Antitumor Potential Effect of Graviola Leaves Extracts on the MDA-MB-231 Cell Line

Tito N. Habib1,*, El-Sabry Abu Amra2, Ahmed R.H. Ahmed3, Gehad S. Mokhtar4

1 Genetics Lab, Zoology Department, Faculty of Science, Sohag University, Sohag 82524, Egypt.
2 Physiology Lab, Zoology Department, Faculty of Science, Sohag University, Sohag 82524, Egypt.
3 Department of Pathology, Faculty of Medicine, Sohag University, Sohag 82524, Egypt.
4 Genetics Lab, Faculty of Medicine, Sohag University, Sohag 82524, Egypt.

E-mail: tito habib99@science.sohag.edu.eg

**Abstract:** Breast cancer is the most common malignancy accounting for 38.8% of all malignancies among Egyptian women. This study aimed to investigate the *A. muricata* leaves extract and its active fraction in inhibiting cell proliferation in the human MDA-MB-231 cell line as a model of Triple Negative Breast Cancer. MDA-MB-231 cell lines were seeded and maintained in RPMI 1640 culture medium. RNAs were isolated from non-treated MDA-MB-231 and treated cell lines with *A. muricata* DMSO extract after 72 hrs incubation using TriPure isolation reagent. qRT-PCR was applied to measure the gene expression of *P53* and *Bcl2* against the β-actin gene as an internal control. Protein analysis of BRCA1, BRCA2, EGFR, p53, Bcl2, Cytochrome C, and Caspase3 gene expression was carried out by ELIZA immunoassay. The level of expression of the pro-apoptotic *P53* gene in the treated cell line with *A. muricata* DMSO extract was overexpressed, indicating its potential efficacy in directing cancer cells towards programmed death. On the other hand, the treated cell line significantly (*P = 0.05*) showed low expression of BRCA1, BRCA2, and EGFR when compared to the negative control. The present study revealed that *A. muricata* leaves extract is a promising inhibitor of cell proliferation in the MDA-MB-231 cell line (TNBC), and has efficacy against BRCA1, BRCA2, and EGFR gene expression. Since this plant is widely consumed by humans and is non-toxic, it could be developed quickly for chemoprevention and intervention in breast cancer patients.

**Keywords:** BRCA1, BRCA2, EGFR, *Annona muricata*, Breast cancer, Apoptosis.

1. Introduction

Mammary epithelial cell division that goes beyond control is a characteristic of breast cancer. Throughout life, there is an increased probability of getting this illness. The average age of diagnosed women is 62 years old, and it is frequently diagnosed in females after the age of 55 [1]. American women have a 12.3% (1 in 8) risk of being diagnosed with breast cancer [2].

The Centers for Disease Control and Prevention estimates that 40,610 per 100,000 women will die from breast cancer in the United States in 2017, representing 14% of all cancer-related deaths [1,3,4]. In addition, breast cancer is considered the most frequent cancer in women worldwide and the United States [2,5,6].

In 2017, there were 252,710 new cases of female breast cancer in the USA, which accounts for 30% of all new cancer cases [2,7]. Breast cancer represents the most common malignancy among Egyptian women, representing 38.8% of all cases in this population. In response to changes in lifestyle forced on by urbanization and economic development, breast cancer is probably increasing in South America, Africa, and Asia (i.e., obesity and physical inactivity) [8].

Although the mortality rate from breast cancer is increasing in these regions. This is primarily due to the lack of access to proper diagnostics and therapy [3-5,9]. There are several challenges to completely curing this disease, resulting in a negative impact on health worldwide. The second leading cause of cancer-related mortality in Egypt, after liver cancer, is estimated to be breast cancer, with a mortality rate of around 11%. Each type has its prognosis, therapeutic management, and breast cancer markers. Some tumors overexpress estrogen receptors (ER), progesterone receptors (PR), and human epidermal receptor 2 (HER2) [9].

Compared with positive breast cancer, triple-negative breast cancer has a less favorable prognosis [10]. The triple-negative breast cancer (TNBC) cell line MDA-MB-231 is highly aggressive, invasive, and poorly differentiated because it lacks the expression of the estrogen receptor (ER) and the progesterone receptor (PR), and the human epidermal growth factor receptor 2 (HER2) amplification [10,11]. The proteolytic degradation of the extracellular matrix makes the MDA-MB-231 cells extremely invasive compared to other invasive cancer cell lines. 15-20% of all incidences of newly diagnosed breast cancer (BC) are of this kind of tumor [12,13]. Young women, mainly those of African American or Hispanic origin, and women with a poor socioeconomic status are more likely to have invasive ductal carcinoma type and triple-negative disease [14,15].

Inhibitors of the epidermal growth factor receptor (EGFR) have been explored as a potential therapeutic target in TNBC and breast cancer [16]. EGFR overexpression in the majority
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of TNBCs [17]. Cell surface EGFR is internalized and degraded after antibody attachment [16]. Several malignancies can already be treated with these targeted therapies. There is evidence that anti-EGFR monoclonal antibodies become more sensitive to cell lines with EGFR amplification. Future research is required to identify patients with EGFR amplification or activating mutations to yield more promising outcomes and improve the attraction of EGFR inhibitors as a therapeutic approach for TNBC patients [17].

An essential step in developing multicellular systems and tissue homeostasis is apoptosis or programmed cell death. Cell shrinkage, blebbing of the plasma membrane, externalization of phosphatidylserine, and a reduction in the potential of the mitochondrial membrane is the most characterized morphological and biochemical abnormality main regulator of this process is the activation of many proteins, including caspases, a family of cysteine proteases that can cleave many cellular substrates to disassemble cell contents or of this process [20,21].

Graviola fruit flesh consists of 80% water, 1% protein, 18% carbohydrates, and a small amount of vitamins B, B2, C, potassium, and dietary fiber [22]. A limited number of published data are found in the literature for the anticarcinogenic potential of Graviola natural extracts. Recent studies have suggested that Graviola also expresses analgesic and anti-inflammatory effects and promotes apoptosis (programmed cell death) and cytotoxicity on cancer cells that may result from the presence of alkaloids, essential oils, and acetogenins [23].

The current study has revealed that caspase-3 is essential for cell death in a remarkable tissue-, cell type-, or death stimulus-specific manner. It is also essential for some of the distinctive morphological changes in cells and specific biochemical events associated with the execution and completion of apoptosis [24]. This work investigates whether the A. muricata DMSO extract, and its active fraction can inhibit TNBC cells from proliferating. The MDA-MB-231 cell line is a model of TNBC that overexpresses EGFR [25] induced cell proliferation and possesses the potential to upregulate P53 and Bax gene expression while down-regulating the expression of BRCA1 and BRCA2 [26,27].

2. Materials and method

2.1. Chemicals

Graviola leaves Extraction and Purification Graviola supplement capsules were purchased from Raintree (Carson City, NV). The capsules consisted of 100% pure Graviola leaf. The capsule contents were suspended in DMSO (50mg/ml). After incubating for 5 min, the suspension was centrifuged, and the supernatant was filtered to remove any remaining particles.

2.2. Cell lines and culture conditions:

The human breast cancer cell lines (MDA-MB-231) (Catalog No. 92020424) were purchased from the ATCC company, Egypt branch (Manassas, VA, USA) and maintained in RPMI 1640 culture medium (Gibco, UK) supplemented with 10% fetal bovine serum (Gibco, UK) and 100 U/mL of penicillin and 100 ng/mL-1 of streptomycin at 37°C in a 5% humidified CO₂ incubator (Jouan, France). Maintenance of cells was performed according to Degli et al. [28]. Maintained cells were split and dispensed in 96-well plates and tissue culture flasks according to the need.

2.3. The cell viability (MTT Assay):

Cell proliferation was conducted according to Degli et al. [28], where MDA-MB231 cancer cells were propagated in a 75 cm² (SA) cell culture (SPL, Korea). Cells were plated at a concentration of 2 X 10⁵ cells/mL in 96-well cell culture plates and incubated at 37°C for 24 h to achieve confluence. The growth medium was decanted, and a fresh medium containing 2-fold serially diluted A. muricata leaves’ extract was dispensed to a pre-cultured plate 24 hrs later; dead cells were washed out using phosphate buffer saline (PBS, pH = 7.2) (Advia-Egypt) and 50µl of MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyltetrazoliumbromide] stock solution (0.5 mg/ml) was dispensed per well. The plate was incubated for 4 h at 37 °C, the supernatant was discarded, and the formazan precipitate was solubilized using 50 µl/well of DMSO; dimethyl sulfoxide (Sigma-Aldrich, USA). Plates were incubated in the dark for 30 min at 37 °C, and absorbance was determined at 570 nm wavelength using a microplate reader (ELX800, Bio-Tek Instruments, Inc, Winooski, VT, USA). The cell viability percentage was calculated using the following formula:

\[
\text{Viability percentage (\%)} = \frac{\text{Mean OD of test dilution}}{\text{Mean OD of control wells}} \times 100
\]

The IC50 value was determined using GraphPad Prism software (v.6, GraphPad Software, La Jolla, CA, USA).

2.4. RNA isolation:

MDA-MB-231 cells were seeded in T-25 flasks in RPMI 1640 culture medium and incubated for two days under the conditions (5% CO₂, 37 °C). After 48 h, the culture medium was changed, and Graviola DMSO leaf extract was added to the corresponding flasks. The medium was changed every 48 h with the corresponding assay medium. After 72 h, the total RNA was isolated using TriPure isolation reagent (Roche, Germany) according to the method previously described by AshaRani et al. [29] and Chomczynski & Sacchi [30]. The growth medium was replaced every 42 h, and cells were passed when they reached 85–95% confluence, as observed by light microscopy.

2.4.1 RNA extraction, cDNA synthesis, and RT-PCR analysis:

RNA was prepared using TRIzol reagent and the Pure Link RNA mini kit (Life Technologies) following the kit’s instructions. RNA was reverse-transcribed, and PCR was performed qPCR as the following:

Total RNA (1 µg) was reverse transcribed using the high-capacity Complementary DNA (cDNA) reverse transcription kit (Life Technologies) according to the manufacturer’s instructions. cDNA samples were diluted at 1:5. RT-PCR reaction mixture was prepared by adding 4 µL cDNA, 10 µL Fast SYBR Green PCR Master Mix (Life Technologies), 0.6 µl of each primer of 10 µM working concentration, and 4.8 µL H₂O. Amplification conditions for P53, Bcl₂, and β-actin
amplification were: 35 cycles of 95 °C for 1 min, annealing was performed at 69 °C (P53), and 57 °C (β-actin) for 1 min, and extension at 72 °C for 1 min and 20 sec.

The PCR products were analyzed using 1.2% agarose gel electrophoresis with ethidium bromide staining and photographed by image Lab 6.0 software (BioRad), and TotalLab TL120 Quant software. In the negative control, template cDNA was replaced by DEPC water. The obtained CT values were normalized to the β-actin gene, which was used as an internal standard. For repressed genes (i.e., a ΔΔ CT value <0.0), relative fold change is depicted graphically as -2(Δx), where x is the absolute value of the ΔΔ CT value. Primers with specific species were purchased from Macrogen, Seoul, Korea. Primers sequences used in the experiment were:

- **P53 gene:**
  F 5'- CCCCTCCTGGCCCCCTGTCATTTTC -3',
  R 5'- GCAGGCCTCACAACCTCCTCAT -3'.

- **Bclgene:**
  F 5'- CCGTGTG GATGACTGAGTAC -3',
  R 5'- GAGACAGGC AGGAGAAATCA -3'.

- **BRCA1 gene:**
  F 5'- AGCAGAAGAACGTGCTCTTTACGG -3',
  R 5'- CAGTCTTCAATGTGGAGGAG -3'.

- **BRCA2 gene:**
  F 5'- CTCCCCACAAAAGGGGACAAAGC -3',
  R 5'- ACAACTCCTCACACTGCG -3'.

- **EGFR gene:**
  F 5'- AACCCTCATGCTGGAAGTAC -3',
  R 5'- TCGTGGACAGGCTTCAAGGACC -3'.

- **β-actin gene:**
  F 5'- GTGACATCCACACCAGAGG -3',
  R 5'- ACAGGATGTCAAATGCG -3'.

The PCR reactions were carried out in final volumes of 50 µL containing 1.5 mM MgCl2, 0.2 mM dNTP, 0.4 µM of each oligonucleotide primer, and Taq DNA polymerase (2.5 U). PCR conditions for P53, Bcl, and β-actin amplification were: 35 cycles of 95 °C for 1 min, 69 °C (P53), and 57 °C (β-actin) annealing for 1 min and 72 °C extensions for 1 min and 20 sec. The PCR products were analyzed using 1.2% agarose gel electrophoresis with ethidium bromide staining. In the negative control, template cDNA was replaced by DEPC water.

2.5. Protein analysis of gene expression by ELISA:

Treated MDA-MB-231 cells were obtained from an American-type culture collection. Cells were grown in RPMI-1640 containing 10% fetal bovine serum at 37 °C, stimulated with compounds to be tested for gene expression, and lysed with Cell Extraction Buffer. Total protein was extracted using RIPA Lysis Buffer (Thermo Fisher Scientific, Inc.). A bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.) was used to quantify proteins: BRCA1, BRCA2, EGFR, P53, Bcl2, Cytochrome C, and Caspase3. This lysate was diluted in Standard Diluent Buffer over the range of the assay and measured for human active P53 content.

MDA-MB-231 cells are plated at a density of 1.2–1.8 × 10^5 cells/well in a volume of 100µl complete growth medium plus 100µl of the tested compound per well in a 96-well plate for 24 hours before the enzyme assay. Averaging was based on two readings for every sample.

ELISA plate reader was used to measure color absorbance at OD 590 nm (Stat Fax 2200, Awareness Technologies, Florida, USA). The number of cells had a direct relationship with the amount of absorption. The correlation between color absorbance at 590 and cell growth was plotted with each study group.

2.6. Statistical analysis:

Data were presented as means ±SE. Significant differences between means are determined by one-way analysis of variance (ANOVA) and a Bonferroni test to compare the control and the treated groups. When P < 0.05, data were considered to be significantly different. The samples were tested in triplicates, and each assay was done in three independent experiments. The SPSS (IBM, Armonk, NY) software was used for analyzing the data and plotting the graphs.

3. Results:

3.1. MTT Assay:

Cell viability (MTT Assay) MDA-MB231 treated with A. maricata leaves’ extract shows a progressively elevated viability %, as long as the concentration of extract decreases, recording an IC50 value in the order of 6.2 µg/ml (Fig. 1). In the meantime, treated cells were enlarged, and rounded with vacuolated cytoplasm and vesicular nuclei containing prominent nuclei and detached from the surface (Fig. 2A-C).

3.2. qRT-PCR analysis:

![Fig. 1: Evaluation of MDA-MB231 cell viability using MTT stain relative to concentration.](image)

![Fig. 2A-C: Microscopic examination of MDA-MB231 treated cells using different dilutions of Graviola leaves extract (A) low concentration (B) IC50](image)
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Fig. 3 shows the mean of CT values of biological quadruplicate samples to Ps3 in MDA-MB231 negative control cell lines relative to treated MDA-MB231 with Graviola extract.

Apoptotic gene expression using qRT-PCR was monitored, revealing a significant ($P < 0.042$) up-regulation of Ps3, Cytochrome C, Caspase3, and Bax, compared with its values in the untreated MDA-MB231 cell culture. EGFR, Bcl2, BRCA1, and BRCA2 showed a significant ($P < 0.021$) down-regulation compared with untreated cell culture (Fig. 4).

![Graphical amplification](image)

**Fig. 3:** A graphical amplification was plotted by qRT-PCR to show the mean CT values of Ps3 in quadruplicate samples of control cell lines (Red) compared with Graviola extract-treated MDA-MB231 (Blue).

**Target genes**

Fig. 4: Evaluation of BRCA1, BRCA2, EGFR, Ps3, Bcl2, Cyc, Caspase 3, and Bax genes’ regulation under the effect of A. muricata DMSO extract on the MDA-MB-231 cell line.

3.3. Active protein content tested by ELISA:

The gene expression process passes through multiple molecular steps within the cell, starting with mRNA transcription followed by post-transcription modifications and functional protein synthesis. As we have seen above, the impact of AMLE on mRNA may or may not extend to the following steps and affect the protein levels. Thus, we measured the levels of functional proteins of the genes as mentioned earlier before and after AMLE treatment using the ELIZA technique.

The ELISA test shows that Ps3 concentration has a significant increase ($P = 0.03$) in the treated breast cancer cell line with **A. muricata** extract (785.9 ± 20.5) when compared to the control cell lines (44.3 ± 10.3). The same tendency was demonstrated by CYC ($P = 0.02$), Caspase 3 ($P = 0.03$), and Bax ($P = 0.05$) in the treated cell lines as 0.544 ± 0.01; 405.8 ± 6.91; 6.34 ± 0.51 compared to the control with values of 0.068 ± 0.01; 46.08 ± 94; 1.85 ± 0.03, respectively (Fig. 4). While there was a significant decrease in EGFR, Bcl2, BRCA1, and BRCA2, proteins of the MDA-MB-231 cell line after treatment with **A. muricata** DMSO extract with the values of 170.9 ± 3.18; 1.73 ± 0.09; 1.98 ± 0.03 compared to control cell lines with 449.3 ± 80.9; 5.11 ± 0.06; 2.15 ± 0.04; 1.61 ± 0.03 values, respectively. The percentage of each active protein was calculated and plotted, as shown in (Fig. 5).

![Percentage graph](image)

**Fig. 5:** The percentage of Ps3, Cyc, Caspase 3, Bax, EGFR, Bcl2, BRCA1, and BRCA2 proteins under the effect of **A. muricata** DMSO extract on the MDA-MB-231 cell lines compared with negative control.

4. Discussion:

Breast cancer is one of the most frequent types of cancer in women, with an incidence that rises dramatically with age, and the highest incidence rates being in older people aged 75 and over [30]. The MDA-MB-231 are epithelial cells isolated from the breast tissue of a 51-year-old white female adenocarcinoma patient.

The idea of testing **A. muricata** for anticancer effects is due to the presence of Annonaceous acetogenins, which are derivatives of long-chain C35-C37 fatty acids-present in various parts (fruits, leaves, stem, pulp, seeds, etc.) [31].

Several of these acetogenins have limited anti-cancer effects via inhibiting mitochondrial complex I [32]. Based upon that, it was rationalized that acetogenins could specifically inhibit the growth of cancer cells that usually have
a higher demand for ATP than normal cells [33,34].

Our historical IC₅₀ value Minimum inhibitory concentration of the extract following the reports of Razak et al. [35] and Pierre et al. [32]. They recorded that the antiproliferative effect of A. muricata crude extract (AMCE) on MCF-7, MDA-MB-231, 4T1, and A549 lung cancer cells, and cell viability was determined by comparing it to the survival of untreated negative cell control cultures, which was normalized to 100%. Graviola extract reduced cell viability and caused cell death by targeting multiple pathways that regulate metabolism, cell cycle, survival, and metastasis [30]. Also, they found that Graviola extract downregulates the expression of molecules related to hypoxia and glycolysis (HIF-1α, NF-kB, GLUT1, GLUT4, hexokinase II, and lactate dehydrogenase-A). Molecular evidence suggests that one of the critical pathways involved in the pathogenesis of TNBC is EGFR. The EGFR is commonly overexpressed or constitutively activated in many types of tumors of epithelial origin, including breast, colon, lung, skin, and ovarian cancer. These cancer cells are characterized by uncontrolled proliferation and survival. Thus, novel treatment methods targeting the EGFR and its downstream pathways, such as JAK/STAT, PI3K/AKT, and MAPKs, have been confirmed in cancer-overexpressed EGFR signaling, such as MDA-MB-231. In addition, natural compounds have been exhibited to induce cancer cell apoptosis by inhibiting the EGFR signaling pathway, including MAPK and PI3K/AKT. Dai et al. [36] reported that Graviola extract selectively inhibits the growth of human breast cancer cells in vitro and in vivo by down-regulation of EGFR expression. The apoptosis process is tightly regulated by several proteins, including the Bcl family of proteins, which contain pro-apoptotic and anti-apoptotic proteins [37]. The pro-apoptotic proteins such as Bax are involved in the cytochrome c release from mitochondria to the cytosol via dimerization and translocation to the outer mitochondrial membrane.

However, Bcl₂ is one of the anti-apoptotic proteins that suppress the translocation of Cytochrome c and Caspase3.

Our results demonstrated that the pro-apoptotic gene (PS3) expression level was overexpressed, indicating its potential efficacy in directing cancer cells toward programmed death. On the other hand, treatment of MDA-MB-231 cells with A. muricata DMSO extract significantly (P = 0.001) showed a low expression of BRCA₁, BRCA₂, Bcl₁, and EGFR when compared to the control as agreement with [38-40].

In the present study, immunological assay by ELISA indicated that A. muricata DMSO extracts significantly suppressed the activation of the EGFR signaling pathways in MDA-MB-231 cells. These results indicated that Graviola extract may reduce cell proliferation in MDA-MB-231 by inhibiting EGFR signaling pathways. The apoptosis process is tightly regulated by several proteins, including the Bcl family of proteins, which contain pro-apoptotic and anti-apoptotic proteins [30]. The pro-apoptotic proteins such as Bax are involved in the cytochrome c release from mitochondria to the cytosol via dimerization and translocation to the outer mitochondrial membrane. However, Bcl₂ is one of the anti-apoptotic proteins that suppress the translocation of cytochrome c [40].

Conclusion:

The present study revealed that A. muricata DMSO extract is a promising inhibitor of cell proliferation in the MDA-MB-231 cell line and has efficacy against BRCA₁ and BRCA₂ gene expression. Since this plant is widely consumed by humans and is non-toxic, it could be developed quickly for chemoprevention and intervention in breast cancer patients. The upregulation of P38, Bax, cytochrome c, caspase-3 genes, and their expressed proteins, and the down-regulation of BRCA₁, BRCA₂, and EGFR in MDA-MB-123 cell line treated with Graviola leaves’ extract might be responsible for apoptotic process modulation and directing tumor cell toward death.

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Author Contributions:

Tito Habib, El-Sabry Abu Amra, Ahmed Hamed Supervision, study design, data analysis, and discussion. Tito Habib, El-Sabry Abu Amra, Gehad Mokhtar, Methodology, data collection, writing -review & editing. All authors reviewed the manuscript before publishing.

Conflicts of Interest:

Conflict of interest relevant to this article was not reported.

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