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ANTI-TUMOR EFFECT OF GRAVIOLA AND/OR CRANBERRY AS A NATURAL ANTI-PROLIFERATIVE AGENT ON COLORECTAL CELL LINES (CACO-2)

Iman Hesham El-Khashab* and Nadia Noble Daoud Aniss

Zoology Department, Faculty of Women for Arts, Science and Education, Ain Shams University.

***Corresponding Author: Iman Hesham El-Khashab**
Zoology Department, Faculty of Women for Arts, Science and Education, Ain Shams University.
Email ID: iman.elkhashab@women.asu.edu.eg

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ABSTRACT
The purpose of this study was to examine the anti-tumor effect of Graviola and/ or Cranberry on colorectal cancer cell lines (Caco2). Graviola and/or Cranberry were obtained and tested in vitro for their anti-proliferative activity against the colorectal cell lines (Caco2) for 24h by the MTT cell viability assay. DNA fragmentation was assessed by gel electrophoresis. Trypan blue assay was performed to determine the viability of cells. DNA content and distribution in different stages of cells and cell death were measured by flow cytometry. The result of this study highlighted the anti-tumor effect of Graviola and or Cranberry on Caco2. Moreover IC50 concentration of Graviola alone or with Cranberry were able to inhibit more than half of the percentage of colorectal cancer cell lines indicating an interesting anti-proliferating activity of Graviola alone or with Cranberry. This study suggests that Graviola alone or with Cranberry could be used as a source of natural anti-proliferative agent and thus could be useful as anti-tumor agent against colorectal cancer cells and is worth further investigation.

KEYWORDS: Colorectal cell lines- Graviola- Cranberry- anti-tumor – cell viability.

INTRODUCTION
Cancer is a very complex disease. Decades of elaborated genetic analysis have revealed that there are nearly one thousand legendary cancer-associated genes in humans (~ 250 oncogenes, ~ 700 tumor suppressors). The world health organization reported that Cancer is the second leading reason behind death globally.[1]

The word cancer derives from the father of medicine, Hippocrates, who used the Greek word Karakinos to explain tumours. Progress in understanding and treating cancer has been slow and its origin from the development of pathological anatomy, ranging from the eighteenth century.

The traditional medical literature reported that physicians recommended the use of some natural, and particularly plant products, that represent an interesting point of comparison with current knowledge.

Colorectal cancer (CRC) is recorded to be the third most typical cancer worldwide after bronchus and breast cancers with two-thirds of all colorectal cancers occurring within the most developed regions of the whole world. CRC affects men and women of all racial and ethnic groups, and is most often found in those aged 50 years or older.[2]

In Egypt, CRC represents 3 per- cent of all malignant tumors. It is the third most common tumor in males after urinary bladder and lymphohemopoietic malignancies, and in females it ranks fifth after breast, lymphohemopoietic, cervical, and urinary bladder cancers.[3] Recent interest in Egyptian CRC has been raised when personal observations and epidemiologic studies revealed a high incidence of the disease among the young Egyptian population.[4]

Natural product play a relevant role in cancer medicine nowadays with substantial numbers of antitumor agents employed in the clinic being either natural or derived from natural product from varied sources like plants, animals and microorganisms. During the last few years, natural-product-based drug discovery has increased based on new technologies, such as combinatorial synthesis and high-throughput screening, and their associated approaches.

It is well known that an increased consumption of fruits and vegetables is associated with a reduced risk of most cancers.[5] For this reason, the potential of natural products in therapies has been widely investigated.[6]

A medical plant, Graviola (*Annona muricata* L.), is a native of North America and rarely grows in India. Its fruits are mainly processed as juices, ice cream, jams and

sweets. The peels, leaves, stems, roots and fruit seeds also are well-known and wide employed in ancient drugs, containing an unlimited style of chemical compounds known for their medicative properties.[7-10] Graviola leaves are wealthy in bioactive compounds and have a high inhibitor capability. Therefore, they can be used in an array of high-value-added products, such as dietary supplements and phytotherapics. It has a large potent anticancerous agents coined as Acetogenins that play a key role towards many sorts of cancer, Acetogenins are potent inhibitors of NADH enzyme of the plasma membranes of cancer cells.

Phytochemical screening of Graviola leaves extracts revealed that they are rich in secondary metabolites such as alkaloids, saponins, terpenoids, flavonoids, coumarins and lactones, anthraquinones, tannins, cardiac glycosides, annonaceous acetogenins and phenols.[7,11] The high presence of synthetic resin compounds confers potent inhibitor capability to the leaves and their extracts (Nawwar et al., 2012). They can avert the onset and/or progress of oxidative disorders, moreover as lower the danger of diseases like cancer, arthritis and other diseases that occur throughout aging.[12,13]

The major bioactive element that are extracted from completely different components of the Graviola plant are referred to as Annonaceous acetogenins. These are derivatives of long chain (C35 or C37) fatty acids derived from the polyketide pathway[14] they are by selection ototoxic to cancer cells, as well as multidrug-resistant tumor cell lines.[15-17] Annonaceous acetogenins induce toxicity to cells by inhibiting the mitochondrial complex I, which is concerned in nucleotide synthesis.[16] As cancer cells have a better demand for nucleotide than the conventional cells, mitochondrial complicated I inhibitors have potential in cancer medical therapy.

Although a few *in vitro* reports have shown the cytotoxic characteristics of Graviola against various cancer cell lines, including colorectal cells[14], the comprehensive *in vivo* effects and mechanistic scientific studies are still lacking.

Cranberries are rich in bioactive constituents reported to influence a variety of health benefits, ranging from improved immune function and decreased infections to reduced cardiovascular disease and more recently cancer inhibition. A review of cranberry analysis targeting cancer discovered positive effects of cranberries or cranberry derived constituents against[18] completely different cancers utilizing a range of in vitro techniques, whereas in vivo studies supported the restrictive action of cranberries toward cancers of the stomach, colon, bladder, prostate, brain and lymph tumor. Mechanisms of cranberry linked cancer inhibition embrace cellular death induction via cell death, necrosis and autophagy, reduction of cellular proliferation and modification of protein and signal transduction pathways.

The cancer restrictive potential of cranberries and cranberry derived product is being elucidated supported multiple in vitro investigations and a little range in vivo studies. A significant amount of research shows cranberry derived constituents decrease cancer cell density, viability and proliferation.[19]

Immortal cell lines are often used in research in place of primary cells as they offer several advantages they are cost effective, easy to use, provide an unlimited supply of material and bypass ethical concerns associated with the use of animal and human tissue. Cell lines also provide a pure population of cells, which is valuable since it provides a consistent sample and reproducible results. Cell lines have revolutionized scientific research and are being used in vaccine production, testing drug metabolism and cytotoxicity, antibody production, study of gene function, generation of artificial tissues (e.g., artificial skin) and synthesis of biological compounds e.g., therapeutic proteins.[20-22]

Both MTT and trypan blue assays are routine and convenient methods for determination of cell viability.[23,24] The MTT assay is a colorimetric assay, which is based on the cleavage of the yellow tetrazolium salt MTT to purple formazan crystals only by viable cells. Usually, it is performed in plates and measured the absorbance using the micro-plates reader. The Trypan blue assay is dye exclusion staining assay, which is based on uptake of trypan blue dye by dead cells due to loss of their membrane integrity, so the dead cells appear darker than the viable cells. It is measured by using a hematocytometer and a microscope or cell counting instruments.

Apoptosis, or programmed death of the cell, is a lively, sequence directed sort of necrobiosis that is completely different from cell necrosis with relation to its morphology, bio-chemistry, medical and biological significance. Many varieties of mammals cells undergo apoptosis throughout typical development or in response to a different stimuli, including desoxyribonucleic acid damage, growth factor deprivation, and tumor suppressor genes.[25,26] Apoptosis may be a widely accepted, vital mechanism that contributes to cell growth reduction.

Thus, the aim of the present study was to assess the therapeutic effect of either Graviola or Cranberry alone or synergetically as medical natural plants in the treatment of colorectal cancer cells.

MATERIALS AND METHODS

- Cell lines: The human cancer cell lines: colorectal adenocarcinoma (Caco2), cell line Caco2 was obtained from the Egyptian Company for Vaccine and Serum (VACSERA, Cairo, Egypt) .The cells were cultured in RPMI 1640 (Sigma, St. Louis, MO) supplemented with 10 percent fetal bovine serum (Sigma, St. Louis, MO) and 100mg/mL streptomycin, 100 U/mL penicillin. Subsequently, the cells were distributed in 96-well plates

at 37°C and 5% CO2 in air and incubated for 24 h. The stock solution was diluted to obtain different concentrations of 10000, 5000, 2500, 1000, 625, 312.5, 100, 78.1 and 10. IC50 values were calculated and the morphological changes were examined under light microscope (Nikon, Japan).

- Graviola and Cranberry: Graviola was supplied from USA nowfoods.com, while Cranberry was purchased from future pharmaceutical Industries.

- **Cell Viability assay (MTT assay):** Cell viability percentage was determined by MTT as modified by Elsayed et al.[27] Briefly, the 96 well tissue culture plate was inoculated with 1 X 105 cells / ml (100 ul / well) and incubated at 37°C for 24 hours to develop a complete monolayer sheet. Growth medium was decanted from 96 well micro titer plates after confluent sheet of cells were formed, cell monolayer was washed twice with wash media. Then, two-fold dilutions of tested sample were made in RPMI medium with 2% serum (maintenance medium). 0.1 ml of each dilution was tested in different wells leaving 3 wells as control, receiving only maintenance medium. Plate was incubated at 37°C and examined. Subsequently, cells were checked for any physical signs of toxicity, e.g. partial or complete loss of the monolayer, rounding, shrinkage, or cell granulation. MTT solution was prepared (5mg/ml in PBS) (BIO BASIC CANADA INC) then 20ul MTT solution were added to each well and placed on a shaking table, 150rpm for 5 minutes, to thoroughly mix the MTT into the media. Incubation (37C, 5% CO2) for 1-5 hours was performed to allow the MTT to be metabolized. Formazan (MTT metabolic product) was resuspended in 200ul DMSO and then placed on a shaking table, 150rpm for 5 minutes, to thoroughly mix the formazan into the solvent. Optical density at 560nm was read and subtracted from background at 620nm.

- **Trypan Blue Staining:** Cells were incubated with trypan blue stain (Invitrogen, 15250–061). Cells that excluded the dye (viable) and cells that retained the dye (dead) were counted on haemocytometer slide under light microscope. Samples were diluted in Trypan blue dye of an acid azo exclusion medium by preparing a 1:1 dilution of the cell suspension using a 0.4% trypan blue solution.

- **Antioxidant Enzymes:** Lipid peroxidation was determined according to Rao and Sresty[28] by estimating the Malondialdehyde (MDA) content. Catalase activity (CAT) was determined according to Bergmeyer[29] and µmoL H2O2 destroyed per min was defined as one unit CAT. Glutathione Reductase (GSR) activity was measured according to Foyer and Halliwell.[30] One enzyme unit is defined as µmoL mL-1 oxidized Glutathione per min. Superoxide Dismutase (SOD) activity was assayed based on the method of Beauchamp and Fridovich[31] and the specific enzyme activity was expressed as units' mg-1 protein g FW.

- DNA fragmentation: To extract DNA, the tissues were lysed by incubation for 5min with 1mL of lysis buffer (1% N-SDS, 20mM Tris-HCl pH 8.0, 5mM EDTA), and the cell lysates were collected and transferred into 15mL corning tubes. Proteins were digested overnight by incubation with 100µg/mL proteinase K at 37◦C, then 7.5M-ammonium acetate and phenol-tris (pH8) chloroform (2:1, v/v) were added to the homogenate. After centrifugation for 5min at 6000rpm at 10 ◦C, the aqueous supernatant was transferred to Ependorf tubes and incubated for 5min at 37◦C to eliminate traces of chloroform. After centrifugation for 5min at 6000rpm at 10 ◦C, the DNA in the aqueous supernatant was precipitated at −10◦C for 4h with ethanol. The mixture was centrifuged for 45min at 6000rpm at 4 ◦C, and the supernatant removed. The pellet was rinsed with 70% ethanol, dried at room temperature for 2h, and resuspended in 200µL of TE 20–1 (20mM Tris-HCl pH 8.0, 1mM EDTA) for DNA quantification by UV spectrophotometry at 254 nm. Loading buffer was added to 10ul of DNA for each treatment, and the samples were analysed by electrophoresis on a 1% agarose gel with a TBE running buffer (44mM Tris–HCl, 44mM boric acid, 50mM EDTA, pH 8.0).[32]

- **Cell Cycle analysis:** Caco2 cells (5×105cells/well) were plated in six-well microplates. The cells were washed twice with PBS, suspended in 300µl of PBS, and finally fixed with 4 ml of ice-cold 70% ethanol. To stain them with propidium iodide (PI), cell sedimentation was performed by centrifugation, the ethanol was removed and the cells were washed once with PBS. The cell pellets were then resuspended in 1 ml of PI/Triton X-100 staining solution consisting of 0.1% Triton X-100 in PBS, 0.2 mg ml−1ribonuclease enzyme A and10 mg ml−1PI, and incubated for 30 min at room temperature. The stained cells were analyzed using a MoFlo flow cytometer (Dako Cytomation, Glostrup) to calculate the percentages of cells occupying the different phases of the cell cycle.[33] The kit used for enzyme assay was ab139418 Propidium iodide flow cytometry kit/BD and the solvent DMSO.

Statistical analysis: Statistical differences among groups were assessed by one-way ANOVA. The results are shown as mean ± S.D. Values of p less than 0.05 were considered statistically significant.

RESULTS

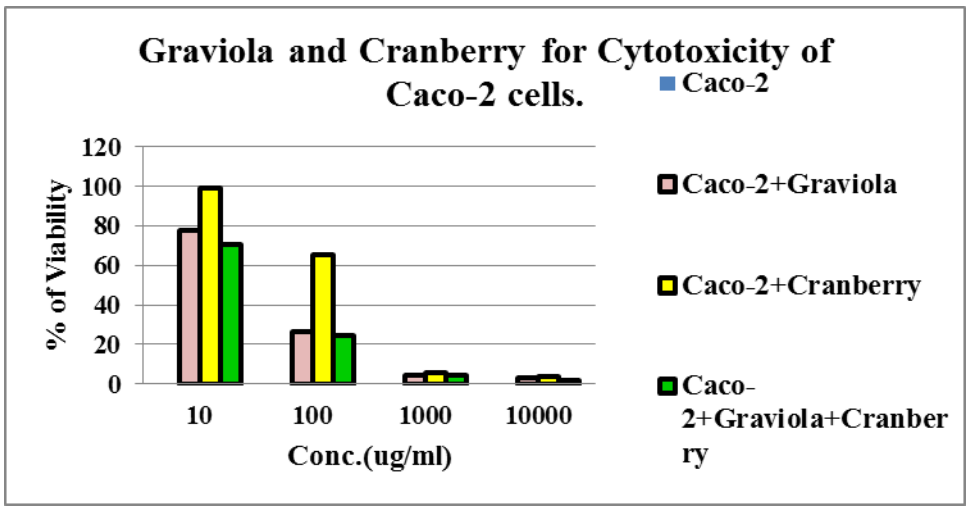
MTT Assay: Anticancer Activity against Caco-2 Colorectal Cancer Cell Line: 24 hours after treatment with Graviola and Cranberry extract, Caco-2 cells viability decreased in a concentration-dependent manner. The viability of Caco-2 cells significantly decreased with high concentrations, compared with untreated cells of the control group ($P < 0.001$). The highest inhibition of the viable cell after treatment with Graviola and Cranberry extract at 10000-µg/mL concentrations was approximately 2.13257%.

Graviola and Cranberry extract inhibited Caco-2 cell line proliferation in a time and concentration-dependent manner. The IC50 was calculated for Graviola and/or Cranberry extract, the effective concentration of Graviola and/or Cranberry extract to determine the IC50 value was obtained by regression analyses of concentration-inhibition curves. The IC50 value was 98.95 µg/mL for graviola extract, 223.87 µg/mL for Cranberry extract and 161.21 µg/mL for the mixed treatment of Graviola and Cranberry extract (Table 1)

After treatment with Graviola and /or Cranberry, the cell displayed cytotoxic activity on morphological changes. Cell line revealed the characteristic features of cell shrinkage, demonstrated the lobulated appearance of apoptotic cells and rounding and partial detachment of cells (Fig.1).

Table (1): Functional assay (MTT) for viability and IC50.

Group	Conc.(ug/ml)	Mean O.D	ST.E	Viability %	IC50
Caco-2	---	0.294	0.004619	100	ug/ml
Caco-2+ Graviola	10000	0.009	0.000577	3.061224	98.95
	5000	0.011333	0.000882	3.854875	
	2500	0.011333	0.000667	3.854875	
	1000	0.013333	0.001202	4.535147	
	625	0.018667	0.000882	6.349206	
	312.5	0.04	0.003215	13.60544	
	100	0.077667	0.007881	26.41723	
	78.1	0.148	0.003215	50.34014	
	10	0.228667	0.007311	77.77778	
Caco-2+ Cranberry	10000	0.009	0.000577	3.667642	223.87
	5000	0.011333	0.000333	3.945875	
	2500	0.015	0.001155	5.102041	
	1000	0.016	0.001528	5.442177	
	625	0.032	0.005033	10.88435	
	312.5	0.061	0.002517	20.7483	
	100	0.193	0.003512	65.64626	
	78.1	0.281333	0.003712	95.69161	
	10	0.299	0.007	99.7007	
Caco-2+ Graviola+ Cranberry	10000	0.006221	0.000333	2.13257	161.21
	5000	0.008333	0.000333	2.834467	
	2500	0.010667	0.000667	3.628118	
	1000	0.015667	0.000882	4.328798	
	625	0.033333	0.004702	11.33787	
	312.5	0.085667	0.003756	12.13832	
	100	0.152	0.003464	24.70068	
	78.1	0.229667	0.00348	48.11791	
	10	0.263667	0.006064	70.68254	



Figure(1):Cytotoxicity of Caco-2 Cells.

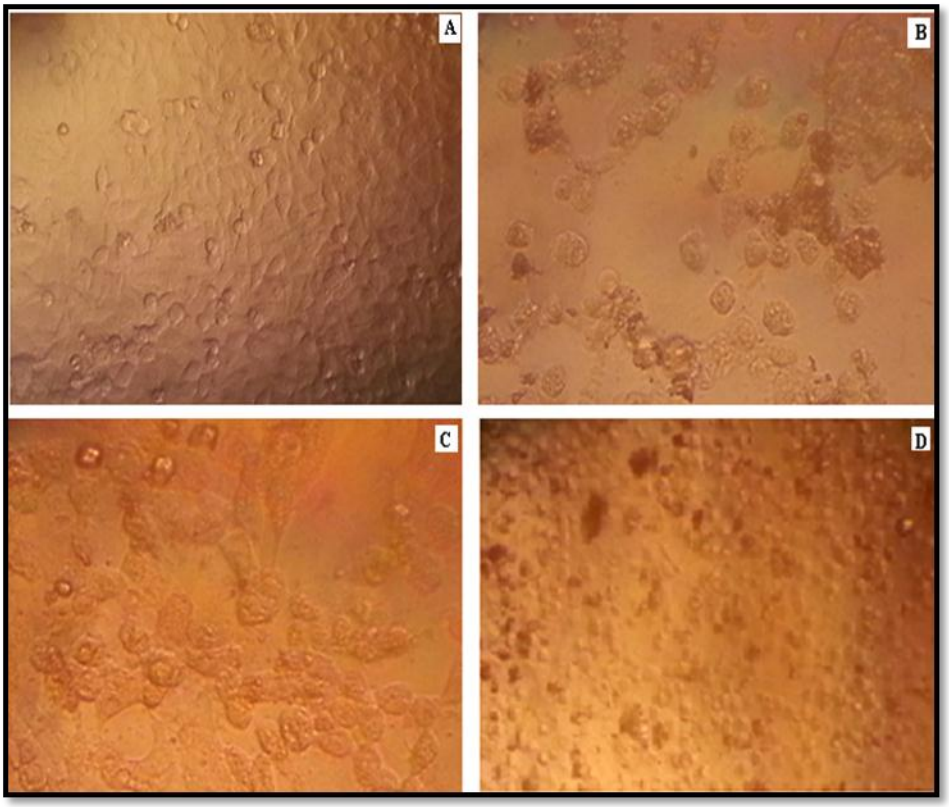


Figure (2) : Photographs of cell morphology of : A) Caco2 cells; B) Caco2 cells treated with Graviola; C) Caco2 cells treated with and D) Caco2 cells treated with both Graviola and Cranberry.

Trypan Blue: According to trypan blue stain the dark cells represent the dead trypan blue positive cells while cells with bright centers were considered live cells. In the present study presence of dead cells were more frequent after Graviola either alone or with Cranberry (Table 2 and Fig. 3).

Table 2: Trypan blue assay.

Test	Dead cells Mean	Live cells Mean	Total cell number*10^4	viability %	Necrosis %
Caco-2	1	23	48	100	2.083333
Caco2+Graviola (98.95 ug)	9	2	22	45.83333	40.90909
Caco2+Cranberry (223.87 ug)	8	4	24	50.11114	33.33333
Caco2+ Graviola+Cranberry (161.21 ug)	5	9	28	58.33333	17.85714

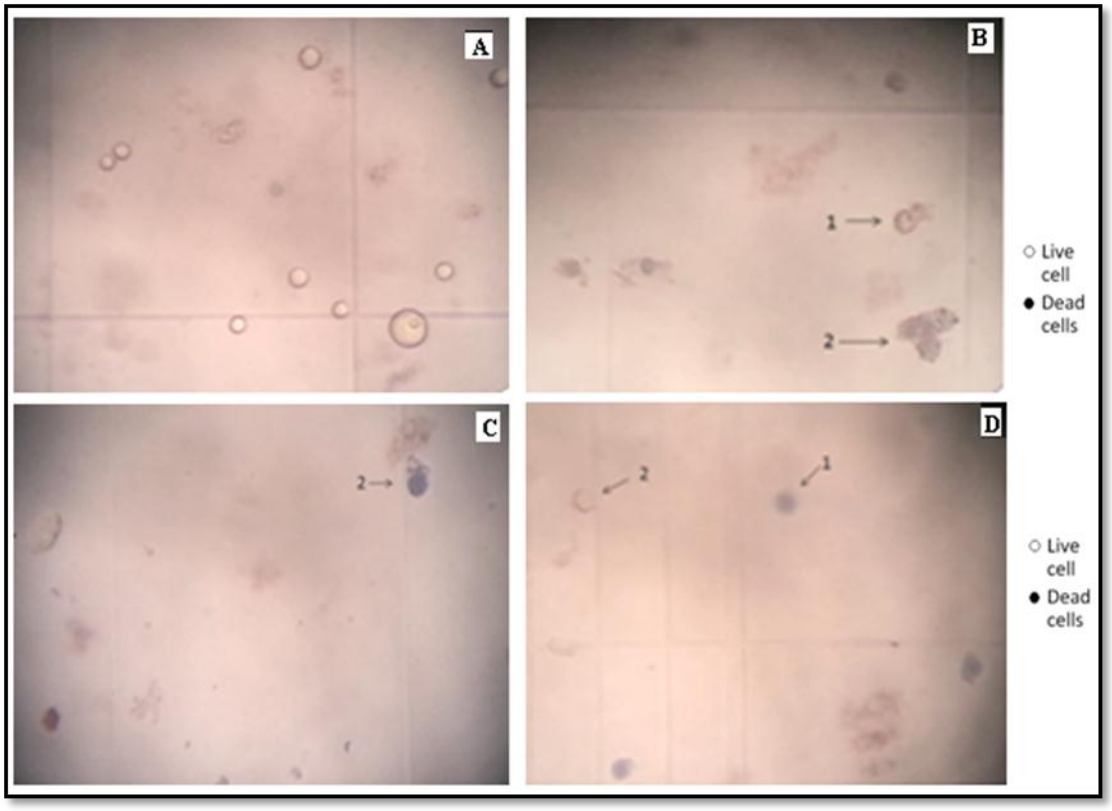


Figure (3): Micrograph representing cells stained with trypan blue showing: A) Caco2 cells; B) Caco2 cells treated with Graviola; C) Caco2 cells treated with Cranberry and D) Caco2 cells treated with both Graviola and Cranberry.

Effect of Graviola and Cranberry on MDA content, GSH content, SOD activity and Catalase in Caco2-Cells.

As shown in Table (3) and Fig. (4), Caco2 cells treated with Graviola and Cranberry showed a highly significant increase in MDA content compared with Caco2 cells group. Graviola or Cranberry was recorded versus a significant decrease in MDA level compared with Caco2 cells group. Nevertheless, group treated with Graviola showed approximately similar values to that of Cranberry levels.

A highly significant decrease in SOD, GSH and Catalase activity of Caco2 cells treated with Graviola and Cranberry was realized compared with the Caco2 cells group. Nevertheless, group treated with Graviola or Cranberry revealed a slight decrease compared with the Caco2 cells group. However, group treated with Graviola showed approximately similar values to that of Cranberry levels.

Table (3): Effect of Graviola and Cranberry on MDA content, GSH content, SOD activity and Catalase in Caco2-Cells.

Parameter Group	MDA µ mol/dL	GSH mg/dL	SOD U/m	CAT U/m
Caco-2 cells	5.08 ± 0.06	8.51±0.38	20.13±0.33	27.11±0.29
Caco_2 + Graviola	7.29±0.01 43.45%	6.40±0.41 -24.77%	15.36±0.77 -23.70%	20.73±0.24 -23.53%
Caco_2+ Cranberry	6.15±0.92 20.92%	6.70±0.50 -21.31%	15.97±0.50 -20.67%	21.05±0.51 -22.33%
Caco_2 + Graviola + Cranberry	9.26±0.24 82.06%	3.40±0.17 -60.03%	11.26±0.26 -44.04%	18.00±0.12 -33.61%

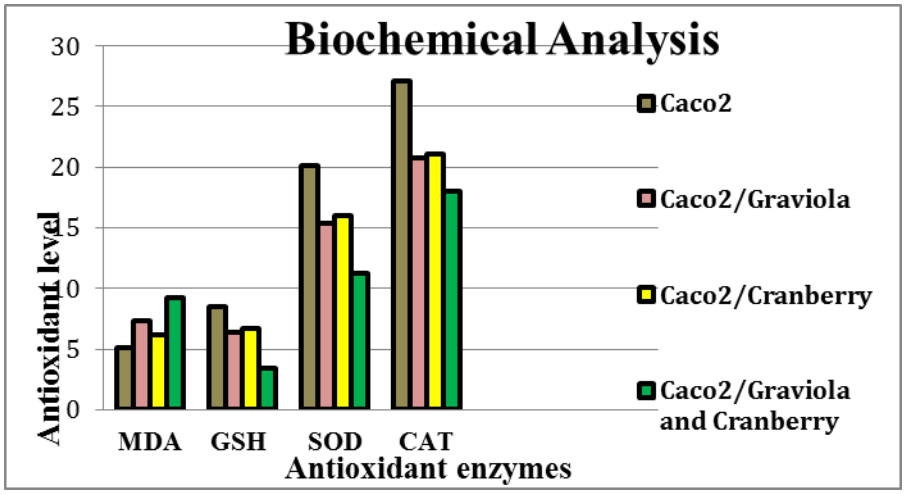


Figure (4): Effect of Graviola and Cranberry on MDA content, GSH content, SOD activity and Catalase in Caco2-Cells.

DNA fragmentation

DNA gel electrophoresis method was used to determine the possible mode of cell death caused by Graviola and /or Cranberry. Thus, cytotoxic effect of Graviola and /or Cranberry treatment allows the appearance of apoptotic DNA fragments on agarose gel, and Graviola and /or Cranberry treatment on colorectal cell lines (Caco-2) was mediated via an apoptotic mechanism (Fig. 5).

Caco-2 cells without treatment showed two bands, A (genomic DNA); B (organelles DNA). Effect of Graviola showed genomic DNA band and disappearance of the organelles DNA band while Effect of Cranberry showed genomic DNA band less than Caco-2 cells and disappearance of the organelles DNA band. Effect of mixed sample showed genomic DNA band similar to the effect of Graviola sample and the disappearance of the organelles DNA band.

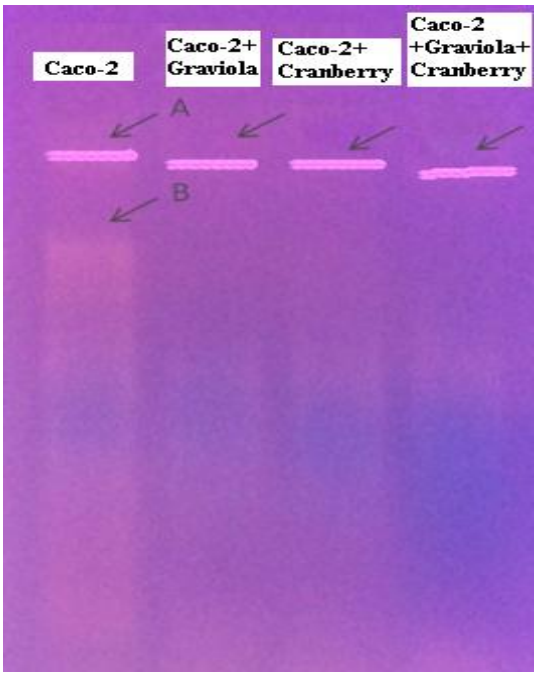


Figure (5): DNA fragmentation.

Cell cycle analysis

Flow cytometric analysis indicated that Graviola and/or Cranberry induced cell- cycle arrest of Caco-2 cells at the G2/M phase and prevented cells from going through the mitotic phase supported by an increase of sub-G1 and G2/M phase cell populations, with a parallel decrease of cells at the G0/G1 phase and S phases compared to the caco-2 cells Table (4) and Fig. (6 and 7). Values were presented as mean $\pm$ S.D. (n=3).

Graviola alone or with Cranberry induced apoptotic pattern within 24h incubation. The present study suggested that Graviola alone or with Cranberry induced accumulation of cells in G2/M phase and may lead to apoptosis in later stage of colorectal cancer cells.

Table (4): Flow cytometric analysis of Caco-2 cells after 24h of Graviola and/or Cranberry treatment showing apoptosis represented by the cells accumulated at G2/M phase. Graviola and/or Cranberry treatment induced apoptosis represented by the high cells accumulated at G2/M phase.

Parameter / Group	G0-G1%	S%	G2/M%	Sub-G1%
Caco-2 cells	60.18±0.93	28.02±0.13	11.16±0.13	10.83±0.13
Caco-2/ Graviola	52.84±0.27 -12.20%	15.14±0.55 -45.94%	20.04±0.13 79.52%	14.07±0.55 29.88%
Caco-2/ Cranberry	53.13±0.13 -11.718%	15.44±0.11 -4489%	19.65±0.10 76.05%	13.95±0.60 28.737%
Caco-2/Graviola and Cranberry	47.17±0.31 -21.626%	18.05±0.10 -35.571%	21.57±0.17 93.25%	17.91±0.03 65.282%

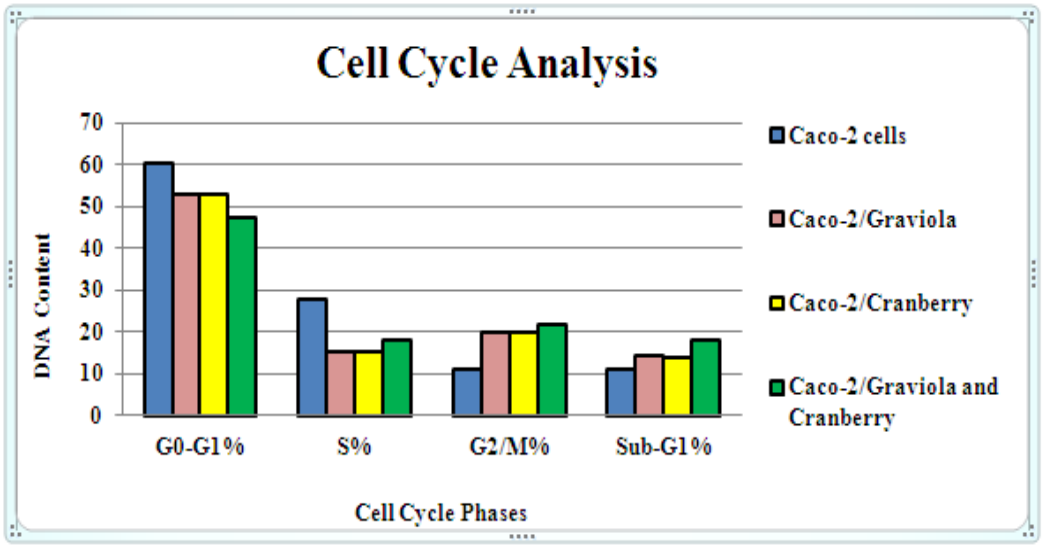


Figure (6): Cell cycle analysis.

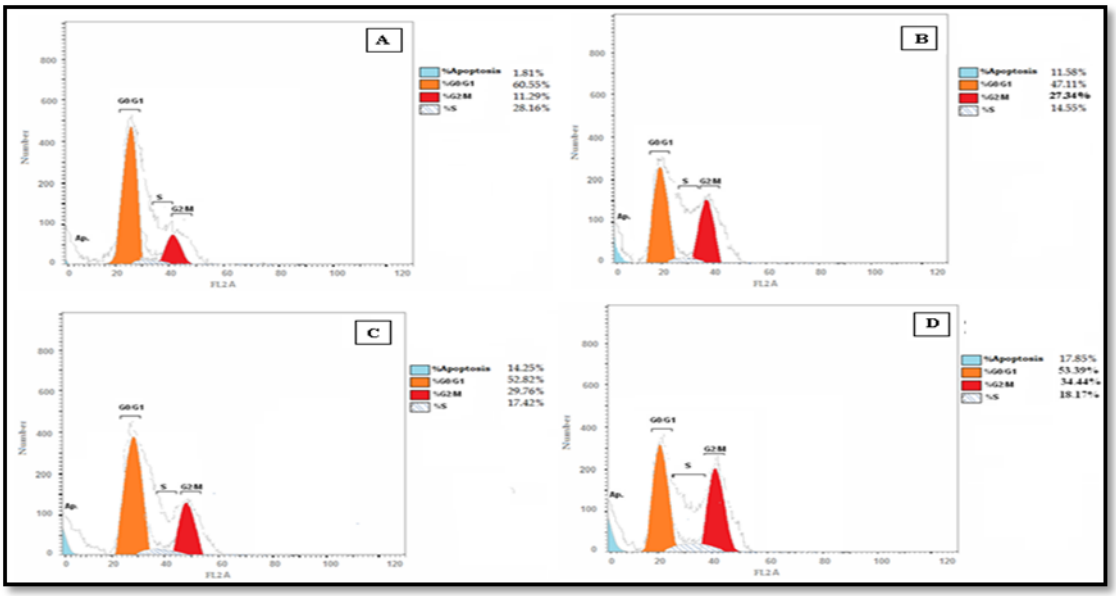


Figure (7): Flow cytometric analysis of Caco-2 cells after 24h of Graviola and/or Cranberry treatment. (B) and (C): Graviola or Cranberry induced cell death (apoptosis) represented by the cells accumulated at G2/M phase

compared with Caco-2 cells alone (A). (D): Graviola and Cranberry treatment induced cell death (apoptosis) represented by the high cells accumulated at G2/M phase compared with Caco-2 cells alone (A).

Detection of Apoptosis:

Flow cytometric analyses in table (4) and fig.(7 and 8) demonstrated high accumulation of healthy cells in Caco-2cells represented 96.31% and low accumulation in all apoptotic stages while in Graviola or Cranberry treatment showed a decrease accumulation of healthy cells represented 83.58% and 86.19% respectively and an increase accumulation in all apoptotic stages. Moreover, Graviola and Cranberry treatment showed a highly decrease accumulation of healthy cells represented 80.22% and an increase accumulation in all apoptotic stages.

Table (4): Flow Cytometric Analysis of Apoptosis.

Parameter / Group	Total Apoptosis	Early Apoptosis	Late Apoptosis	Necrosis
Caco2	10.83±0.13	3.68±0.02	3.87±0.2	1.68±0.03
Caco2/Graviola	14.07±0.55 29.815%	5.10±0.11 38.555%	5.53±0.14 42.89%	2.53±0.12 49.79
Caco2/Cranberry	13.95±0.60 28.737%	4.96±0.7 34.67%	5.15±0.12 33.15%	2.48±0.46 47.12%
Caco2/Graviola and Cranberry	17.91±0.55 65.282%	6.83±0.14 85.59%	6.99±0.17 80.62%	3.17±0.51 88.04%

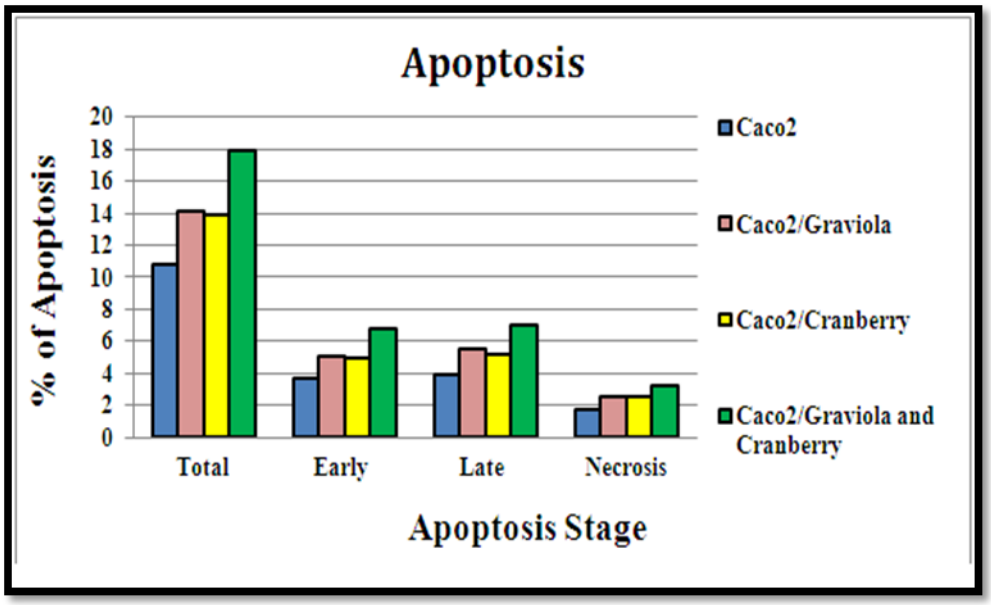


Figure (7): Flow Cytometric Analysis of Apoptosis.

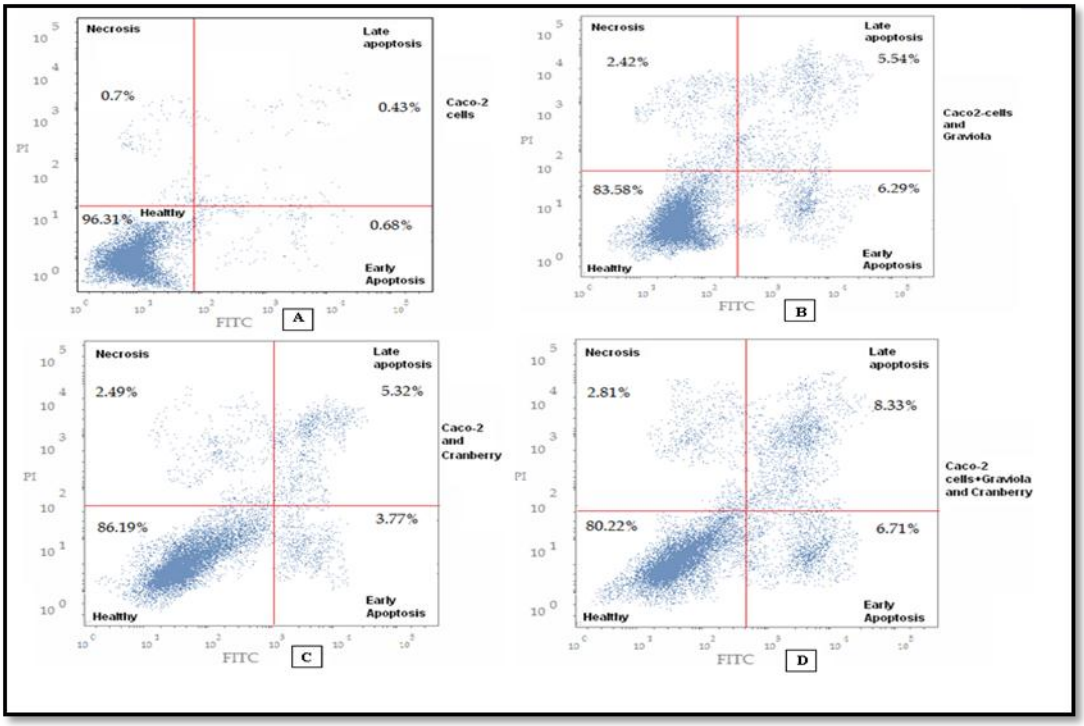


Figure (8): Flow Cytometric Analysis of Apoptosis showed (A): high accumulation of healthy cells in Caco-2cells and low accumulation in all apoptotic stages. (B) and (D): Graviola or Cranberry treatment showed a decrease accumulation of healthy cells and an increase accumulation in all apoptotic stages. (C): Graviola and Cranberry treatment showed a highly decrease accumulation of healthy cells and an increase accumulation in all apoptotic stages.

DISCUSSION

Cancer is a complicated malady that manifests itself in an exceedingly range of forms, all marked by the identical uncontrolled proliferation of cells (34). Despite continuous development of synthetic drugs, the plant kingdom still remains an attractive source of novel anti-cancer drugs. It is estimated that a high dietary intake of vegetables and fruits (> 400 g/day) could prevent at least 20% of all cancer cases.[35]

It has long been suggested that inflammation is a major driver of carcinogenesis, contributing to tumor initiation, promotion, growth, invasion and metastasis.[36] Several phytochemicals exhibit anti-inflammatory activity.

Presently, treatment with Graviola or Granberry managed to attenuate cell viability in a concentration-developed manner. Nevertheless, both herbs presented best viability decline then either alone followed by Graviola. Similar results were recorded by Mohamed et.al.[37] using Apollon and Ahmed et.al.[38] using Ashwagandha.

However, there has been little report about the evaluation of MTT and Trypan blue assay on Caco2 cell viability after Graviola or Cranberry. In this study, it was found that the MTT and Trypan blue assay gave similar results for cell viability contrary with the study done by Khalili et. al.[39] After treatment with Graviola and/or Cranberry the cell displayed cytotoxic activity on morphological changes appeared as cell shrinkage and lobulated appearance of apoptotic cells this may be due to considerable reduction in cell volume in their first stage of apoptosis. Moskwa et. al.[40]

In the current study, Caco2 cells treated with Graviola and/or cranberry represented a significant increase in MDA level and significant decrease in GSH, SOD and CAT levels compared with Caco2 cells. These results are in agreement with Martirosyan et. al[41] who demonstrated that Histone deacetylase inhibitors (HDACI) generate ROS in leukemia cells. Moreover Bhalla et. al,[42] showed that Histone deacetylase inhibitors (HDACI) down regulate the expression of several antioxidant genes including GSH and SOD coding genes.

ROS has the ability to influence the mitochondrial function, mediate the elevation of intracellular Ca2+, and lead to the activation of the caspase cascade.[43] Fuduka et. al.[44] found that the oxidative stress reduces Na channel availability, which could explain the significant increase in MDA level in Caco-2 cells treated with Graviola and /or Cranberry.

The obtained results suggest that down regulation of Graviola and/or Cranberry is a key step in reactivating the apoptotic machinery, which lead eventually to the death of malignant cells.

Moskwa et. al. [40] had declared that the easily identified morphological forms of apoptosis are when some cells were in the early stages of apoptosis with chromatin being condensed into the nuclear membrane forming semilunar structures.

In addition, the cytotoxic effect of Graviola and/or Cranberry was performed against colorectal cancer cell line (Caco2). From our result, Flow cytometric analysis indicated that Graviola and/or Cranberry induced cell-cycle arrest of Caco-2 cells at the G2/M phase and prevented cells from going through the mitotic phase. Also similar results are obtained by Berndt et al.[45] who illustrated the significant increase of cells in the G2/M phase 24 h after quercetin treatment, suggesting that in 143B cells (The human osteosarcoma cell line), the inhibition of cell proliferation is directly associated with a G2/M cell-cycle arrest.

The cell cycle machinery and components of check-point pathways have already provided a wealth of targets for novel anticancer drugs. Many of compounds under study as anti-tumor agents act at multiple steps in the cell cycle, and their effects may be cytostatic or cytotoxic, depending on the cell cycle status of the target cells. In particular combination of drugs, applied in a specific sequence, may be used to fight a tumor cell population into a state where it is most susceptible to cytotoxic effects of traditional chemotherapeutic agents.[46]

Graviola and /or Cranberry has been shown to induce apoptosis in colorectal cell lines However exact molecular mechanism of Graviola and/or Cranberry action is not yet clearly understood.

CONCLUSION: Accordingly the current study conferred that Graviola and/or Cranberry possessed a promising anticancer potential effect against colorectal cell lines. This could be related to the ability of Graviola and/ or Cranberry to exert preferential cytotoxic effect and motivate apoptosis of colorectal cancer cells with further exploration. So, more trials should be further carried to evaluate the benefit of the proposed treatment.

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REFERENCES

1. David S. Wishart Is Cancer a Genetic Disease or a Metabolic Disease? EBio Medicine, 2015; 2(6): 478-479.
2. Ahmed G. et al. Colorectal cancer in Egypt is commoner in young people: Is this cause for alarm? Alex. J of Medicine, 2014; 50(3): 197-201.
3. Mokhtar N. Cancer pathology registry 1985–1989, National Cancer Institute. Cairo: Al Asdekaa Press, 1991.
4. Soliman AS, Bondy ML and Levin B. Colorectal cancer in Egyptian patients under 40 years of age. Int J Cancer, 1997; 71: 26 –30.
5. Jansen RJ, Robinson DP, Stolzenberg-Solomon RZ, Bamlet WR, de Andrade M, Oberg AL, Hammer TJ, Rabe KG, Anderson KE, Olson JE, Sinha R, Petersen GM. Fruit and vegetable consumption is inversely associated with having pancreatic cancer. Cancer Causes Control. 2011; 22:1613–1625.
6. Stan SD, Singh SV, Brand RE. Chemoprevention strategies for pancreatic cancer. Nat Rev Gastroenterol Hepatol, 2010; 7: 347–356.
7. Gavamukulya Y, Abou-Elella F, Wamunyokoli F, and AEI-Shemy H. Phytochemical screening, anti-oxidant activity and in vitro anticancer potential of ethanolic and water leaves extracts of Annona muricata (Graviola) Asian Pac. J. Trop. Med, 2014; 7(1): S355-S363.
8. Machado ART, Lage GA, Medeiros FS, Filho JDS and Pimenta LPS. Total α,β-unsaturated-γ-lactone acetogenins in Annona muricata by proton NMR spectroscopy. Appl. Mag. Res, 2015; 46: 153-160.
9. Matos FJA and Lorenzi H. Plantas Medicinais no Brasil - Nativas e Exóticas - 2a Edição – Nova Instituto Plantarum de Estudos da Flora, Odessa, SP, 2008.
10. Gajalakshmi S, Vijayalakshmi S. and Devi RV. Phytochemical and pharmacological properties of Annona muricata: a review. Int. J. Pharm. Pharm. Sci, 2012; 4: 3-6.
11. Moraes, IVM, Ribeiro PRV, Schmidt FL , Canuto KM, Zocolo GJ, Brito ES, Luo R, Richards KM , Tran K and Smith RE UPLC–QTOF–MS and NMR analyses of graviola (Annona muricata) leaves Rev. Bras. Farmacogn, 2016; 26: 174-179.
12. Almela L, Sánchez-Muñoz B, Fernández-Lópe JA, Roca MJ and Rabe V. Liquid chromatographic–mass spectrometric analysis of phenolics and free radical scavenging activity of rosemary extract from different raw material. J. Chromatogr. A, 2006; 1120: 221-229.
13. Tabart, J.; Kevers, C.; Sipel, A.; Pincemail, J.; Defraigne, J.-O. and Dommes J.-O. (2007): Optimisation of extraction of phenolics and antioxidants from black currant leaves and buds and of stability during storage.
14. Food Chem, 2007; 105(3): 1268-1275.
15. Kim GS, Zeng L, Alali F, Rogers LL, Wu FE, Sastrodihardjo S, McLaughlin JL. Muricoreacin and murihexocin C, mono-tetrahydrofuran acetogenins, from the leaves of Annona muricata. Phytochemistry, 1998; 49: 565–571.
16. Oberlies NH, Jones JL, Corbett TH, Fotopoulos SS, McLaughlin JL. Tumor cell growth inhibition by several Annonaceous acetogenins in an in vitro disk diffusion assay. Cancer Lett, 1995; 96: 55–62.
17. McLaughlin JL. Paw and cancer: annonaceous acetogenins from discovery to commercial products. J Nat Prod, 2008; 71: 1311–1321.

18. Liaw CC, Chang FR, Lin CY, Chou CJ, Chiu HF, Wu MJ, Wu YC. New cytotoxic monotetrahydrofuran annonaceous acetogenins from Annona muricata; J Nat Prod, 2008; 65: 470–475.
19. Walsh JM, Ren X, Zampariello C, Polasky DA, McKay DL, Blumberg JB, Chen CY. Liquid chromatography with tandem mass spectrometry quantification of urinary proanthocyanin a2 dimer and its potential use as a biomarker of cranberry intake. 2016; J. Sep. Sci, 39: 342–349. doi: 10.1002/jssc.201500922.
20. Katherine MWeh, Jennifer C and Lara A Kresty. Cranberries and Cancer: An Update of Preclinical Studies Evaluating the Cancer Inhibitory Potential of Cranberry and Cranberry Derived Constituents. Antioxidants (basel), 2016; 5(3): 27.
21. Go´mez-Lecho´n MJ, Donato MT, Castell JV, Jover R. Human hepatocytes as a tool for studying toxicity and drug metabolism. Curr Drug Metab, 2003; 4: 292–312.
22. MacDonald C. Development of new cell lines for animal cell biotechnology. Crit Rev Biotechnol, 1990; 10: 155–78.
23. Schurr MJ, Foster KN, Centanni JM, Comer AR, Wicks A, Gibson AL. Phase I/II clinical evaluation of StrataGraft: a consistent, pathogen-free human skin substitute. J Trauma, 2009; 66: 866–73, discussion 873-4.
24. Jauregui HO, Hayner NT, Driscoll JL, Williams-Holland R, Lipsky MH, GalleN PM. Trypan blue dye uptake and lactate dehydrogenase in adult rat hepatocytes freshly isolated cells, cell suspensions, and primary monolayer cultures. In-vitro, 1981; 17: 1100-1110.
25. Mosmann T. Rapid colorimetric assay for cellular growth and survival: applica on to prolifera on and cytotoxicity assays. J Immunol Methods, 1983; 65: 55-63.
26. Thompson CB. Apoptosis in the pathogenesis and treatment of disease, 1995; Science, 267: 1456-62.
27. Vaux DL, Strasser A. The molecular biology of apoptosis. Proc Natl Acad Sci USA, 1996; 93: 2239-44.
28. Elsayed EA, Sharaf-Eldin MA and Wadaan MA. In vitro evaluation of cytotoxic activities of essential oil from Moringa oleifera seeds on HeLa, HepG2, MCF-7, CACO-2 and L929 cell lines. Asian Pacific J. Cancer Preven, 2015; 16(11): 4671-5.
29. Rao KVM and Sresty TVS. Antioxidative parameters in the seedlings of pigeonpea (Cajanus cajan (L.) Millspaugh) in response to Zn and Ni stresses, 2000; Plant Sci, 157(1): 113-128.
30. Bergmeyer HU. (Ed.), 2nd ed. Methods of Enzymatic Analysis, Academic press, New York, 1974; 1: 438.
31. Foyer C and Halliwell B. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism, 1976; Planta., 133: 21-25.
32. Beauchamp C and Fridovich I. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Anal. Biochem, 1971; 44: 276-287.
33. Abid-Essefi S, Baudrimont I, Hassen W, Ouanes Z, Mobio TA, Anane R, Creppy EE and Bacha H. DNA fragmentation apoptosis and cell cycle arrest induced byzearalenone in cultured DOK, Vero and Caco-2 cells: Prevention byVitaminE. Toxicology, 2003; 192(2-3): 237-48.
34. Pozarowski, P. and Darzynkiewicz, Z. Analysis of cell cycle by flow cytometry. 2004; Methods Mol Biol., 281: 301-11.
35. Anand P, Kunnumakkara AB, Sundaram C, Harikumar KB, Tharakan ST, Lai OS, Sung B, Aggarwal BB. Cancer is a preventable disease that requires major lifestyle changes. Pharm Res, 2008; 25: 2097–2116. doi: 10.1007/s11095-008-9661-9.
36. Key TJ, Schatzkin A, Willett WC, Allen NE, Spencer EA, and Travis RC. Diet, Nutrition and the prevention of cancer. Public health Nutri, 2004; 7(1A): 187-200.
37. Multhoff G, Molls M and Radons J. Chronic inflammation in cancer development. Front Immunol, 2011; 2: 98.
38. Mohamed LG, Sabit H, Sabet S,Salem R and El Ghareeb A.Targeting Apollon via siRNA Induces Apoptosis in Different Human Malignancies. Academic Journal of Cancer Research, 2014; 7(4): 233-238.
39. Ahmed W, Mofed D, Zekri AR, El-Sayed N, Rahouma M, Sabet S.Antioxidant activity and apoptotic induction as mechanisms of action of Withania somnifera (Ashwagandha) against a hepatocellular carcinoma cell line. J Int Med Res, 2018; 46(4): 1358-1369.
40. Khalili MA, Hadi NA, Naim R, Baig AA, Mahmud K. Study on antioxidant capacity and anticancer activity of Bismillah leaf (Vernonia amygdalina). W J Pharm Res, 2014; 3: 14-29.
41. Moskwa J, Borawska MH, Markiewicz-Zukowska R, Puscion-Jakubik A, Naliwajko SK. Polish natural bee honeys are anti-proliferative and anti-metastatic agents in human glioblastoma multiforme U87MG cell line. PLoS ONE, 2014; 9: e90533.
42. Martirosyan A, Leonard S, Shi X, Griffith B, Gannett P and Strobl J. Actions of a Histone Deacetylase Inhibitor NSC3852 (5-Nitroso-8-quinolinol) Link Reactive Oxygen Species to Cell Differentiation and Apoptosis in MCF-7 Human Mammary Tumor Cells. 2006; J. Pharmacol. Exp. Ther, 317(2): 546-552.
43. Bhalla S, Balasubramanian S, David K, Sirisawad M, Buggy J, Mauro L, Prachand S, Miller R, Gordon LI and Evens AM. PCI-24781 induces caspase and reactive oxygen species-dependent apoptosis through NF-kappaB mechanisms and is synergistic with bortezomib in lymphoma cells, 2009; Clin Cancer Res., 15; 15(10): 3354-65.

44. Paradies G, Petrosillo G, Pistolese M and Ruggiero F. Reactive oxygen species affect mitochondrial electron transport complex I activity through oxidative cardiolipin damage, 2002; Gene., 286: 135–141.
45. Fukuda K, Davies SS, Nakajima T, Ong BH, Kupershmidt S, Fessel J, amaranth V, Anderson ME, Boyden PA, Viswanathan PC, Roberts LJ and Balser JR. Oxidative mediated lipid peroxidation recapitulates proarrhythmic effects oncardiac sodium channels.Circulation Res, 2005; 97: 1262-1269.
46. Berndt K, Campanile K, Muff R, Strehler E, Born W and Fuchs B. Evaluation of Quercetin as a Potential Drug in Osteosarcoma, 2013; Anticancer Res, 33(4): 1297-1306.
47. Shapiro GI, Harper JW. Anticancer drug targets: cell cycle and checkpoint control. The Journal of Clinical Investigation, 1999; 104: 1645-53.