THE ANTICANCER EFFECT OF GRAVIOLA FRUIT EXTRACT IN COMPARISON WITH ANTICANCER-EFFECTIVE DRUGS

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Abstract
Malignant tumors are the world's second leading cause of mortality. Graviola, Soursop, or Guanabana, is a member of the Annonaceae family. It is native to the warmest tropical areas in South and North America. It is now widely distributed throughout tropical and sub-tropical parts of the world, including Nigeria, Malaysia, and India. Current chemotherapy treatments like 5-fluorouracil (5-FU) and Carboplatin often have limited success and fatal outcomes. Several studies have demonstrated the considerable antiproliferative effects of different extracts of Graviola and their separated acetogenins (AGEs) against various cancer cell lines; therefore, it may be a possibility as an alternative or adjuvant to chemotherapies. In this study, The GC-MS analysis shows that Many compounds with pharmaceutical properties were present in the methanolic extract as well as aqueous extract. The Kys-30 esophageal cancer cell line, SW480 colorectal cancer cell line, and Vero normal cells were treated with Graviola fruit extracts(GFE), chemotherapy drugs (5FU and Carboplatin), and GFES combined with chemotherapy drugs, then the cytotoxic effect of Graviola fruit extract (GFES) alone and in combination with drugs which Measured by MTT assay showed a growth-inhibitory effect as it reduced the viability of the cancer cell lines. The cytotoxicity assay results in the Vero cell line showed that the GFES had higher cell viability under the various tested concentrations. Therefore, the GFES is non-toxic to the treated Vero cell line. According to the results, Graviola extract is toxic to cancer cells, unlike normal cells, confirming its safety.
Keywords: Graviola, cancer, Cytotoxicity, Esophageal cancer, colorectal cancer.

INTRODUCTION

Malignant tumors are the world's second leading cause of mortality(1). Colorectal cancer is the second most common cancer globally and the third most often diagnosed malignancy(2). It is expected that more than 2.2 million new cases and 1.1 million deaths will occur due to CRC in 2030 (3). Esophageal cancer is likewise a fatal disease with a dismal prognosis, accounting for around 570,000 cases and nearly 500,000 deaths yearly globally(4). Medicinal herbs have long been used in traditional medicine for their anticancer, antibacterial, antioxidant, antidiabetic, analgesic, and anticonvulsant properties(5). Soursop (English), Graviola (Portuguese), Guanabana (Latin American Spanish), and other indigenous names. This plant is a member of the genus Annona and belongs to the family Annonaceae. Annona has around 70 species, the most cultivated of which is A. muricata (6). Many plant substances have shown promising medicinal benefits, including Graviola extracts. Graviola-derived chemicals have been related to several anticancer activities, including cytotoxicity, apoptosis, necrosis, and repression of proliferation on a variety of cancer cell lines, including breast, prostate, colorectal, lung, leukemia, renal, pancreatic, and hepatic(7), Oral, melanoma, cervical, and ovarian cancers throughout addition, this plant's bark, fruit, leaves, root, and seeds are all used as herbal treatments throughout the tropics. Furthermore, research has shown that Graviola is more toxic to cancer cells than healthy ones. Because Graviola extracts have a variety of pharmacological activities, the current study explored the lethal induction in four colorectal and esophageal cancer cells caused by Graviola fruit extracts, as well as the safety of the extracts on Vero cells.

MATERIALS AND METHODS

Plant collection. Graviola fruit was purchased from a neighboring market in Iraq. Dr. Neddaa Adnan of the Plant Herbarium, Department of Biology, College of Science, University of Babylon, identified the plant. To prepare the methanolic extract, the Graviola fruit was dried in an oven at 50 ˚C and then crushed using an electric blender; the resulting powder is stored at 4°C until use.

Methanolic extract

In a conical flask, 40g of Graviola powder was soaked in 100 ml of 99% methanol (room temperature) and plugged with cotton wool. After 48 hours, the mixture was filtered using cheesecloth and then through Whatman No.1 filter paper. The residual solution was kept in the fridge for subsequent phytochemical testing(8).

Aqueous extract

30g of plant extract powder was added to 100 ml of distilled water (room temperature) inside a conical flask and plugged with cotton wool. After 48 hours, the mixture was filtered using cheesecloth and then through Whatman No.1 filter paper. The remaining solution was stored in a refrigerator for further phytochemical screening (8).

Preparation of cell lines

Thawing of SW480, Kys-30, and Vero cell lines: The frozen cell line vial was removed from the liquid nitrogen container with caution and directly placed into a beaker containing pre-warmed (37°C) sterile DDW. The vial was removed from the water before the ice floccule dissolved completely, then it was wiped with 70% ethanol. Without delay, the cell suspension content of the vial was pipetted under a laminar flow cabinet into a 15 ml sterile plastic centrifuge tube containing 10 ml of pre-warmed serum-free medium. Centrifugation was done at 1000 rpm for 5 minutes, and the supernatant was aspirated and decanted. The cells pellet was re-suspended into 5ml warm (37C˚) serum medium and transferred into a 25 ml size cell culture flask, incubated at 37C˚, and the serum medium was replaced on the next day.

(GC-MS) analysis

The GC-MS analysis was conducted using Gas Chromatography (GC, Agilent Technologies 7890A) coupled with a mass-selective detector (MSD, Agilent 7000). It was equipped with a polar Agilent HP-5ms (5%-phenyl methyl poly siloxane) capillary column (30 m × 0.25 mm ID and 0.25 µm film thickness). Helium was used as the carrier gas with a linear velocity of 1 mL/min.

Cytotoxicity assay:

Cytotoxicity was assessed using a nonradioactive, colorimetric assay with the 3 - (4,5 – dimethylthiazol – 2 - yl) - 2,5 - diphenyltetrazolium bromide (MTT) method (9). The SW480, Kys-30, and Vero cells seeded in 96-well plates at a concentration of 1×105 cells per well were treated with serial concentrations (31.25–1000 µg/ml) of tested extracts and drugs, SW480 (AGFE, MGFE, and Carboplatin), Kys-30 (AGFE, MGFE, and 5FU), and Vero (AGFE, MGFE, and Carboplatin), and incubated in a humidified 5% CO2 atmosphere at 37°C. After an incubation period of 48 hours, 10µl of 5 mg/ml MTT was added to each well, and the incubation was continued for another 4 hours. After discarding the supernatant, the formazan crystals were dissolved in 100 µl of DMSO. It was found that the small percentage of DMSO present in the wells (maximum 0.1%) did not affect the experiment. The optical density values were then determined at 570 nm using a microplate reader.

To determine the IC50 concentration at which the drug induces approximately 50% of cancer cell death after 48 hours of exposure, as well as to obtain the dose-response curve, a series concentration of Carboplatin was assessed on the colorectal cancer cell line, and 5FU was assessed on the Kys-30 esophageal cancer cell line(9).

Statistical Analysis

The data from the MTT assay, conducted with three replicates, underwent statistical analysis to calculate the mean value and its respective standard error. The percent change between the control and experimental values was calculated. The data was analyzed statistically using "Two-Way Analysis of Variance (ANOVA)." The data, together with graphs/bar diagrams, are presented in appropriate places in the text.

RESULTS AND DISCUSSION

GC-MS analysis

In the present study, the GC-MS method was used to identify phytoconstituents in the methanolic and aqueous extracts of Graviola. In the current investigation, Many compounds with pharmaceutical properties were present in the methanolic extracts.

Table 1: the phytochemical identity of Graviola fruit methanolic extract

No	RT (min)	Graviola methanolic extract	Area%
1	4.507	3,4-Dihydropyran	1.57
2	5.005	2(3H)-Furanone, 5-methyl-	9.07
3	6.079	Ether, allyl heptyl	3.27
4	16.841	1-Propanamine, 2-methyl-N-(2-methylpropyl)-	1.58
5	22.844	Longiborneol	1.11
6	26.004	Methyl palmitate	2.43
7	28.551	9,12-Octadecadienoic acid, methyl ester, (E,E)-	2.78
8	28.619	Cyclohexane, 1,2-diethenyl-, cis-	1.25
9	28.702	Methyl oleate	0.81
10	29.148	Methyl 16-methylheptadecanoate	0.89
11	29.335	Tributyl aconitate	13.54
12	29.729	Butyl citrate	2.53
13	30.746	Tributyl acetylcitrate	2.68
14	30.948	4-Hydroxy-.beta.-ionone	1.22
15	32.256	N,N'-Diacetyl-1,7-heptanediamine	3.17
16	34.202	1-Tetradecene	1.31
17	34.316	11-Tricosene	0.78
18	35.556	8.beta.,12-Epoxy-13,14,15,16,17,19-hexanorlabdane	1.28
19	36.397	Piperidin-2-one-5-carboxylic acid, 5,6-didehydro-, methyl(ester)	1.68
20	36.962	Ethane, 1,2-diiodo-	3.51
21	37.206	1,4-PHENYLENE DI(2-NAPHTHOATE)	0.99
22	37.289	1,3,5-Triazin-2(1H)-one, 4,6-bis(methylamino)-	0.89
23	37.414	Erucylamide	2.46
24	37.471	Ethane, 1,2-diiodo-	2.74
25	37.564	Oxiraneoctanoic acid, 3-octyl-, methyl ester	1.45
26	37.787	3-(p-chlorophenyl)amino-thixo-2,5-dimethyl-thiophene	1.48
27	38.083	N-ethyl-1,3-dithioisoindoline	1.15
28	38.259	Pyrimidine, 2-(4-nitro-2-thienyl)-	2.18
29	42.825	Campesterol	6.33
30	43.287	Stigmasta-5,22-dien-3-ol, (3.beta.,22E)-	4.16
31	44.2	γ-Sitosterol	19.71

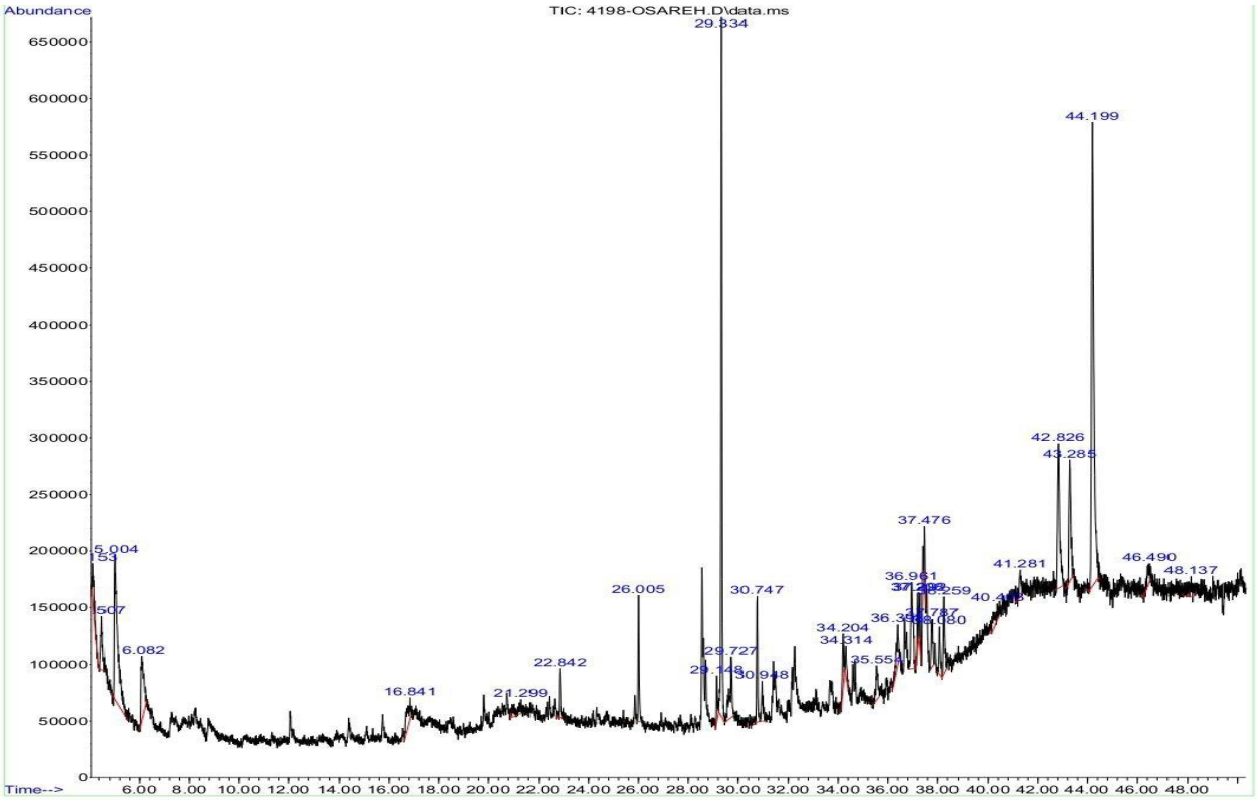


Fig. 1. GC-MS chromatogram of methanolic extract of Graviola fruit.

The most abundant compound in the GME was γ-sitosterol .γ-sitosterol is an important plant sterol and has been reported for the first time in Girardinia heterophylla. Gamma-sitosterol effectively reduces hyperglycemia in STZ-induced diabetic rats by boosting insulin secretion and inhibiting gluconeogenesis. It can be used in Diabetes mellitus. Docking studies of the ligand γ-sitosterol with four different target proteins showed that this is a good molecule that docks well with various targets related to Diabetes mellitus. Thus, γ-sitosterol can be considered for developing into a protein antidiabetic drug. It has also been reported that γ-Sitosterol might influence the quantity and function of elements in the extrinsic apoptotic pathway in human lung and breast adenocarcinoma cells.

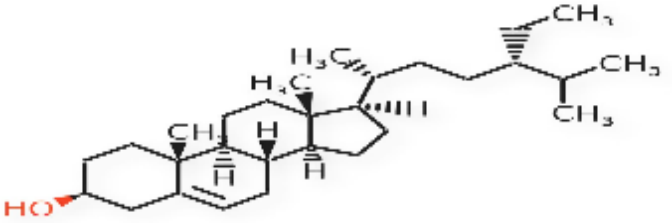


Fig. 2: Chemical structure of γ –sitosterol

Many compounds with pharmaceutical properties were present in the aqueous extracts. The most abundant compounds in aqueous extract were stigmasterol,22,23-dihydro-, 2-Ethylacridine, 2-Hydroxy-2-cyclopenten-1-one, and stigmasterol.

Table 2: phytochemical identification of Graviola fruit aqueous extract

No	RT (min)	Graviola aqueous extract	Area%
1	5.021	2-Hydroxy-2-cyclopenten-1-one	11.18
2	6.121	2-Heptanone, 7,7,7-trichloro-	4.03
3	16.903	Lactose	2.83
4	22.413	4,9-Dodecanedione	2.38
5	26.009	Methyl palmitate	5.32
6	29.148	Heptadecanoic acid, 15-methyl-, methyl ester	3.06
7	32.246	9H-Indeno[2,1-c]pyridin-9-one, 7-phenyl-	2.42
8	38.259	Cyclotrisiloxane, hexamethyl-	4.89
9	40.252	Arsenous acid, tris(trimethylsilyl) ester	2.10
10	42.825	2-Ethylacridine	12.08
11	43.292	Stigmasterol	11.03
12	44.2	Stigmasterol, 22,23-dihydro-	38.68

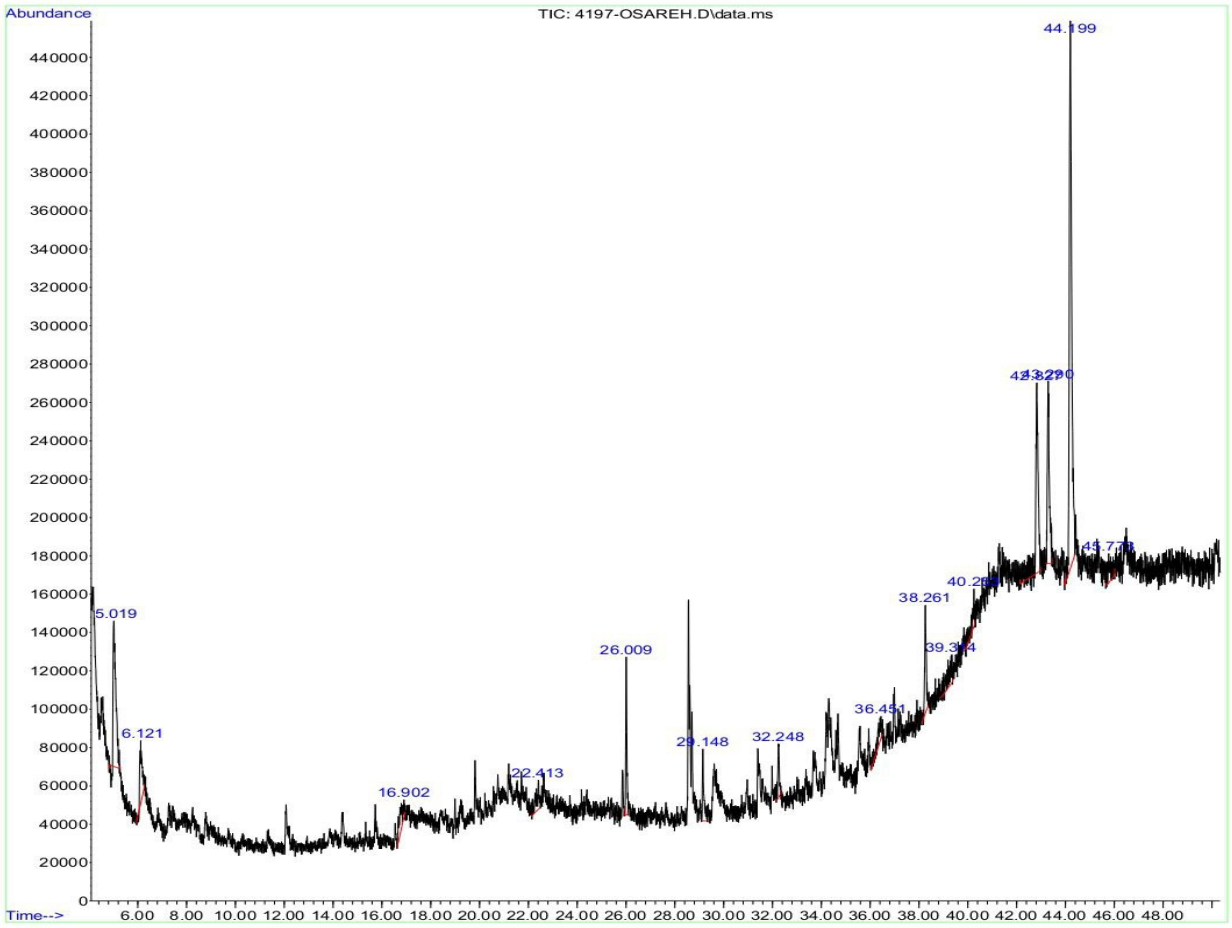


Fig. 3. GC-MS chromatogram of aqueous extract of Graviola fruit.

Cytotoxicity of Graviola extract.

Measuring the killing effectiveness of Graviola methanolic extract (GME) and Graviola aqueous extract (GAE) was conducted in two cancer cell lines: Kys-30 esophageal cancer cell line, SW480 colorectal cancer cell line, and Vero normal cells. The results presented specified that Vero cells were treated with serial concentrations (31.25-1000 µg/ml) of GME and GAE, showing no significant decrease in cell viability with all graviola extract concentrations after 48 hours (Fig3).

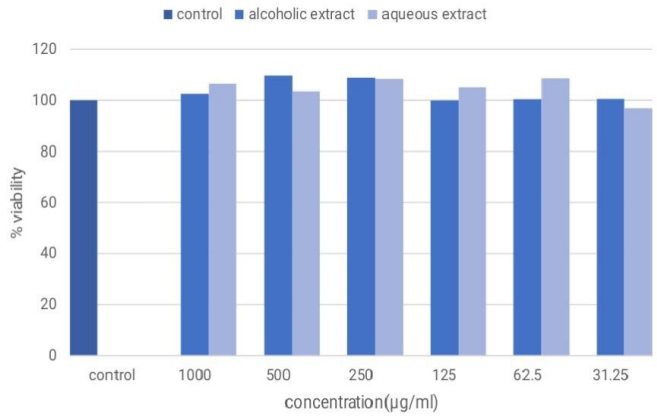


Fig. 4, Cell viability percentage of Vero cell line at different concentrations of Graviola extracts after incubation for 48 hours by MTT assay.

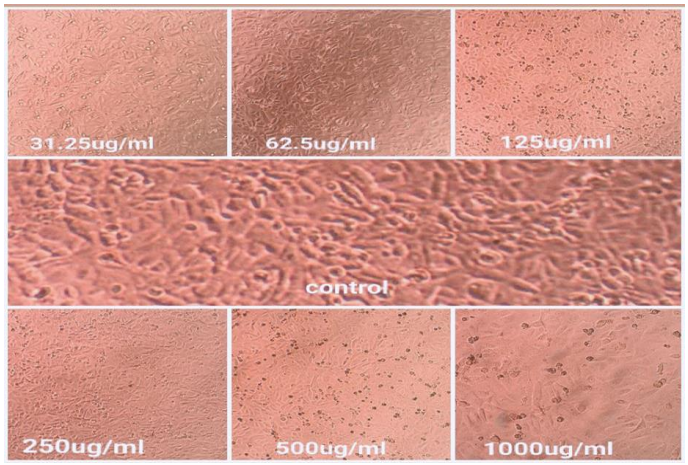


Fig. 5. Vero cell line at different concentrations (31.25-1000 µg/ml) of GME.

Results in (Fig.6) show that GME effectively reduced the viability of esophageal cancer cells with significant effects ($p \leq 0.01$)at 1000 and 500 µg/ml, while the aqueous extract(gray columns) has a significant ($P \leq 0.05$) decrease in viability at the concentration of 31.25 µg/ml only. In addition, Graviola extracts had less effect on SW480 colorectal cell growth as only 125 µg/ml is effective with significant effects ($p \leq 0.01$) for GME and a significant ($P \leq 0.01$) decrease in the viability at the concentration of 1000 µg/ml for GAE (Fig.8).

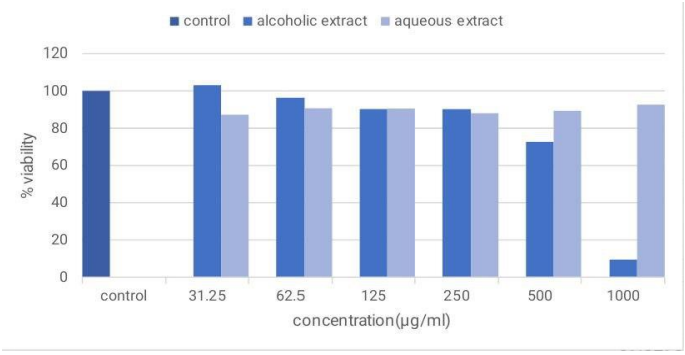


Fig.6. Cell viability percentage of Kys-30 Esophageal cell line at different concentrations of Graviola extracts after incubation for 48 hours by MTT assay.

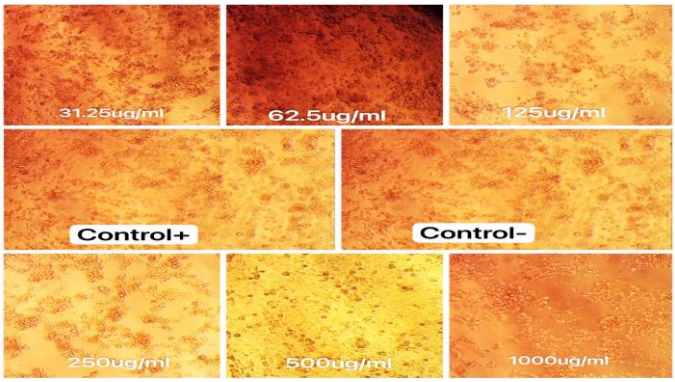


Fig.7. Esophageal cancer cell line at different concentrations (31.25-1000 µg/ml) of GME.

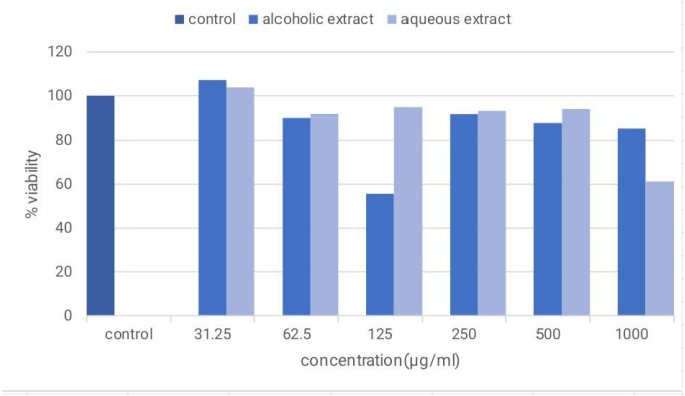


Fig.8. Cell viability percentage of sw480 colorectal cell line at different concentrations of Graviola extracts after incubation for 48 hours by MTT assay.

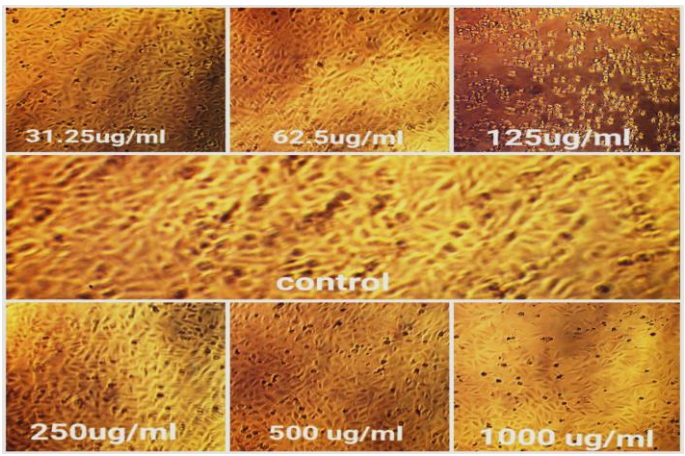


Fig.9. SW480 colorectal cell line at different concentrations (31.25-1000 µg/ml) of GME.

Graviola has been established to have therapeutic benefits against several human malignancies and disease agents in vitro culture and preclinical animal model systems. Various extracts from the plant have been used in traditional herbal medicine and have proven to possess a broad range of properties, including antioxidant, anti-inflammatory, anti-arthritic, hepato-protective, gastro-protective, antidiabetic, antimalarial, antibacterial, antiprotozoal, insecticidal, larvicidal, and wound-healing. It is interesting as well that the extract of this plant provides notable protection against various malignancies, including leukemia, colon, breast, HNSCC, and prostate cancers.

The leaf, stem, bark, fruit, and seeds of A. muricata contain a multitude of metabolically active compounds known as annonaceous acetogenins. So far, 14 different assessed acetogenins have displayed ATP-blocking characteristics and are more effective against multidrug-resistant cancer cells.

Acetogenin induces apoptosis by inhibiting NADH: ubiquinone oxidoreductase (Complex I) in cancer cell's electron transport system, preventing ATP generation. As a result, cancer cells lose energy and weaken, eventually leading to cell death (10). Natural acetogenins are effective inhibitors of mitochondrial complex I when the methyl group is added to the lactone moiety (11). The expression of Bax and Bcl-2 proteins determines the tumor response to anticancer treatments.

A study (12) reported that aqueous leaf extract possesses antiproliferative activity against BPH-1 (human benign prostatic hyperplasia) cells, where cell viability decreased from 100% to 47% with increasing doses from 0 to 1.5 mg/ml. The extracts of the leaf have been found to reduce prostate size, which may be due to apoptosis.

The ability to halt cell cycle progression in cancer cells can considerably improve natural products' anticancer capacities. Graviola extracts have been proven to induce G1 cell cycle arrest. A study (13) concluded that treating HCT116 and HT-29 cells increased apoptotic pathway activity, as indicated by increased ROS generation, detectable cytochrome C, and initiator and executioner caspases. Flow cytometry also demonstrated an elevation in Bax protein levels, which indicates apoptosis.

In vitro studies of A549 (human lung adenocarcinoma) cell lines revealed that A. muricata leaf extract possesses cytotoxic activity, and the IC50 values for hexane, ethyl acetate, and methanol extracts were 21.05 ± 0.42 g/mL, 5.09 ± 0.4 g/mL and ≤100 g/mL respectively, caused cell cycle arrest at the G0/G1 phase and apoptosis(14). The results above also agree with the result of the study of (Priya MR, Kamala), which is a toxicity study aimed at synthesizing gold nanoparticles (AuNPs) using a medicinal plant(Graviola) with anticancer properties to incorporate the therapeutic activity within the NPs. The VERO cell line was used to conduct an in vitro cytotoxicity assay. The cytotoxicity assay results showed that the AuNPs have higher cell viability under all the various tested concentrations. "The synthesized gold nanoparticles (AuNPs) are non-toxic when tested on the treated Vero cell line."(15).

Combination of GEs with drugs

After treating the two cell lines with different concentrations of anticancer drugs(Kys-30 with 5FU) and (SW480 with Carboplatin), the half-maximal inhibitory concentration (IC50) for each drug was calculated. The IC50 for 5FU and Carboplatin was 21 and 3µg/ml, respectively (Fig. 10).

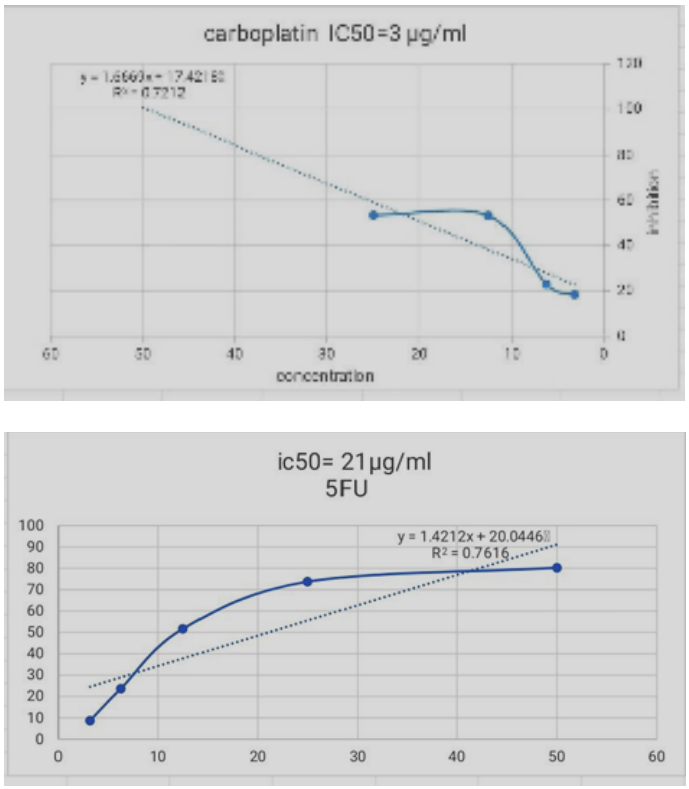


Fig.10. Dose-response curve of 5-FU and Carboplatin against Kys-30 and SW480 cell lines, respectively, after a 48 exposure period.

Results in (fig.11) show that booth Graviola extracts with 5fu have a significant (P≤ 0.01) decrease in the viability with all the concentrations, in comparison with the control group after an incubation period of 48 hours.
(fig.11) also show that the alcoholic extract with Carboplatin has a significant (P≤ 0.05), (P≤ 0.01) decrease in the viability of SW480 cells at the concentrations of(1000,500,250,125)µg/ml, other concentrations (62.5,31.25)µg/ml do not have a significant decrease in the viability of SW480 cells, in comparison with the control group after an incubation period of 48 hours. On the other hand, aqueous extract shows a cytotoxic effect at concentrations of(62.5) µg/ml.

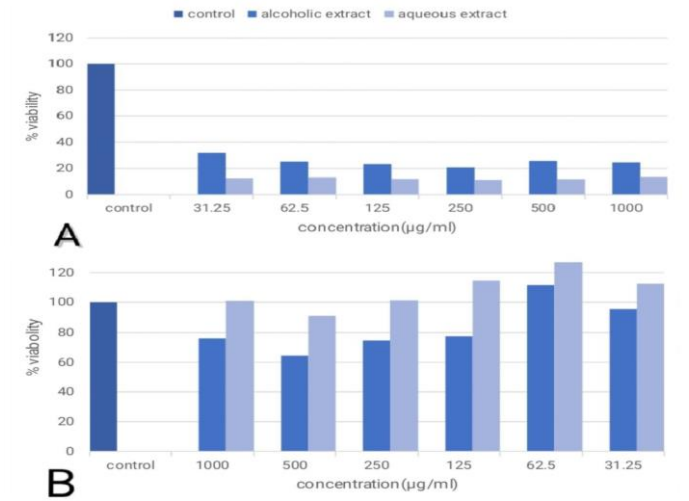


Fig.11: The Cell viability percentage of (A) esophagus (Kys-30) and colorectal (SW480) cancer cell lines after being treated with serial concentrations (31.25- 1000 µg/ml) of Graviola extracts combined with 5FU and carboplatin drug.

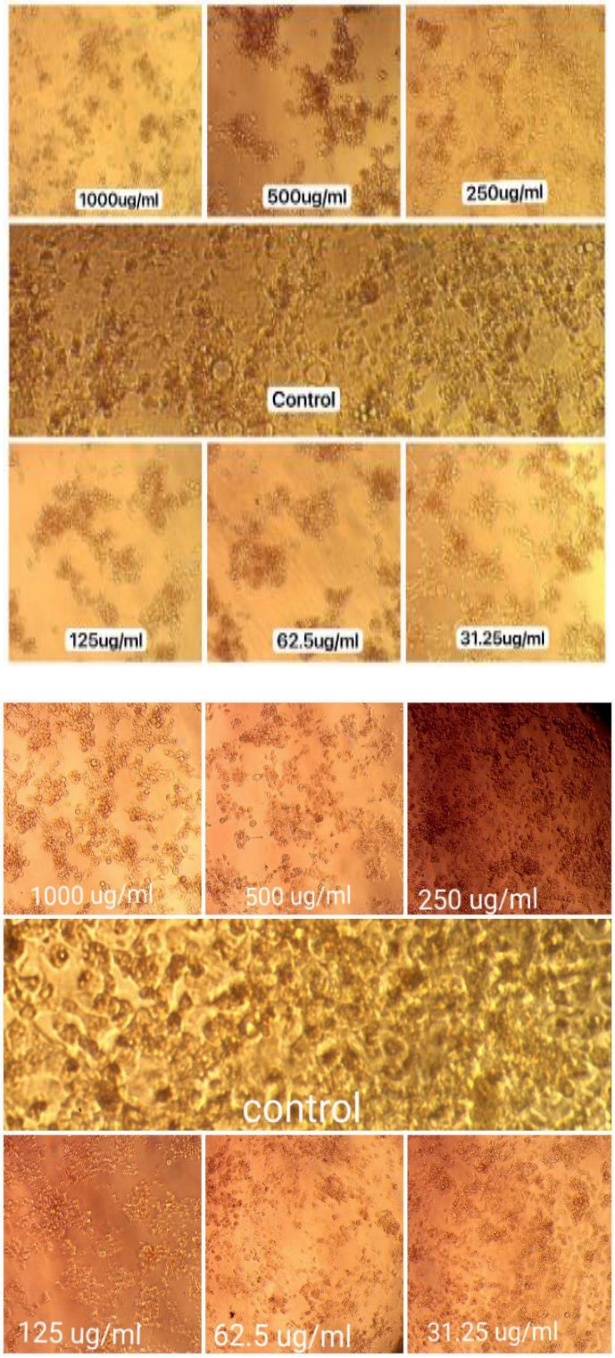
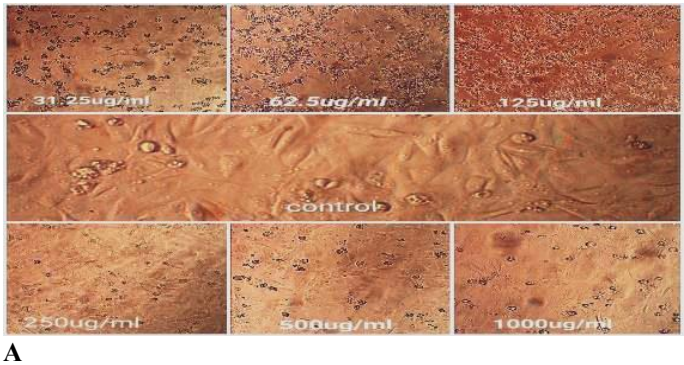


Fig.9. The KYS-30 esophagus cancer cells line after treated with serial concentrations (31.25- 1000 µg/ml) of Graviola extracts combination with 5FU drug.



A

Fig.10. The SW480 colorectal cancer cells line after being treated with serial concentrations (31.25- 1000 µg/ml) of Graviola extract combination with carboplatin drug.

Cancer is commonly treated with chemotherapy-based drugs such as paclitaxel (Taxol), doxorubicin, 5fu, topotecan, Carboplatin, and cisplatin. However, these treatments are considered to have significant adverse effects on patient health due to their potential to destroy healthy tissues. Hence, searching for new anticancer agents safe for human health is essential.

Plant products may combine with standard drugs or biological molecules to produce synergistic, additive, or antagonistic effects(16). Graviola fruit pulp extract has a potent antitumor effect on several cancer cells, which creates great interest in further explorations of the mechanisms underlying its antiproliferative activity(17). Numerous studies have reported that the major secondary metabolites in the Annonaceae family, known as Annonaceous acetogenins, are potent cytotoxic inhibitors of complex I mitochondrial by inhibiting the ubiquinone-linked NADH oxidase of the electron transport chain that is implicated in ATP synthesis (18). In a specific study, Caco-2 colorectal cells treated with Graviola and/or Cranberry showed significantly increased malondialdehyde (MDA) levels and significantly decreased levels of reduced glutathione (GSH), superoxide dismutase (SOD), and catalase (CAT) compared to normal Caco-2 cells (19). They suggested that HDACIs generate reactive oxygen species (ROS) in leukemia cells based on a previous study(20).

ROS can influence mitochondrial function, mediating the elevation of intracellular Ca2+ and leading to the activation of the caspase cascade. In addition, oxidative stress reduces Na+ channel availability, which could explain the drastic increase in MDA levels in Caco-2 cells treated with Graviola and/or Cranberry. Furthermore, Martirosyan et al. (2006) investigated the functions of a histone deacetylase inhibitor (5-nitroso-8-quinolinol), connecting ROS formation to cell differentiation and apoptosis in MCF-7 human mammary tumor cells.

A study by Addai et al. reported that 5-FU increased the activity of TNF-β and caspase-8 in the extrinsic apoptotic path while decreasing the activity of cyto-c, SMAC, bad, and HTRA in the intrinsic pathway. However, it decreased the activity of IAP (survivin, livin', and XIAP), which inhibits caspase-3 and 9.29, Causing up-regulation of death receptors (DR6 and TRAILR-2-3).32 The combination of graviola chloroform extract and 5-FU increased the expression of Fas, TNF-α, TNF-β, sTNF-R1, and caspase-8 in the extrinsic apoptotic pathway. It also decreased the expression of anti-apoptotic proteins (Bcl2 and Bcl-w) and increased the expression of pro-apoptotic proteins (Bad and BID). It also increased the expression of SMAC and HTRA in the intrinsic pathway and induced death receptors such as DR-6 and TRAILR-1-2-3. This combination stimulates caspase-independent apoptosis by activating the tumor suppressor genes P53 and P27 (21).

Another study concluded that Graviola leaf extract GLE has antiproliferative and cytotoxic properties in many cancer cell lines with little damage to non-transformed cells. Furthermore, they discovered that known inhibitors of Na/K and sarcoplasmic reticulum ATPase pumps might cause cell death in various cancer cell lines. This may explain the antagonistic effects of GFE on 5FU in our study when administered in combination(22).

CONCLUSIONS

Our results show that GEs significantly decrease the cell number of SW480 and Kys-30 cell lines. Normal cells are not affected, which proves safety. The study result indicated that the cytotoxicity of the mixture of (extract and Carboplatin) is more than the cytotoxicity of Carboplatin alone on the SW480 cell line.

The result also concluded the anticancer activity of a mixture of (GEs and 5FU) shows a more cytotoxic effect toward esophageal cancer cell line Kys-30 compared with the cytotoxicity of each one alone. Thus, the Graviola fruit is a promising alternative or complementary supplement for reducing esophageal and colorectal cancer growth.

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