Anticancer and Apoptogenic Effect of Graviola and Low-Dose Radiation in Tumor Xenograft in Mice

Ghada A. El Tawiil¹, Eman Ali Noaman¹, Mostafa A. Askar¹, Neama Mohamed El Fatih¹, and Hebatallah E. Mohamed¹

Abstract

Background: Annona muricata (graviola) has been claimed for its potential against various diseases including cancer. Objective: The present study aimed to investigate the anticancer effect of graviola extract on Ehrlich solid tumor (EST) mice along with or without a low dose of γ radiation (LDR). Methods: Mice were treated with graviola 50 mg/kg body weight orally for 30 days after EST induction and exposed to γ-ray (2 Gy/week for 3 weeks). Cell cycle, CD44, TGF-β, Bcl-2, and annexin V were determined in tumor tissue. Results: The result obtained showed a significant decrease (P < .05) of tumor size in 28 graviola-treated EST-bearing mice group (EG) or graviola-treated and irradiated EST-30–bearing mice (EGR) groups versus the EST group. The large number of cells in the sub-G0/G1 population and low cell number at S and M phases signify tumor cell apoptosis and inhibition of cell division in EG or EGR groups. Additionally, significant increases in the expression of CD44 and TGF-β were recorded in EST mice as compared with EG or EGR mice. Furthermore, EST mice exhibited a decrease in the apoptotic marker annexin v and increase in antiapoptotic Bcl-2 compared with EG and EGR mice. Conclusion: It could be suggested that graviola exerts its antitumor effect throughout the regulation of the tumor cell cycle as well as inducing apoptotic signals. The combined treatment of graviola and LDR augments their effect on tumor proliferation.

Keywords

graviola, anticancer, apoptogenic, low-dose radiation

Submitted July 10, 2019; revised December 18, 2019; accepted December 23, 2019

Introduction

Cancer is still the number one killer in the world, and most treatments for the disease depend on the use of cytotoxic substances and other drugs as chemotherapy that have a toxic effect on normal cells. Therefore, an alternative approach to cancer treatment should involve the drugs or other substances that block the growth and spread of cancer by interfering with specific molecules targeted drugs that are involved in the development of cancer growth. Furthermore, the new generation of drugs should be non-toxic to the normal cells and reasonable for the patients.¹

Actually, more than 47% of anticancer therapies on the market are natural products, their derivatives or natural substances synthetic simulate,²,³ and over 25 000 identified phytochemicals have been shown to possess effective anticancer activities.²,⁴ Graviola is a small deciduous tree of the Annonaceae family broadly distributed in tropical countries, that is well known by several names including Annona muricata, soursop, Brazilian paw, and guanabana.⁴,⁵ Annona muricata is a natural plant source of anticancer phytochemicals, with more than 212 phytochemicals identified in the various extracts of graviola, found in fruits, seeds, bark, roots, and pericarp. It is used to treat a number of illnesses and diseases like cough, skin disorders, diabetes, and cancer.⁵,⁶
The diverse constituents’ metabolites of graviola such as acetogenins are understood to play a central role in its anticancer properties on different human cell lines, as are many other constituents such as flavonoids, sterols, alkaloids, and others.5,7 Different studies in non–skin cancer cell lines show that graviola suppresses cancerous cells without harmful effects on normal cells. This therapeutic action of graviola might be due to its ability to selectively inhibit cancer.5,8

Several studies have shown that low-dose radiation (LDR) has the potential to stimulate response to anticancer drugs and reduce side effects by improving therapeutic efficiency through enhancement of different phenomena such as adaptive response and cell-cell communication.9,10 Additionally, response of the immune system11,12 and inhibition of metastasis subsequently have been observed with LDR.13 These findings suggest that LDR stimulates natural molecular barriers against oxidative damage that were observed in earlier mouse studies showing that the levels of natural barriers increased in liver cells after whole-body exposure to LDR,14 helping protect or treat sporadic cancer. This study was aimed to explore the basis of graviola’s antitumor and apoptogenic effect, as well as the role of LDR as an adjuvant in increasing the action of graviola and vice versa. Among these, we determined CD44 growth rate, mitotic index by cell cycle, apoptotic index by annexin V, and proliferation by transforming growth factor β (TGF-β) and Bcl-2 in tumor tissue.

Materials and Methods

The study was performed in the National Center for Radiation Research and Technology (NCRRT), Atomic Energy Authority, Cairo, Egypt.

Animals

Female Swiss albino mice weighing 27 to 30 g were obtained from the breeding unit at the NCRRT, Cairo, Egypt. Mice (10 animals/cage) were housed and maintained under proper environmental conditions, that is, controlled air, temperature, and relative humidity. Mice were provided with pellet diet and free access to water. Animal experiments were consistent with the ethics guidelines of the Public Health Guide for the Care and Use of Laboratory Animals (National Research Council),15 in accordance with the recommendations for the proper care and use of laboratory animals approved by the Animal Care Committee of NCRRT, Cairo, Egypt.

Chemicals

All chemicals used in the present investigation were of analytical grade and purchased from Sigma Chemical Company (St Louis, MO). Graviola supplement capsules (50 mg) were purchased from Raintree (Carson City, NV).

Graviola Preparation

Graviola, a product of Raintree, consists of capsules weighing 50 mg, consisting of 100% pure graviola leaf/stem powder without binders or fillers. The capsule contents were suspended in dimethyl sulfoxide (50 mg/mL). After incubating for 5 minutes, the suspension was centrifuged and the supernatant was filtered to remove any remaining particles. Subsequent dilutions were prepared in Dulbecco’s modification of Eagle’s medium supplemented with 10% of fetal bovine serum. Stock solutions and respective dilutions were freshly prepared prior to treatment.

Irradiation Procedures

Whole-body γ irradiation was performed with a Canadian Cs116 Gammacell 40 biological irradiator at the NCRRT, Cairo, Egypt, at a dose rate of 0.006 Gy/s. After 10 days of tumor inoculation, mice were irradiated with a fractionated dose of 2 Gy delivered per week with a total cumulative dose of 6 Gy after 3 weeks, according to the experimental design.

Solid Tumor Induction

Ehrlich ascites carcinoma (EAC) cell line was obtained from the Pharmacology and Experimental Oncology Unit of the National Cancer Institute, Cairo University, Giza, Egypt. The tumor cell line was maintained in the experimental female Swiss albino mice by weekly intraperitoneal injection of 2.5 × 106 cells/mouse. EAC cells are of mammary origin.16 The viability of the cells was 99% as judged by trypan blue exclusion assay. The xenograft model of Ehrlich solid tumor (EST) was induced in female Swiss albino mice by viable EAC cells (about 2.5 × 106/mouse) in 0.2 mL isotonic saline implanted subcutaneously (sc) into the right thigh of the hind limb of each mouse.17,18 The tumor developed in 100% of the mice with a palpable solid tumor mass (>1 cm3) was achieved within 7 days postimplantation. The day of tumor implantation was assigned as day 0. On the seventh day, animals were randomized into 4 experimental groups.

Survival Rate Analysis

The day of implantation was considered as the 0 point of the experiment. Mice were monitored for recording and analysis of the survival rate daily for 37 days by registering mortalities occurring in EST-bearing untreated and treated groups according to Abdin et al.19 After 1 week of tumor growth, oral gavage treatment of phosphate-buffered saline (PBS)–suspended graviola extract was given daily for 30 days. The doses of graviola extract at 50 mg/kg for these studies were based on previous in vivo studies, and on the recommended dose for human consumption.20-23
Experimental Design and Treatment Protocols

Sixty control mice were divided into 4 groups as follows: (1) Normal control group (C; 10 mice): animals received 2 mL/kg PBS orally at corresponding times along the experimental time course; (2) EST group (20 mice): animals were inoculated intramuscularly with 0.2 mL (containing 2.5 × 10^6 EAC cells) in the right thigh once and received 2 mL/kg PBS orally at corresponding times along the experimental time course; (3) graviola-treated EST-bearing mice group (EG; 15 mice): EST-bearing mice were gavaged with graviola at a dose of 50 mg/kg body weight/day for 30 days; (4) graviola-treated and irradiated EST-bearing mice (EGR; 15 mice): EST-bearing mice were administered graviola at a dose of 50 mg/kg body weight/day orally for 30 days and exposed to fractionated whole-body low-dose γ radiation at a dose level 2 Gy once a week for 3 weeks.

Mice were sacrificed after 37 days of tumor growth and 30 days of treatment with graviola and LDR. Changes in tumor growth were screened in each treatment group. Body weights and tumor volume of mice were measured weekly and before the treatment. Tumor weight was measured after removal at the end of the experiment. For tumor volume monitoring and tumor growth inhibition determination, the volume of solid tumor was measured by using micro calipers (Vernier, Shanghai, China) after 7, 14, 21, and 28 days from inoculation of EAC. The tumor volume was calculated after 28 days by the following equation:

\[
\text{Tumor volume (mm}^3\) = \frac{1}{2} \times (\text{length [mm]} \times \text{width [mm]})^2
\]

Tumor growth inhibition rate (%) was determined according to the following equation:

\[
\text{Tumor growth inhibition} (\%) = \left( \frac{\text{average tumor volume of treated group}}{\text{average tumor volume of the control group}} \right) \times 100
\]

Tumor Tissue Sample Collection and Preparation

After 37 days and 16 hours of fasting, animals of each group were sacrificed under gentle ether anesthesia and tumor tissues were dissected immediately and weighed, then washed with PBS for biochemical analyses.

Flow Cytometry Assays

Cell Cycle. One hundred microliters of cell suspension (1 × 106 cell/mL) was prepared by isolation of mononuclear cells from the processing of tissue with Tris-EDTA buffer. One hundred microliters of cell suspension was added to PI buffer (propidium iodide with RNase) and incubated for at least 1 hour in the dark at +4°C. After incubating, the cells were acquired by using BD Flow cytometry Accurie C6Plus version (Becton Dickinson, San Diego, CA). The DNA content of 10 000 cells was analyzed by FACS Caliber flow cytometry to calculate the percentages of cells occupying the different phases of the cell cycle by using the BD Accuri C6 Plus software files, which can import data into flow cytometry software for seamless software and FlowJo software.25 Samples were run in triplicate, and each experiment was repeated 3 times.

Cell Surface Markers. One hundred microliters of cell suspension (1 × 106 cell/mL) was prepared by isolation of mononuclear cells from the processing of tissue with Tris-EDTA buffer. The cells were washed with PBS/BSA (bovine serum albumin) with 2 mL and then centrifuged at 2000 rpm for 5 minutes. The supernatant was discarded, and the pellet resuspended in 100 µL of PBS. Seven microliters of a marker (CD44 [BD Pharmingen, Cat. No. 564392]; TGF-β [BD Pharmingen, Cat. No. 555053]; and BcL-2 [BD Pharmingen, Cat. No. 610539]) was mixed well, and the tube was incubated for 30 minutes at room temperature in the dark. Cells were washed twice with 2 mL PBS/BSA, and centrifuged at 2000 rpm for 5 minutes and the supernatant discarded. Finally, cells were resuspended in 200 µL of 4% paraformaldehyde in PBS for fixation until acquired by using BD Flow cytometry Accurie C6 Plus version (Becton Dickinson). The DNA content of 10 000 cells was analyzed by FACS Caliber flow cytometry to calculate the percentages of cell surface markers by using the Intuitive Software. BD Accuri C6 Plus software files can be exported in FCS 3.1 format for seamless data import into flow cytometry analysis programs such as FCS Express software and FlowJo software.25 Samples were run in triplicate, and each experiment was repeated 3 times.

Annexin V. One hundred microliters of cell suspension (1 × 106 cell/mL) was prepared by isolation of mononuclear cells from the processing of tissue with Tris-EDTA buffer. One microliter of cell suspension was added to a 5 mL test tube then 5 µL PI (PE label). The incubation time was for at least 15 minutes in the dark at room temperature. After the incubation time, the cells were resuspended in 200 µL 1× binding buffer. The cells were ready to acquire on flow cytometry by using BD Flow cytometry Accurie C Plus version (Becton Dickinson). The DNA content of 10 000 cells was analyzed by FACS Caliber flow cytometry to calculate the percentages of cells occupying the different phases of the cell cycle by using the Intuitive Software. BDFCS 3.1
format for seamless data be exported in programs such as FCS Express software and FlowJo software. Samples were run in triplicate, and each experiment was repeated 3 times.

**Statistical Analysis**

Statistical analysis of data was performed by 1-way analysis of variance (ANOVA) using the Graph Pad Prism software package 5.0 for Windows from Graph pad Software Inc. Results were expressed as the mean ± standard error of mean for the 10 mice in each group. Values of $P < .05$ were considered statistically significant.

**Results**

Graviola and combination of graviola with LDR improves survival rate and induces tumor volume regression in EST.

The change in body weight gain, mortalities, tumor volumes, tumor weight, and % of tumor volume inhibition of each group were recorded during the experimental period. As shown in Table 1 and illustrated by Figure 1, the results displayed final body weight gain resulting in nonsignificant changes in the groups under investigation when compared with the initial body weight in all groups, but when subtracting tumor weight from final body weight, the results recorded amelioration in the body weight in groups treated with graviola alone or accompanied with radiation exposure compared with the group bearing tumor without treatment.

Concerning animal mortalities, none were observed in the normal control group, and $\approx 20\%$ died throughout the experimental period in the EST group, and $\approx 10\%$ in the EG and EGR groups.

Table 1 and Figure 1 clarified the great variation in the tumor volumes and % tumor growth inhibition between the examined groups. At the end of 37 days of the experiment, the average tumor volume reached up to $1.32 \pm 0.9$ cm$^2$ in the EST group. Graviola treatment significantly reduced the tumor volume up to $1.32 \pm 0.9$ cm$^2$ and inhibited tumor growth by 14.4% with $P$ value $< .05$ compared with the EST group. Combination of both therapies (graviola and
LDR) significantly reduced the tumor volume to $1.02 \pm 0.08$ cm³ and inhibited tumor growth by 22.7% with $P$ value <.01 and <.05 compared with the EST and EG groups, respectively.

Treatment with graviola or as an adjuvant with LDR suppresses proliferation by inhibiting CD44, TGF-β, and Bcl-2 signaling in EST. The flow cytometry analysis was used to demonstrate the expression of CD44, TGF-β, and Bcl-2 in Table 2 and Figure 2. The results clarified that the mean values of CD44, TGF-β, and Bcl-2 of the normal control group were 5.2 ± 0.7, 5.4 ± 0.9, and 15.2 ± 1.4, respectively. Meanwhile, the mean values of CD44, TGF-β, and Bcl-2 expression were significantly increased to 75.7 ± 3.9, 66.8 ± 3.8, and 76.4 ± 4.5, respectively, in the EST group compared with the normal control group with $P$ value <.0001. Otherwise, the graviola-alone treatment significantly increased the early and late apoptotic cell population downregulates significantly in the tumor group to 8.9 ± 1.9 and 1.8 ± 0.5 with $P$ value <.0001 and <.03, respectively, and a significant increase in the necrotic cell population to 7.2 ± 1.8 with $P$ value <.02, respectively, compared with the tumor group. Otherwise, the graviola-alone treatment significantly increased the early and late apoptosis to 19.8 ± 2.6 and 12.2 ± 3.1, respectively, with $P$ value <.0001 compared with the tumor group. Also, combination therapy resulted in a significant elevation in early and late apoptosis to 21.1 ± 2.9 and 15.5 ± 3.8.

### Table 2. Effect of Graviola and LDR on Expression of CD44, TGF-β, and Bcl-2.

| Parameters | Control | EST    | EG     | EGR     |
|------------|---------|--------|--------|---------|
| CD44       | 5.2 ± 0.7 | 75.7 ± 3.9 | 28.2 ± 2.1 | 22.7 ± 2.2 |
| $P$         | —       | .0001b | .001c  | .001c   |
| TGF-β      | 5.4 ± 0.9 | 66.8 ± 3.8 | 31.6 ± 3.4 | 28.7 ± 2.5 |
| $P$         | —       | .0001b | .001c  | .001c   |
| Bcl-2       | 15.2 ± 1.4 | 76.4 ± 4.5 | 31.1 ± 4.1 | 25.2 ± 2.8 |
| $P$         | —       | .0001b | .001c  | .001c   |

Abbreviations: LDR, low-dose radiation; EST, Ehrlich solid tumor; EG, EST-bearing mice group; EGR, irradiated EST-bearing mice group.

*a*Values are expressed as the mean ± standard error of mean.

*b* $P$ is significantly different compared with normal control.

*c* $P$ is significantly different for EG and EGR groups compared with the EST group.

*d* $P$ is significantly different for EGR group compared with the EG group.
respectively, with \( P \) value < .0001 compared with the tumor group, and with \( P \) value < .01 compared with the graviola-alone therapy in late apoptosis.

**Discussion**

Murine-type tumors are common malignant tumors. The primary therapy for those tumors includes surgery, radiotherapy, chemotherapy, immunotherapy, and targeted therapy. Though these therapies have been very successful in the treatment of early carcinoma, the prognosis for advanced and recurrent diseases remains very guarded. The basic target in the use of anticancer agents is to inhibit the propagation of tumor cells or destroy them without damaging the normal cells.\(^{26,27}\) One such strategy to control cancer growth and metastasis without affecting normal cells is the use of phytochemicals along with low-dose ionizing radiation. It could be mentioned that graviola might be such a novel dietary agent that was initially identified with anticancer potential through preliminary screening of hundreds of food extracts and compounds for growth inhibition of cancer cells.\(^{2,26}\) Researchers have demonstrated that LDR-induced accelerated epigenetic changes include miRNA expression that involved stimulating DNA repair, suppressing cell lethality, intercellular induction of apoptosis, and suppressing cancer progression.\(^{28,29}\)
In this study, we found that graviola treatment only (EG group) at a dose of 50 mg/kg body weight/day for 30 days or as an adjuvant with LDR (EGR group) at a dose level 2 Gy once a week for 3 weeks decreased tumor volume and inhibited tumor growth. According to a study on breast cancer, treatment with *Annona muricata* fruit extract for 5 weeks induced tumor regression. In another study, the therapeutic effects graviola treatment in mice bearing 4T1-induced tumors were assessed, and it was found that the mean tumor volume of the group treated was smaller than the untreated group.

Our study using low-dose γ rays with graviola in animals bearing EST demonstrated a decrease in the growth rate of tumors as well as inhibition of metastasis; these findings could be attributed to the enhancement of anticancer immunity. These results are associated with expression of key cancer

### Table 3. The Percentage of Cells Appearing in Each Phase of the Cell Cycle as Analyzed by Flow Cytometry.

| Parameters       | Control       | EST           | EG            | EGR           |
|------------------|---------------|---------------|---------------|---------------|
| Sub G1%          | 6.5 ± 0.9     | 3.1 ± 0.4     | 6.1 ± 0.8     | 6.3 ± 0.7     |
| P                | —             | .0001b        | .001c         | .001c         |
| G1 %             | 76.3 ± 3.3    | 6.8 ± 0.8     | 54.3 ± 3.1    | 58.9 ± 2.4    |
| P                | —             | .0001b        | .0001c        | .0001c        |
| S%               | 12.4 ± 1.5    | 35.6 ± 2.1    | 23.1 ± 2.3    | 21.4 ± 1.4    |
| P                | —             | .0001b        | .0001c        | .0001c        |
| G2/M %           | 1.3 ± 0.3     | 52.5 ± 3.2    | 13.3 ± 1.1    | 8.6 ± 1       |
| P                | —             | .0001b        | .0001c        | .0001c        |

**Abbreviations:** EST, Ehrlich solid tumor; EG, EST-bearing mice group; EGR, irradiated EST-bearing mice group.

*Values are expressed as the mean ± SEM.*

*P is significantly different in relation to the normal control.*

*P is significantly different for EG and EGR in relation to the EST group.*

*P is significantly different for EGR group compared with the EG group.*

**Figure 3.** Flowchart showing the cell cycle analysis in experimental groups.
progression markers such as downregulation and maintenance of CD44, TGF-β, and Bcl-2.

CD44 is the most common cancer stem cell marker in various types of cancer, including breast cancer.\textsuperscript{32} CD44 plays an indispensable role in activating survival pathways that protect cancer cells from apoptosis.\textsuperscript{33} We have found that graviola treatment alone or as adjuvant with LDR inhibits tumor growth and represses CD44 expression in vivo without causing toxicity symptoms. Furthermore, combining treatment (graviola and LDR) appeared more efficient compared with treatment with graviola alone. This could be interpreted as suggesting graviola and LDR might prevent hyaluronic acid (HA) and CD44 binding, the event that constitutively activates phosphoinositide 3-kinase (PI3K)/AKT signaling. Several studies reveal that PI3K/AKT signaling progresses the cancer cascade and also enhances P-glycoprotein activity in doxorubicin-resistant human breast carcinoma cells.\textsuperscript{34} The expression of P-glycoprotein coded by the multidrug resistance (MDR) gene reduces drug uptake or causes efflux of the drug out of cancer cells and also increases HA binding with CD44.\textsuperscript{35} Furthermore, HA-CD44 binding activates protein kinase C, which in turn phosphorylates the stem cell maintenance transcription factor resulting in the upregulation of

### Table 4. The Percentage of Viable Cells, Necrotic Cells, Early Apoptosis, and Late Apoptosis Appeared as Analyzed by Flow Cytometry.\textsuperscript{a}

| Groups   | Viable Cells | Necrotic Cells | Early Apoptosis | Late Apoptosis |
|----------|--------------|----------------|-----------------|---------------|
| Control  | 97.8 ± 3.9   | 0.6 ± 0.1      | 1.1 ± 0.3       | 0.2 ± 0.07    |
| EST      | 84.6 ± 4.5   | 7.2 ± 1.8      | 8.9 ± 1.9       | 1.8 ± 0.5     |
| P        | .002\textsuperscript{b} | .0001\textsuperscript{b} | .0001\textsuperscript{b} | .03\textsuperscript{b} |
| EG       | 62.6 ± 4.4   | 5.3 ± 1.3      | 19.8 ± 2.6      | 12.2 ± 3.1    |
| P        | .0001\textsuperscript{c} | .0001\textsuperscript{c} | .0001\textsuperscript{c} | .0001\textsuperscript{c} |
| EGR      | 59.5 ± 3.9   | 3.9 ± 1.8      | 21.1 ± 2.9      | 15.5 ± 3.8    |
| P        | .0001\textsuperscript{c} | 0.02\textsuperscript{c} | .0001\textsuperscript{c} | .0001\textsuperscript{c} |

Abbreviations: EST, Ehrlich solid tumor; EG, EST-bearing mice group; EGR, irradiated EST-bearing mice group.

\textsuperscript{a}Values are expressed as the mean ± standard error of mean.

\textsuperscript{b}P is significantly different in relation to the normal control.

\textsuperscript{c}P is significantly different between EG and EGR in relation to the EST group.

\textsuperscript{d}P is the significantly different for EGR group compared with the EG group.

![Figure 4. Flowchart showing Annexin V in experimental groups.](image)
It is worth mentioning that knocking down of CD44 from colon cancer cells leads to reduced expression of anti-apoptotic molecules like Bcl-2, Bcl-XL, and increased level of apoptotic molecules like Bax and caspases-3/8/9.47 Moghadamtousi et al reported that graviola induced apoptosis through activation of caspases 3/7 and 9, upregulation of BAX, and downregulation of Bcl-2 at the mRNA and protein levels.50 Graviola leaf might induce apoptosis in MCF-7 cells by an intrinsic pathway related to cytochrome c migration to the cytosol as the result of Bax protein binding and Bcl-2 suppression. The ability to block cell cycle progression in cancer cells can effectively elevate the anticancer potential of natural products.51 In mice that received graviola alone or graviola combined with LDR, the increase of G0/G1 phase cells was accompanied by a significant decrease of S-phase and G2/M phase cells (Figure 3), which means that graviola or graviola/LDR might block the G1/S transition and induce dose-dependent G0/G1 cell cycle arrest. Significant increases of cell population at the sub G0/G1 phase could suggest the incidence of cell cycle arrest in the EG and EGR mice groups. Moghadamtousi et al stated that graviola extracts were shown to have the potential to induce G1 cell cycle arrest.52 The significant increases in the G1/G2 population in EGR mice more than EG mice could be interpreted in the view that DNA mismatch repair (MMR) proficiency appears to be associated with radiosensitivity. LDR enhances sensitivity in G2/M checkpoint and p53-caspase-3-dependent apoptosis as well as persistent RAD48 leading to cell cycle arrest and regulation.53 Evidently, graviola extracts have the capacity to regulate the cell cycle machinery, leading to cell cycle arrest and inhibition of cell proliferation.54 Also, graviola leaves could inhibit the proliferation of human breast cancer cells by arresting the cell cycle and inducing apoptosis.55 A dysfunction or inability of cells to execute to remove cancer cells was observed in multiple cancer types as defects within the intrinsic pathway in breast cancer progression.55 Flow cytometric analysis of annexin V/FITC distinguished a separate population of early apoptotic, late apoptotic/necrotic cells, and living cells as a result of the employment of the high affinity binding of annexin V to phosphatidylserine (PS),52 which is exposed at the surface of apoptotic cells and has broadly been used as a probe to measure apoptosis in vivo and in vitro.56 As depicted in Table 4 and Figure 4, the total apoptotic percentages (early apoptotic and late apoptotic cells) in EG mice and EGR mice are higher than EST mice, rather than the necrotic cells percentage in EG and EGR groups, which were lesser than in EST mice. Corsten et al reported induction of cancer cells apoptosis in a time-dependent manner, whereas the combined treatment of graviola and LDR augment apoptosis.56 In other studies, graviola extract exaggerates the inhibition of growth capabilities as well as surging apoptosis in lung A549 and breast MDAMB-468 cells.30,57
It could be suggested that graviola inhibits tumor growth via its capabilities to downregulate the proliferation signals (CD44 and TGF-β), suppress cell cycle G0/G1 phase, and arrest G2/M phase, as well as to stimulate cancer cells apoptosis through stimulation of PS expression and suppression of antia apoptotic Bcl-2. It seems that combining LDR with graviola is more efficient in controlling tumor growth compared with graviola alone. Therefore, this combination between graviola and LDR might be considered as a promising anticancer strategy and could represent a beneficial base for clinical applications. However, further studies applied to different types of tumors and cancer cells in response to the graviola/LDR treatment are strongly recommended to complete the picture and confirm the results emerging from the present study.

**Declaration of Conflicting Interests**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Funding**

The author(s) received no financial support for the research, authorship, and/or publication of this article.

**ORCID iD**

Neama Mohamed El Fatih https://orcid.org/0000-0001-6689-4607

**References**

1. Ioannis P, Anastasis S, Andrea Y. Graviola: a systematic review on its anticancer properties. *Am J Cancer Prev*. 2015;3:128-131. doi:10.12691/ajcp-3-6-5
2. Newman DJ, Cragg GM. Natural products as sources of new drugs from 1981 to 2014. *J Nat Prod*. 2016;79:629-661. doi:10.1021/acs.jnatprod.5b01055
3. Schmidt B, Ribnicky DM, Poulev A, Logendra S, Cefalu WT, Raskin I. A natural history of botanical therapeutics. *Metabolism*. 2008;57(7 suppl 1):S3-S9. doi:10.1016/j.metabol.2008.03.001
4. Moghadamtousi SM, Fadaeinasab M, Nikzad S, et al. *Annona muricata* (Annonaceae): a review of its traditional uses, isolated acetogenins and biological activities. *Int J Mol Sci*. 2015;16:15625-15658. doi:10.3390/ijms160715625
5. Gavamukulya Y, Wamunyokoli F, El-Shemy HA. *Annona muricata*: is the natural therapy to most disease conditions including cancer growing in our backyard? A systematic review of its research history and future prospects. *Asian Pac J Trop Med*. 2017;10:835-848. doi:10.1016/j.ajpm.2017.08.009
6. Coria-Tellez AV, Montalvo-Gonzalez E, Yahia EM, et al. *Annona muricata*: a comprehensive review on its traditional medicinal uses, phytochemicals, pharmacological activities, mechanisms of action and toxicity. *Arab J Chem*. 2016;11:4-83. doi:10.1016/j.arabjc.2016.01.004
7. Torres MP, Rachagani S, Purohit V, et al. Graviola: a novel promising natural-derived drug that inhibits tumorigenicity and metastasis of pancreatic cancer cells in vitro and in vivo through altering cell metabolism. *Cancer Lett*. 2012;323:29-40. doi:10.1016/j.canlet.2012.03.031
8. Prabhakaran K, Ramasamy G, Doraisamy U, Mannu J, Rajamani K, Murugesan JR. Polyketide natural products, acetogenins from graviola (*Annona muricata* L.), its biochemical, cytotoxic activity and various analyses through computational and bio-programming methods. *Curr Pharm Des*. 2016;22:5204-5210. doi:10.2174/1381612822666160531163144
9. Liu SZ, Jin SZ, Liu XD. Radiation-induced bystander effect in immune response. *Biomed Environ Sci*. 2004;17:40-46.
10. Chandna S, Dwarakanath BS, Khaitan D, Mathew TL, Jain V. Low-dose radiation hypersensitivity in human tumor cell lines: effects of cell-cell contact and nutritional deprivation. *Radiat Res*. 2002;157:516-525. doi:10.1667/0033-7587(2002)157[0516]
11. Ren H, Shen J, Tomiyama-Miyaji C, et al. Augmentation of innate immunity by low-dose irradiation. *Cell Immunol*. 2006;244:50-56. doi:10.1016/j.cellimm.2007.02.009
12. Hosoi Y, Sakamoto K. Suppressive effect of low dose total body irradiation on lung metastasis: dose dependence and effective period. *Radiother Oncol*. 1993;26:177-179. doi:10.1016/0167-8140(93)90101-d
13. Liu SZ. Cancer control related to stimulation of immunity by low-dose radiation. *Dose Response*. 2007;5:39-47. doi:10.2203/dose-response.06-108.Liu
14. Kojima S, Takai E, Tsukimoto M. ATP released from low-dose gamma ray-irradiated cells activates intracellular antioxidant systems via purine receptors. *Anticancer Drugs*. 2011;8:108-113. doi:10.3793/jaam.8.108
15. Clark JD, Gebhart GF, Gonder JC, Keeling ME, Kohn DF. Special report: the 1996 guide for the care and use of laboratory animals. *ILAR J*. 1997;38:41-48. doi:10.1093/ilar.38.1.41
16. Kandil E, Aziz NA. Synergistic efficacy of γ-radiation together with gallium trichloride and/or doxorubicin against Ehrlich carcinoma in female mice. *Tumor Biol*. 2016;37:1825-1834. doi:10.1007/s13277-015-3954-5
17. Awara WM, El-Sisi AE, El-Sayad ME, Goda AE. The potential role of cyclooxygenase-2 inhibitors in the treatment of experimentally-induced mammary tumour: does celecoxib enhance the anti-tumour activity of doxorubicin. *Pharmacol Rep*. 2004;50:487-498. doi:10.1016/j.phrs.2004.04.002
18. Osman AM, Ahmed MM, Khyyal MT, el-Merzabani MM. Hyperthermic potentiation of cisplatin cytotoxicity on solid Ehrlich carcinoma. *Tumori*. 1993;79:268-272.
19. Abdin AA, Soliman NA, Saied EM. Effect of propranolol on IL-10, visfatin, Hsp70, iNOS, TLR2 and survivin in amelioration of tumor progression and survival in solid Ehrlich carcinoma-bearing mice. *Pharmacol Rep*. 2014;66:1114-1121. doi:10.1016/j.pharep.2014.07.010
20. Adewole SO, Caxton-Martins EA. Morphological changes and hypoglycemic effects of *Annona muricata* Linn (Annonaceae) leaf aqueous extract on pancreatic b-cells of streptozotocin-treated diabetic rats. *Afr J Biomed Res*. 2006;9:173-180. doi:10.4314/ajbr.v9i3.48903
21. Adeyemi DO, Komolafe OA, Adewole SO, Obuotor EM, Abiodun AA, Adenowo TK. Histomorphological and mor-
phometric studies of the pancreatic islet cells of diabetic rats treated with extracts of *Annona muricata*. *Folia Morphol (Warsz)*. 2010;69:92-100.

22. Hansra DM, Silva O, Mehta A, Ahn E. Patient with metastatic breast cancer achieves stable disease for 5 years on graviola and xeloda after progressing on multiple lines of therapy. *Adv Breast Cancer Res*. 2014;3:84-87. doi:10.4236/abcr.2014.33012

23. de-Sousa OV, Vieira GD, de-Jesus RG, de Pinho J, Yamamoto CH, Alves MS. Antinociceptive and anti-inflammatory activities of the ethanol extract of *Annona muricata* L leaves in animal models. *Int J Mol Sci*. 2010;11:2067-2078. doi:10.3390/ijms11052067

24. Jensen MM, Jorgensen JT, Binderup T, Kjaer A. Tumor volume in subcutaneous mouse xenografts measured by microCT is more accurate and reproducible than determined by 18F-FDG-microPET or external caliper. *BMC Med Imaging*. 2008;16:16. doi:10.1186/1471-2342-8-16

25. Foo JB, Yazan LS, Tor YS, et al. Induction of cell cycle arrest and apoptosis in caspase-3-deficient MCF-7 cells by *Dillenia suffruticosa* root extract via multiple signalling pathways. *BMC Complement Altern Med*. 2014;14:197. doi:10.1186/1472-6882-14-197

26. Reddy L, Odhav B, Bhoola KD. Natural products for cancer prevention: a global perspective. *Pharmacol Ther*. 2003;99:1-13.

27. Rady I, Bloch MB, Chamcheu RCN, et al. Anticancer properties of graviola (*Annona muricata*): a comprehensive mechanistic review. *Oxid Med Cell Longev*. 2018;2018:1826170. doi:10.1155/2018/1826170

28. Pan D, Du Y, Hu B. The role of epigenetic modulation in the cellular response to ionizing radiation. *Int J Radiol*. 2015;2:7-14. doi:10.17554/j.issn.2313-3406.2015.02.13

29. Portess DI, Bauer G, Hill M, Hill MA, O’Neill P. Low-dose irradiation of nontransformed cells stimulates the selective removal of precancerous cells via intercellular induction of apoptosis. *Cancer Res*. 2007;67:1246-1253. doi:10.1158/0008-5472.CAN-06-2985

30. Dai Y, Hogan S, Schmelz EM, Ju YH, Canning C, Zhou K. Selective growth inhibition of human breast cancer cells by graviola fruit extract in vitro and in vivo involving downregulation of EGFR expression. *Nutr Cancer*. 2011;63:795-801. doi:10.1080/01635581.2011.563027

31. Najmuddin SUFS, Romli MF, Hamid M, Alitheen NB, Rahman NMNA. Anti-cancer effect of *Annona muricata* Linn leaves crude extract (AMCE) on breast cancer cell line. *BMC Complement Altern Med*. 2016;16:311. doi:10.1186/s12906-016-1290-y

32. Yan Y, Zuo X, Wei D. Concise review: emerging role of CD44 in cancer stem cells: a promising44 biomarker and therapeutic target. *Stem Cells Transl Med*. 2015;4:1033-1043. doi:10.5966/sctm.2015.0048

33. Lakshman M, Subramaniam V, Rubenthiran U, Jothy S. CD44 promotes resistance to apoptosis in human colon cancer cells. *Exp Mol Pathol*. 2004;77:18-25. doi:10.1016/j. yexmp.2004.03.002

34. Misra S, Ghatak S, Toole BP. Regulation of MDR1 expression and drug resistance by a positive feedback loop involving hyaluronan, phosphoinositide 3-kinase, and ErbB2. *J Biol Chem*. 2005;280:20310-20315. doi:10.1074/jbc.M500737200

35. Misra S, Ghatak S, Zoltan-Jones A. Regulation of multidrug resistance in cancer cells by hyaluronan. *J Biol Chem*. 2003;278:25285-25288. doi:10.1074/jbc.C300173200

36. Zöller M. CD44: can a cancer-initiating cell profit from an abundantly expressed molecule. *Nat Rev Cancer*. 2011;11:254-267. doi:10.1038/nrc3023

37. Vira D, Basak SK, Veena MS, Wang MB, Batra RK, Srivatsan ES. Cancer stem cells, microRNAs, and therapeutic strategies including natural products. *Cancer Metastasis Rev*. 2012;31:733-751. doi:10.1007/s10555-012-9382-8

38. Ouhtit A, Madani S, Gupta I, et al. TGF-b2: a novel target of CD44-promoted breast cancer invasion. *J Cancer*. 2013;4:566-572. doi:10.7150/jca.6638

39. Seoane J. The TGFbeta pathway as a therapeutic target in cancer. *Clin Transl Oncol*. 2008;10:14-19

40. Tremme J, Bauer G. Low-dose gamma irradiation enhances superoxide anion production by nonirradiated cells through TGF-β1-dependent bystander signaling. *Radiat Res*. 2013;179:422-432. doi:10.1667/RR3161.2

41. Kim S, Lee J, You D, et al. Berberine suppresses cell motility through downregulation of TGF-β1 in triple negative breast cancer cells. *Cell Physiol Biochem*. 2018;45:795-807. doi:10.1159/000487171

42. Liu R, Xiong S, Zhang L, Chu Y. Enhancement of antitumor immunity by low-dose total body irradiations associated with selectively decreasing the proportion and number of T regulatory cells. *Cell Mol Immunol*. 2010;7:157-162. doi:10.1038/cmi.2009.117

43. Woodburn JR. The epidermal growth factor receptor and its inhibition in cancer therapy. *Pharmacol Ther*. 1999;82:241-250.

44. Han B, Park D, Li R, et al. Small-molecule Bcl2 BH4 antagonist for lung cancer therapy. *Cancer Cell*. 2015;27: 852-863. doi:10.1016/j.ccell.2015.04.010

45. Fumarola C, Bonelli MA, Petronini PG, Alfieri RR. Targeting PI3K/AKT/mTOR pathway in non small cell lung cancer. *Biochem Pharmacol*. 2014;90:197-207. doi:10.1016/j.bcp.2014.05.011

46. Sola S, Morgado AL, Rodrigues CM. Death receptors and mitochondria: two prime triggers of neural apoptosis and differentiation. *Biochim Biophys Acta*. 2013;1830:2160-2166. doi:10.1016/j.bbadis.2012.09.021

47. Park YS, Huh JW, Lee JH, Kim HR. shrRNA against CD44 inhibits cell proliferation, invasion and migration, and promotes apoptosis of colon carcinoma cells. *Oncol Rep*. 2012;27:339-346. doi:10.3892/orm.2011.1322

48. Xue X, Yu JL, Sun DQ, et al. Curcumin induces apoptosis in SGC-7901 gastric adenocarcinoma cells via regulation of TGF-β-dependent bystander signaling. *Radiat Res*. 2010;1830:2160-2166. doi:10.1016/j.bbadis.2012.09.021

49. Xu X, Yu JL, Sun DQ, et al. Curcumin induces apoptosis in SGC-7901 gastric adenocarcinoma cells via regulation of mitochondrial signaling pathways. *Asian Pac J Cancer Prev*. 2014;15:3987-3992.

50. MoghadamTousi SZ, Rouhollahi E, Karimian H, et al. The chemopotentiation effect of *Annona muricata* leaves against...
Integrative Cancer Therapies

51. Mantena SK, Sharma SD, Katiyar SK. Berberine, a natural product, induces G1-phase cell cycle arrest and caspase-3-dependent apoptosis in human prostate carcinoma cells. *Mol Cancer Ther*. 2006;5:296-308. doi:10.1158/1535-7163.MCT-05-0448

52. Moghadamtousi SZ, Karimian H, Rouhollahi E, Paydar M, Fadaeinasab M, Kadir HA. *Annona muricata* leaves induce G1 cell cycle arrest and apoptosis through mitochondria-mediated pathway in human HCT-116 and HT-colon cancer cells. *J Ethnopharmacol*. 2014;156:277-289. doi:10.1016/j.jep.2014.08.011

53. Martin LM, Marples B, Coffey M, et al. DNA mismatch repair and the DNA damage response to ionizing radiation: making sense of apparently conflicting data. *Cancer Treat Rev*. 2010;36:518-527. doi:10.1016/j.ctrv.2010.03.008

54. Sun S, Liu J, Kadouh H, Sun X, Zhou K. Three new anti-proliferative annonaceous acetogenins with mono-tetrahydrofuran ring from graviola fruit (*Annona muricata*). *Bioorg Med Chem Lett.* 2014;24:2773-2776. doi:10.1016/j.bmcl.2014.03.099

55. Yajid AI, Ab-Rahman HS, Wong MP, Zain WZW. Potential benefits of *Annona muricata* in combating cancer: a review. *Malays J Med Sci*. 2018;25:5-15. doi:10.21315/mjms2018.25.1.2

56. Corsten M, Hofstra L, Narula J, Reutelingsperger CP. Counting heads in the war against cancer: defining the role of annexin A5 imaging in cancer treatment and surveillance. *Cancer Res*. 2006;66:1255-1260. doi:10.1158/0008-5472.CAN-05-3000

57. Moghadamtousi SZ, Kadir HA, Paydar M, Rouhollahi E, Karimian H. *Annona muricata* leaves induced apoptosis inA549 cells through mitochondrial-mediated pathway and involvement of NF-κB. *BMC Complement Alternat Med.* 2014;14:299. doi:10.1186/1472-6882-14-299