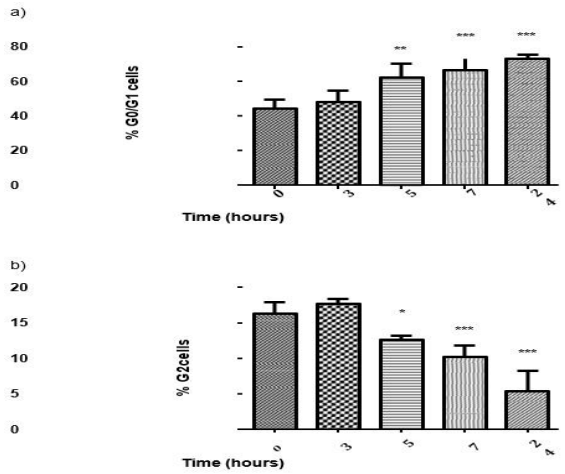

Figure 1: In MCF-7 cells, treatment with extract causes a G0/G1 cell cycle arrest. Two distinct populations of cells, (a) cell arrest were distinguished based on their fluorescence patterns. As compared to the control group (time=0 hours), the data marked with an asterisk (*; p0.05), a double asterisk (**; p<0.01), or a triple asterisk (***; p<0.001) were statistically significant. The results are from three separate trials with triplicate readings.

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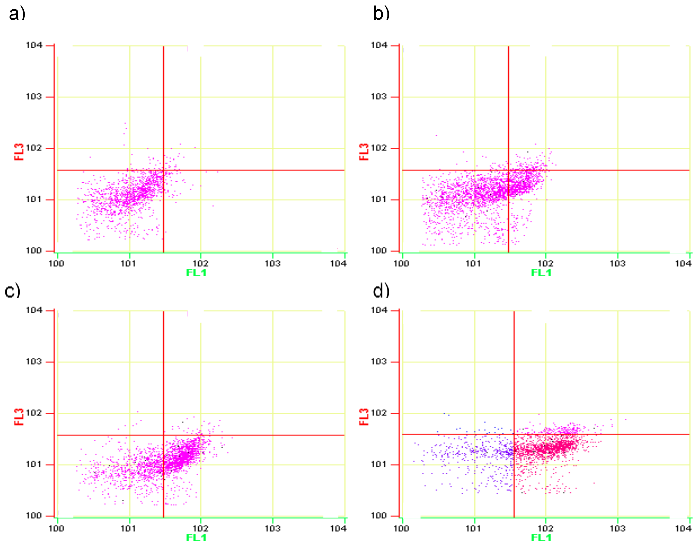


Figure 2. b) MCF-7 cells treated with extract undergo programmed cell death. (a), 24 hours (b), 48 hours (c), and 72 hours (d). The results are typical of three separate trials with identical replications.

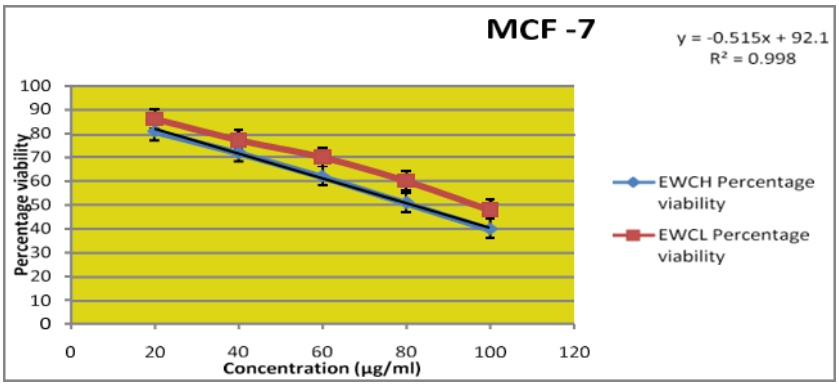
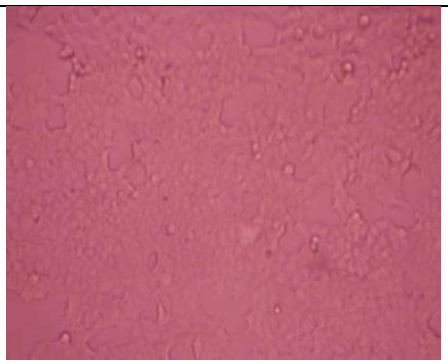
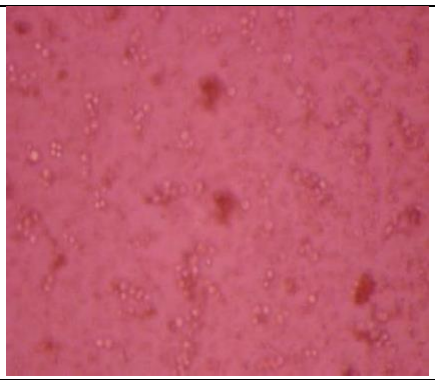


Figure 3: In vitro cytotoxicity study was evaluated for aqueous extraction of plant extract in a human breast cancer cell line.

	
MCF 7 control	MCF 7+standard

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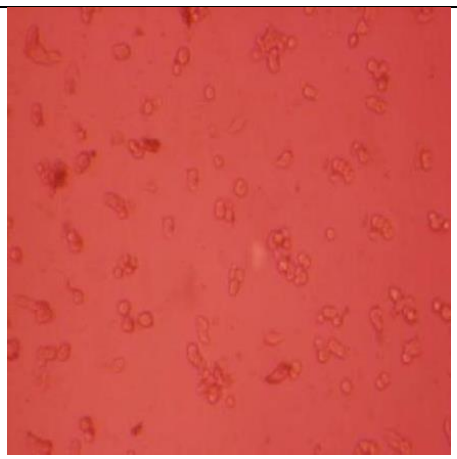
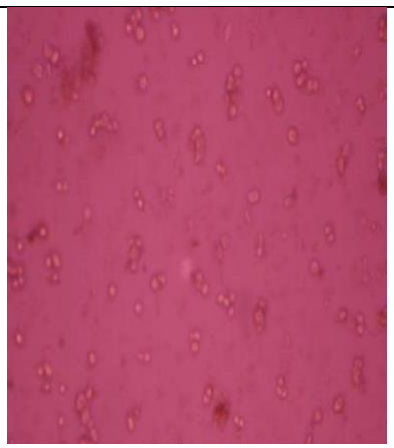


MCF 7+ 100 mcg/ml



MCF 7+ 200 mcg/ml

Figure 4: cell viability of human breast cancer cell line

4. Discussion

Annona cherimola has been shown to be effective against cancer in animal studies [26], but there is a lack of information on its usage in human medicine. This chapter's goal was to describe the work done to determine the best solvents for extracting chemicals from Annona cherimola, and the subsequent testing of those extracts, with an emphasis on the aqueous extract, for possible bioactivity against breast cancer cells.

When compared to MDA-MB-231 cells, the MCF-7 cell line has a distinct phenotype. Tumorigenic in vivo and isolated from pleural effusions of individuals with metastatic breast cancer[27]. Cell migration, invasion, and mobility are all affected by epithelial-to-mesenchymal transition markers changing [28]. Nevertheless, whereas the tumour suppressor genes p53 is present and active in MCF-7 cells, it is produced in a mutant and inactive state in MDA-MB-231 cells. The cellular response to chemotherapy is profoundly affected by p53 because it controls apoptosis and cell cycle arrest. It is not unexpected, however, that extract therapy has a variable cytotoxic capability against the various breast cancer cell lines due to these variances.

Excessive reactive oxygen species (ROS) formation or suppression of conserved and adaptive anti-oxidant mechanisms are two examples of how modulation of intrinsic redox systems may be harmful to cellular viability. Excess reactive oxygen species (ROS) damage DNA, which has been linked to the development of tumours and other diseases [29]. Mutations caused by 8-oxo-dG, an oxidative DNA damage, are common in tumour genetics and are among the most mutagenic. Byproducts of regular cellular metabolism include reactive oxygen species, which are tightly controlled by the body's own anti-oxidant defences. Nevertheless, ionising radiation, cigarette smoke, and other external sources of reactive oxygen species (ROS) may produce an oxidative stress milieu that can lead to disease. Established tumours have a greater baseline level of ROS, making them more vulnerable to the fatal damage caused by oxidative stress [30]. Plant extracts have been shown to have a high concentration of redox-modulating chemicals. Nevertheless, no improvement in cell survival was seen when extract-induced ROS generation was reduced. This data disproves the hypothesis that elevated ROS levels contribute to the cytotoxicity seen in MCF-7 cells after exposure to extracts.

Extract-induced cell cycle arrest and apoptosis

Due to their rapid division, cancer cells are especially vulnerable to environmental factors that disrupt the cell cycle, such as Genetic damage or a lack of nutrients. The faster the rate of cell division, the more often histones are lost during replication, leaving the DNA unprotected and the cell susceptible to DNA-damaging chemicals, cell cycle arrest, and finally death; all of these may be used to treat cancer. Apoptosis, the programmed cell death that occurs when cells are unable to repair DNA damage caused by ionizing radiation or chemotherapy, is a common outcome of cancer treatment.

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Due to their roles in slowing tumour growth (and disease progression) and the controlled, non-damaging elimination of mutant cell apoptosis are significant objective of cancer chemotherapy. This chapter explores the mechanisms by which extract administration decreases cell viability by examining its impact on the cell cycle and the activation of apoptosis. We also examine the impact on key proteins involved in regulating cell cycle checkpoints and apoptosis.

The cell cycle checkpoints play an important role in controlling the cell cycle and protecting the genome from harmful mutations. The cell uses these checkpoints, which are strategically placed at different phases of the cell cycle, to prevent replication unless under the most favourable circumstances. The cell cycle is stopped and DNA repair is initiated in response to a genomic attack [31] to prevent the spread of potentially cancerous lesions. Many subtypes of breast cancer cells may be induced to enter a growth arrest response by exposure to DNA-damaging chemicals [32]. Several cancers are characterised by a dysregulation of DNA repair pathways [33], making this a promising therapeutic target. Hence, substances that disrupt the cell cycle by damaging DNA may cause irreparable damage, resulting in apoptotic cell death. The findings also suggest that p53 stability and enhanced transcriptional activity leading to p21 activation are linked to cell cycle arrest and apoptosis. When p53 is separated from its negative regulator MDM2 as a result of stress, p53 may be stabilised. Apoptosis was triggered 24 hours after extract delivery, after a time-dependent G0/G1 growth halt, suggesting that a threshold is reached after 24 hours of therapy. Similar effect has been seen with various plant extracts; for instance, Hu et al. [34] demonstrated that an extract of Ganoderma lucidum causes G1 arrest in MCF-7 cells after 12 hours, followed by substantial apoptosis after 36 hours. It is possible that the extract includes compounds that cause DNA damage, which would explain why the onset of apoptosis is delayed when the extract is incubated for longer periods of time. Autophagy and apoptosis or autophagic cell death [35] may also be triggered by persistent activation of DNA repair pathways. In response to stress, p53 quickly dissociates from its negative regulator MDM2, leading to a stabilization of the p21 protein and its transcriptional regulation [36]. The findings provided in this chapter show that treatment with aqueous extract increases p21 and p53 expression over time. The expression levels of p53 were dramatically elevated, correlating with the considerable amount of G1 arrest seen at this time as well. At about 24 hours, there was an uptick in both p53 and G1 arrest, which suggests that the former may be influencing the latter. Expression of p21 also altered in response to extracting treatment, demonstrating that cell cycle arrest is caused by higher p53 levels [37]. The elevated levels of bax protein after extract treatment were also obvious. Our findings show that p53 transcriptional activation and/or protein stabilization may contribute to extract-induced G1 arrest and apoptosis in MCF-7 cells, which leads to a loss of cell viability.

5. Conclusion

The triple-negative breast cancer cell line MCF-7 is the only one for which the aqueous leaf extract of Annona cherimola has been shown to exhibit anti-proliferative and proapoptotic effects. The release of cytochrome c, upregulation of p21, and downregulation of the Bax/Bcl-2 ratio were identified as the mitochondrial route underlying this process. Further research into the extract's effectiveness in vivo will include fractionating it to isolate the bioactive components responsible for the reported effects on MCF-7.

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