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Comparative Study of Anti-Cancer Properties of crude ethanolic ginger rhizome extract and Luteolin in EAC-Bearing Mice

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Abstract

Nowadays, the use of plant medicine is a growing trend due to its decreased toxicity and potency. Moreover, several based bioactive compounds exert significant impact against various life-threatening diseases. In the light of the previous we investigated the anti-cancer properties of Ginger (G) and Luteolin (LUT) in Ehrlich Ascites Carcinoma (EAC) b mice. 56 Swiss albino mice were used and divided into seven groups (8 per group) as follows: control group (C), control Ginger (G), control Luteolin (LUT), Ehrlich Ascites Carcinoma group (EAC), (EAC+G) group, (EAC+LUT) group, and (EAC+G+LUT) group. The (EAC), (EAC+G), (EAC+LUT) and (EAC+G+LUT) groups were intraperitoneally injected by Ehrlich cells $3x10^6$ cells to induce Ehrlich tumor. The (G) and (LUT) groups were treated with ginger oral dose (200 mg/kg/day) and LUT (100 mg/ body weight/ day), respectively. Body weight was measured, and blood samples were taken for liver enzymes analysis. After that, the mice were sacrificed, and their liver tissues were obtained for histopathological investigation. Flow cytometry was utilized for cell cycle analysis, evaluation of Caspase-3, Ki67, and BCl-2. Also, Real-time PCR was used for VEGF expression. In this study, liver enzymes and histopathological examination were significantly improved upon treatment with Ginger and Luteolin. Moreover, the combined treatment of Ginger and Luteolin exhibited a potential antiproliferative activity against EAC more than single therapy, they exerted their effect by initiating apoptosis through regulating Caspase-3, Bcl-2, inhibit proliferation and arrest the cell cycle through inhibition of Ki-67.

Keywords: Ginger; Luteolin; EAC; Ki67; Bcl-2; Caspase-3.

1. Introduction

Cancer is the most frequent disease affecting people around the world of different ages and sexes. Cancer treatments are focused on improving a patient's outcome and minimizing the undesirable side effects. The use of natural products or plants extract has been recognized as a promising approach, due to their efficiency in treatment and prevention of various diseases. Moreover, they have significant value as they act as anti-cancer agents with limited or no side effects [1, 2]. Ginger (*Zingiber officinale Rosc*) belongs to the Zingiberaceae family of tropical and subtropical plants that originated in Southeast Asia. Ginger has been used globally for 2500 years in traditional medicine as a cure for headache, diarrhea, cold, stomach disorders and nausea. It contains phenolic compounds which have anti-cancer, antioxidant, and anti-inflammatory properties as paradol, gingerol, and shogoal[3, 4].

Luteolin (LUT) is a flavonoid found in a number of fruits, vegetables, and medicinal herbs, which possess potential antioxidant and anti-inflammatory properties. LUT is widespread in vegetables and fruits as: onion leaves, broccoli, apple skin, carrot, celery, cabbages, and parsley. Chinese traditional medicine uses LUT-rich foods to treat hypertension and inflammation. Recent studies demonstrated that LUT had anti-tumor activity on many tumors such as leukemia, pancreatic tumor, breast, esophageal, colon, prostate, and gastric tumors. It induces apoptosis pathways, cell cycle arrest, and inhibits cell proliferation pathways of cancer cells in different human tumors [5-9] LUT also, inhibits corneal angiogenesis; prevents vascular disorders of eye, and treatment of cataract[10-12]. Rodent induced tumors become the basis of most cancer studies as their biochemical and genetic characteristics can be studied. Ehrlich ascites carcinoma EAC is the most

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common experimental tumor modeling. It is considered as a homogenous example of malignant tissue which grows at predictable rate and is available in large quantities. Some plant extracts are found to be effective against EAC, and this is reported by many [13-15] researchers
In the light of the previous facts, we studied the anti-cancer properties of ginger extract and LUT against Ehrlich Ascites Carcinoma (EAC) *in vivo*. To assess the mechanism of action by which these compounds exerted their effects, cell cycle analysis, Bcl2, Ki67, VEGF and Caspase 3 expression was evaluated.

2. MATERIALS AND METHODS

2.1. Experimental Animals

A total of 56 healthy Female Swiss albino mice weighing 22 – 28 g were purchased from VACSERA, Egypt. The mice were housed in a 12-hour light/12-hour dark cycle at ambient room temperature of 22 - 25 ^{0}C and relative humidity conditions. Commercial diet containing all necessary nutritive elements, and tap water was provided *ad labium* for two weeks before starting the experiments.

2.2. Chemicals

Chemicals were purchased from (Kemet Medical Company - Sigma Aldrich Chemical Company) and used directly without further purification.

2.3. Luteolin Treatment

LUT was purchased from Sigma -Aldrich Chemical Company, Egypt. LUT powder was suspended in distilled water (a dose of 50 mg/kg body weight), and mice were orally given LUT with a gastric tube for two weeks [16]

2.4. Ginger treatment

Ginger rhizomes were purchased from local aromatherapy. Around 100 g fresh ginger was peeled into pieces of different sizes and soaked for 72 hours in 2 L 70% ethanol. The extract was filtrated and evaporated at room temperature for 5 days to give a semi-solid extract. A dose equivalent to 200 mg of the crude extract per kg body weight was calculated and dissolved in distilled water and mice were orally given ginger extract with gastric tube for 2 weeks [17].

2.5. Ehrlich Ascites Carcinoma Cells (EAC)

Ehrlich ascites carcinoma (EAC) cell was purchased from the National Cancer Institute, (Cairo, Egypt). The cells were kept *in vivo* in mice, by serial IP transplantation of 2 x 10^6 cells/mouse/0.2 ml every 7 days. The treatment started after 2 weeks of transplantation.[18]

2.6. Experimental protocol

Rats were divided into seven groups of 8 mice, as follows:

- **Group 1:** served as control group, given commercial diet and tap water only **(negative control) or (C)**
- **Group 2:** mice orally given a dose of 200 mg/kg. b.w. /day of Ginger crude ethanoic extract for two weeks besides normal diet and water. **(Negative Ginger) or (G)**
- **Group 3:** mice orally given a dose of 50 mg/kg. b.w./day LUT for 2 weeks beside normal diet and water. **(Negative LUT) or (LUT)**
- **Group 4:** mice were intraperitoneally injected once by Ehrlich cells 2x10^6 cells to induce Ehrlich tumor development. **(EAC)**
- **Group 5:** mice were intraperitoneally injected once by Ehrlich cells 2x10^6 cells and kept for two weeks till tumor volume reaches 1 mm^3. After that, mice were given an oral dose of 200 mg/kg. b.w./day of Ginger for extra two weeks. **(EAC+G)**
- **Group 6:** mice were intraperitoneally injected once by Ehrlich cells 2x10^6 cells and kept for two weeks till tumor volume reaches 1 mm^3. After that, mice were given an oral dose of 50 mg/kg. b.w./day of LUT for extra two weeks**. (EAC+LUT)**
- **Group 7:** mice were intraperitoneally injected once by Ehrlich cells 2x10^6 cells and kept for two weeks till tumor volume reaches 1 mm^3. After that, mice were given an oral dose of 200 mg/kg. b.w./day of ginger and 50 mg/kg. b.w./day LUT for an extra two weeks**. (EAC+G+L)**

2.6.1. Ethical Approval

This work was done according to "Guide for the care and use of laboratory animals, National Academies" [19]. All experiments were carried out in accordance with the rules of Mansoura University's Institutional Animal Ethics Committee in Mansoura, Egypt approval number (Sci- Ch-M, 2021-109).

2.6.2. Sample collection and preparation for flow cytometry:

Overnight fasting mice were anesthetized using ketamine/xylazine (0.1 ml/100g b.w IP), and blood specimens were taken through heart puncture, deposited in EDTA containers, and centrifuged at 3000 rpm for 20 minutes. Plasma was isolated, and each specimen was marked and stored at -20 ^{0}C till analysis. In addition, each mouse's tumor was

excised, weighed, and the volume of each tumor was recorded. Each tumor was stored at -20°C until it was used in flow cytometric analysis.

Cell suspension from tumor tissues was prepared using phosphate buffer saline (PBS), debris and clumps were eliminated, the suspension was centrifuged at 2000 rpm for 10 min and the supernatant was discarded. 3 ml PBS was added into the pellet and mixed well.

A fluorochrome-labeled antibody was mixed with the cell suspension, and inside the flow cytometer, cells pass individually through an interrogation point due to the laminar flow of the surrounding isotonic fluid. At that point, laser beam emits light which pass through each cell, then the scattered light is collected, filtered, and digitized for analysis. The obtained data were displayed as dimensional dot plot or histogram formats [20, 21]. Liver from each mouse was excised, cleaned, and preserved in 10% formalin for histopathological investigation.

Table 1: primer sequence of VEGF and β-actin

	Forward (5′-3′)	Reverse (5′-3′)	Gen Bank
VEGF	TGCAGATTAT GCGGATCAAA CC	TGCATTCAC ATTTGTTGT GCTGTAG	AB021221
-actinβ	TCCACCTTCCA GCAGATGTG	GCATTTGCG GTGGACGAT	NM_001101

2.6.3. Assessment of caspase-3, Bcl-2 and Ki67

100 µl of sample mixed with 1ml cold PBS in a centrifuge tube and left for 10 min at 37°C and centrifuged for 5 min and the supernatant discarded. Cells were stained with 5 µl anti-caspase-3/anti-Bcl-2/ anti-Ki-67 antibody then incubated in the dark for 15 min at RT (25°C). After incubation cells were washed and fixed with paraformaldehyde and intensity of caspase-3/ Bcl-2/ Ki-67 was measured by flow cytometry [22].

2.6.4. DNA Content and cell cycle analysis:

Prepared cells suspension was centrifuged for 5 min at 1000 rpm and supernatant was decanted thoroughly. The pellet was suspended in 5 ml of PBS and centrifuged for 5 min at 1000 rpm. Pellet was suspended in 1 ml of propidium iodide (PI) solution and kept in the dark at room temperature for 30 min or at 37°C for 10 min. Then, the cell fluorescence was measured after samples were left in the flow cytometer. Maximum of PI excitation bound to DNA is at 536 nm, and emission at 617 nm [23, 24].

2.6.5. Quantitative Real-time PCR for estimation of VEGF

Vascular Endothelial Growth Factor (VEGF) specific gene was estimated semi-quantitatively as stated below: TRIzols Reagent (15596026, Life Technologies, USA) was used for total RNA purification from samples according to manufacturer protocol. Then, 1 µg of total RNA was reverse-transcribed into single-stranded complementary DNA by using QuantiTects Reverse Transcription Kit (Qiagen, USA) using a random primer hexamer in a two-step RT-PCR reaction as mentioned in manufacturer's protocol. Amplicons were amplified using a CreaCon thermal cycler. DNA ladder 1.5 kbp DNA ladder (PeqGold 1.5 Kb, Peqlab, GMH) was used to estimate the final amplified product length. According to manufacture protocol, Green Taq (DreamTaq) master mix (Thermo Scientific) was used for gene amplification. Table 1 showed specific VEGF and β-actin primers. Thermal cycler (Creacon, Holland), conditions were applied according to Chung et al., (2012) as follow: 95° C, 15 sec and 60° C, 1 min for 40 cycles after denatured at 95° C for 10 min Final product for specific amplicon (approx, 81 bp) was photograph and detection using Dig-doc, UVP, INC, England [25] .

PCR products were loaded on 1.5% (w/v) Agarose gel using complete 15x15 cm agarose gel package and stained with Ethidium bromide.

Data analysis

Gel documentation system (Geldoc-it, UVP, England) was applied for data analysis using Total lab analysis software, ww.totallab.com, (Ver.1.0.1)

2.6. Histopathological Examination of Liver

Liver samples were cut into slices and fixed in 10% neutral buffer formalin for 24 h. After fixation, the samples were dehydrated by ethyl alcohol 70%, 80%, and 90% and 95% ascendingly for 30 min, each, then in two changes of absolute ethyl alcohol for 30 min, each. Tissues were cleared in xylene for 20 min, (two changes), then embedded in paraffin wax. Sections 4-5 µm thick were cut using microtome, mounted on glass slide, and stained with hematoxylin and Eosin staining [26].

2.7 Biochemical Investigation of blood samples

The serum level of liver enzymes ALT, AST, total protein (TP), and total bilirubin (TB) were measured with commercial kits, according to manufacturer's protocol.

2.8. STATISTICAL ANALYSIS

Mean values and standard deviation were calculated according to conventional methods. Significance between different groups was determined using one-way analysis of variance (ANOVA) and Tukey-Kramer multiple comparison post hoc test, $P<0.01$ was considered significant. The data was collected

and analyzed using a statistical package for social sciences (SPSS), Version 18.

3. RESULTS

Our study was carried out to evaluate the expression of Caspase-3, Bcl-2, and Ki-67 by FC in the studied groups to assess the anti-cancer activity of Ginger and LUT against EAC bearing mice. Also, we used FC to detect cell cycle analysis and real-time PCR for VEGF.

3.1. Caspase-3 expression level

As shown in figure 1, there was no significant change in Caspase-3 expression in either control or control treated groups. In contrast, Caspase-3 expression was significantly decreased in group (EAC) compared to control group (C) with mean± SEM (8.96±2.0) and (13.6± 0.7) respectively. *(P* < 0.001). Upon treatment with LUT and Ginger or combined therapy, there was a marked increase in Caspase-3 expression when compared to EAC group.

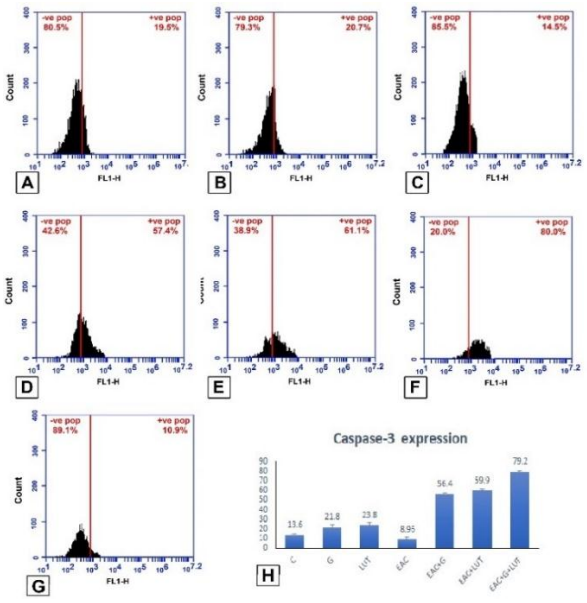

Figure 1. Flow cytometry histograms showed different expression of Caspase-3 among the studied groups (A to G represents groups 1 to 7) and (H) their correlation.

3.2. Bcl-2 expression level

As shown in figure 2, there was no significant change in Bcl-2 expression in either control or control treated groups. In contrast, Bcl-2 expression was significantly increased in group (EAC) compared to control group (C) with mean± SEM (59.7± 2.3) and (31.7± 1.5), respectively (P < 0.001). Upon treatment with LUT and Ginger or combined therapy, there was a marked decrease in Bcl-2 expression when compared to EAC group.

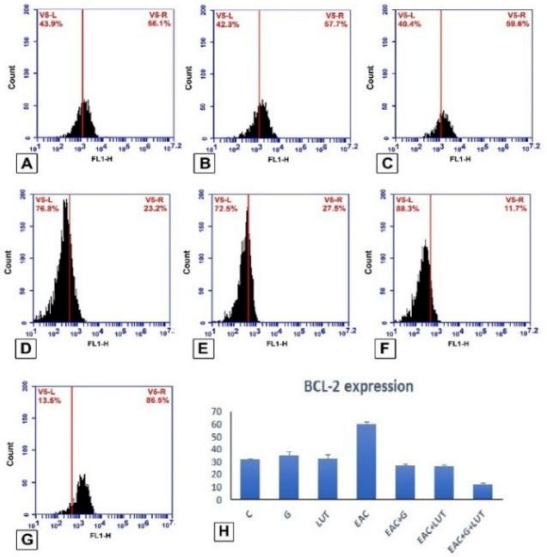

Figure 2. Flow cytometry histograms represent different expression of Bcl-2 among the studied groups (A to G represents groups 1 to 7) and (H)their correlation.

3.3. Ki-67 expression level

As shown in figure 3, there was no significant change in Ki-67 expression in either control or control treated groups. In contrast, Ki-67 expression was significantly increased in group (EAC) compared to control group (C) with mean± SEM (65.3±2.7) and (18.2±1.2), respectively *(P* < 0.001). Upon treatment with LUT and Ginger or combined therapy, there was a marked decrease in Ki-67 expression when compared to EAC group.

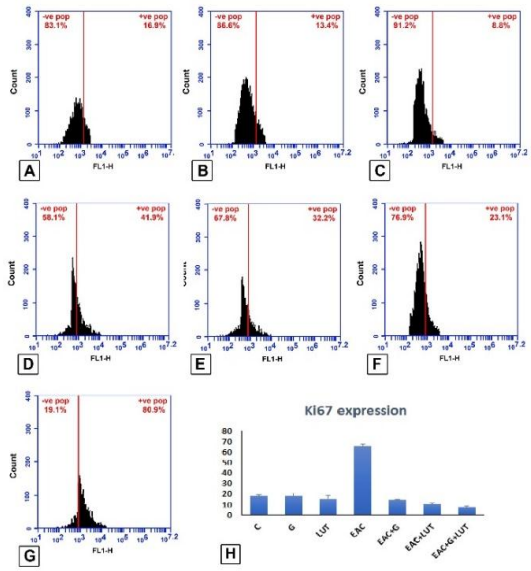

Figure 3. flow cytometry histograms showed different expressions of Ki-67 in the studied groups (A to G represents groups 1 to 7) and (H) their correlation.

3.4. Effect of treatment on body weight gain

The daily body weight (B.W) of each mouse was observed throughout ten days, and the gain in weight due to tumor burden is calculated. Figure 4 clearly shows a superior body weight gain in the EAC group than the initial weight ($p< 0.001$) compared to the control group. A slight increase in (B.W) of group (EAC+G), (EAC+LUT), and (EAC+G+LUT) compared to other groups.

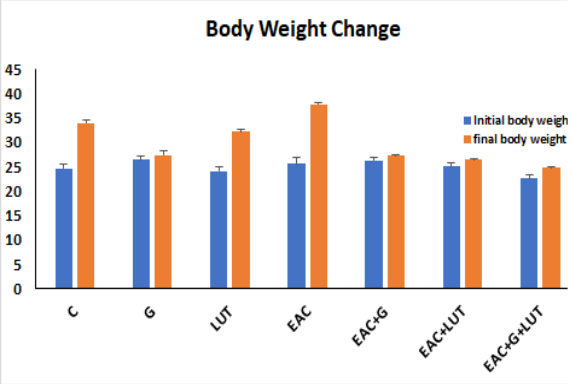

Figure 4. histogram represents the correlation between the initial and final body weight.

3.5. Impact of treatment on hepatic enzymes

As illustrated in table 2, (EAC) group showed a high increment in the hepatic enzymes and biomarkers compared to control (C) group. However, in treated groups (EAC+G), (EAC+LUT) and (EAC+G+LUT), there was a significant decrease in AST, ALT, TP, and TB compared with (EAC) group (P<0.001). The healthy mice treated with ginger (G) and (LUT) groups showed a non-significant difference in the hepatic enzymes compared with normal group (C). Ginger therapy exhibited the best improving activity on the hepatic enzymes and biomarkers more than LUT or combined treatment.

Table 2: Levels of serum ALT, AST, TP, and TB in different studied groups

Groups	ALT	AST	T. P	T. B
C	6.7 ± 0.3	82.7 ± 3.9	2.6 ± 0.4	0.9 ± 0.1
G	7.6 ± 0.2	68.0 ± 4.2	2.2 ± 0.3	0.5 ± 0.07
LUT	4.5 ± 0.7	77.5 ± 0.7	1.4 ± 0.1	0.6 ± 0.07
EAC	42.5 ± 3.5	165.0 ± 7.0	5.5 ± 0.7	1.5 ± 0.7
EAC+G	4.0 ± 1.0	39.0 ± 1.0	0.9 ± 0.07	0.1 ± 0.07
EAC+LUT	8.5 ± 0.7	83.0 ± 4.9	2.7 ± 0.3	0.6 ± 0.2
EAC+G+LUT	8.0 ± 1.4	52.5 ± 3.0	3.5 ± 0.7	0.4 ± 0.1

3.6. Cell cycle analysis

As shown in table 3, the subG1 level of apoptosis decreased in EAC group with mean± SEM (23.6 ±3.8) than treated groups (EAC+G), (EAC+LUT) and (EAC+G+LUT). On the other hand, a superior increase was noticed in (EAC+G+LUT) group with mean± SEM (75.7±2.2) than (EAC+G) group, with mean± SEM (30.4±1.9), and (EAC+LUT) group (50.2±9.8), P< 0.001.

However, G0\G1 increased in (EAC) group with mean± SEM (49.1 ± 1.3) and (EAC+G) group with mean± SEM (59.5 ±12.8) but decreased in (EAC+LUT) group, (40.3±5.4) and another significant decrease was observed in (EAC+G+LUT) group with mean± SEM (16.3±0.8), P< 0.001. For S-phase, a significant increase was noticed in (EAC) group (29.7± 4.3) compared to (EAC+G+LUT) group (7.8± 2.7) and (EAC+LUT) group (8.5±3.1). While G2\M was decreased in (EAC+G+LUT) group (0.8± 0.2) than EAC group (1.8±0.4) and also than (EAC+LUT) group (1.5±1.2) but increase in (EAC+G) group (2.8±0.6) P< 0.001.

Table 3: Stages of the cell cycle.

Groups	Sub G1	G0\G1	S-Phase	G2\M
EAC	23.6 ± 2.2	49.2 ± 0.8	29.7 ±2.5	1.8 ± 0.26
EAC+G	30.4 ±1.12	59.6 ±7.4	16.9 ± 0.9	2.87 ± 0.38
EAC+LUT	50.2 ± 5.68	40.3 ± 3.1	8.5 ± 1.79	1.57 ± 0.73
EAC+G+LUT	75.7 ± 1.32	16.3 ± 0.46	7.9 ± 1.58	0.8 ± 0.15

3.7. Quantitative Real time-PCR Analysis

As shown in figure 5, VEGF specific fragment intensity was increased in EAC group, while decreased in groups (EAC+G), (EAC+LUT), and (EAC+G+LUT). No significant change was observed in control or control treated groups.

Histopathological Inferences of Different Groups

As shown in figure 6, normal hepatocytes with typical architecture in control groups. Whereas in Ehrlich group (D) showing focal infiltration of inflammatory cells, hepatic vacuolation, and loss of hepatic architecture. Hepatic section from positive groups treated with Ginger displayed improvement of hepatic architecture compared to untreated.

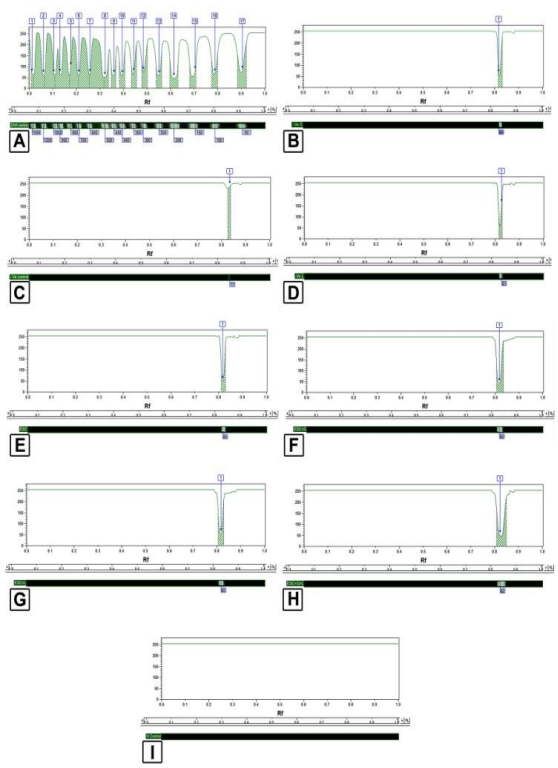

Figure 5. Vascular Endothelial Growth Factor (VEGF) amplicon for DNA ladder(A) and the studied 7 groups B, C, D, E, F, G, H, and I. and intensities of samples

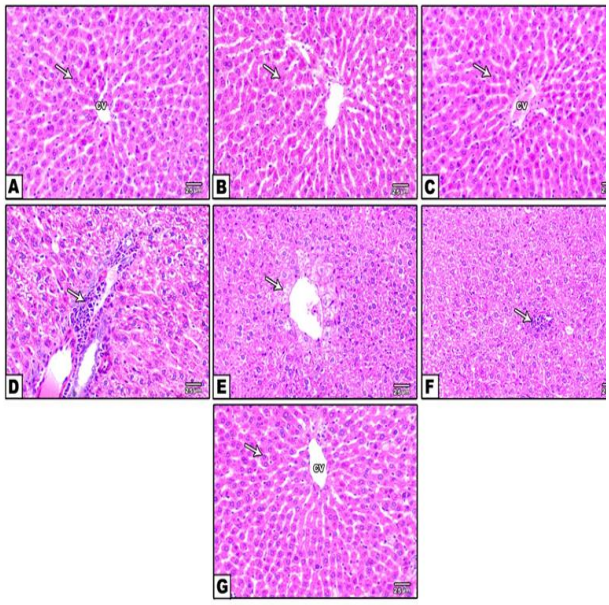

Figure 6. H&E histopathological examination of liver tissues of control and treated mice groups. A: Normal healthy control mice liver showing normal cellular architecture. B and C: negative ginger control and negative LUT control mice liver demonstrating characteristics of normal liver. D: positive EAC group show disorganization of the architecture of the liver. E, F treated groups with G and LUT, respectively, show mild improvement of liver tissues. G: combination group treated with G+LUT together show near-normal improvement.

4.DISCUSSION:

Recent studies proved the efficacy of medicinal plants and antioxidant compounds in cancer treatment; among these are Ginger and LUT. There are extensive studies on the ability of Ginger phenolic compounds to exhibit a high therapeutic effect against the oxidative stress-related disease, including cancer [15, 27-29] In this work, the antitumor effect of both Ginger as a phenolic-rich compound and LUT as a potential flavonoid was studied against EAC-bearing mice. EAC animal model is characterized by its stability and consider the best choice in testing anti-cancer drugs among animal modeling.

According to previous studies [30-32], the anti-tumor effects were estimated by noticing tumor volume and the gain in weight of the bearing- animal. And our study showed an increase in EAC group due to increase in ascites volume, also a slight increase in the control healthy (C) group while, (EAC+G) and (EAC+LUT) groups which treated with Ginger and LUT, respectively, showed a decrease in the weight gain compared to EAC group. But (EAC+G+LUT) group provides the best reduction in weight gain compared to EAC, EAC+G and (EAC+LUT) groups. Thus, Ginger and LUT are superior to native Ginger or LUT alone as anti-tumor agents upon using the same conditions.

It is observed that liver enzymes in EAC group were significantly increased compared to (C) group; this is due to impairment of liver function, which led to the spill of these enzymes into the bloodstream. The high level of AST, ALT indicates hepatic disorder and our histopathological examination confirmed that. These results were comparable to previous studies[1, 33]. Moreover, the treated groups (EAC+G), (EAC+LUT) and (EAC+G+LUT) showed near-normal levels of the hepatic enzymes. Therefore, G and LUT help in maintaining liver functions.

Cells undergo a series of developing, organized, and predictable steps from birth to division into daughter cells, which is called the cell cycle. There are 2 major stages of the cell cycle: interphase and M-phase. The longest phase is the interphase, and it consists of 3 stages: G1, S, and G2. Whereas M-phase or mitotic phase account only for 10% of the total time of the cell cycle. It is crucial for researchers to detect the cell cycle by flow cytometry to find DNA content. A unique signal will be produced by the DNA content of cells in the G1 phase, which will enhance during

the S phase and have a multiplied intensity after the S phase is completed. Any error that occurs during the cell cycle will lead to cancer. Apoptosis, or programmed cell death, is a vital, controlled, and energy-dependent mechanism that selectively kills cells with errors [34]. It involved physiologic and pathologic events and displayed DNA fragmentation, shrinkage of cellular content, membrane bleeding, and activation of Caspases. Any disturbance in the apoptosis level causes cancer seriously, so apoptosis detection is essential in disease diagnosis, progression, and even therapy efficacy. Resistance to apoptosis by cancer cells contributes to metastasis and resistance to treatment [35-37]. Our flow cytometry results for cell cycle showed that, the sub-G1 apoptosis level significantly increased in the treated (EAC+G), (EAC+LUT) and (EAC+G+LUT) groups than the EAC group, with a superior increase in (EAC+G+LUT) group, which was treated with Ginger and LUT.

On the other hand, S-phase and G2\M phase decreased in the treated (EAC+G), (EAC+LUT) and (EAC+G+LUT) groups than EAC group, with a superior decrease in (EAC+G+LUT) group than in (EAC+G) and (EAC+LUT) groups. Our results proved that Ginger and LUT enhance apoptosis of cancer cells and arrest cell cycle at G2\M phase, by downregulation of Bcl-2 anti-apoptotic protein and up-regulation of caspase-3 as in figure (1,2), and this is in great agreement with previous studies [38-40].

Caspases are cysteinyl proteases family enzymes whi ch function as apoptosis effectors. They play a key ro le in apoptosis initiation and execution [41]. Caspase-3 is one of the most important effector caspases, as it is involved in both the death receptor and mitochondrial pathways, which are both launched by caspase-8 and caspase-9, respectively. Moreover, it induces a caspase-activated DNase endonuclease, which causes DNA fragmentation, which is the most important aspect of apoptosis [42]. It acts as an essential executioner of caspases with caspase 6 and 7, which induce apoptosis and maintain survival. Besides, it cleaves many cellular substrates, including structural proteins and DNA repair enzymes[43, 44].

However, the anti-apoptotic protein BCL-2 was involved in different types of cell death as autophagy, necrosis, and apoptosis. It blocks the action of pro-apoptotic proteins Bax and Bak of the same family. It also has a vital role in insulin secretion and control metabolic activity. About 50% of human cancer upregulate BCL-2. Moreover, it is expressed in both solid and hematologic tumors [45, 46]. Our results showed that Caspase-3 expression was increased in the treated groups (EAC+G) (EAC+LUT) (EAC+G+LUT), as in figure 1 than in control groups (C), (G) and (LUT). These results are in great concepts with other previous studies [47-49] which demonstrated that Caspase-3 was activated in the treated animals, due to stimulation of apoptosis and anti-tumor immunity of the host cells. Besides, (EAC+G+LUT) group, which treated with Ginger and LUT, showed a superior increase in Caspase-3 expression, which means they can act together as potent anti-cancer agents. We also found the anti-apoptotic expression of Bcl-2 is increased in Ehrlich group as a cellular way to avoid apoptosis [50, 51]. Additionally, the (EAC+G), (EAC+LUT), and (EAC+G+LUT) groups all shown better reductions in BCL-2 expression, indicating that they can work in concert as effective anti-cancer agents.

On the other hand, the Ki-67, which represents a biomarker for cell proliferation, explains its superior high level in (EAC) group than other groups but decreased in the treated groups. Based on previous studies [49, 52], Ki-67 could estimate the proliferation rate in EAC, and our results prove that too.

Angiogenesis is a physiological process in which new blood vessels are formed from the existing vasculature, starting in utero throughout old age. It includes the migration, growth, and differentiation of endothelial cells, in the wall of blood vessels [53, 54]. It is a vital process in both disease and health as no active tissue can survive without blood supply. Tissues need blood capillaries to exchange metabolites and nutrients. Therefore, oxygen plays a vital role in the regulation of angiogenesis. Chemical signals in the body control this process; some of these signals stimulate angiogenesis as vascular endothelial growth factor (VEGF), whereas others are inhibitors. The balance between the inhibitor and stimulating factors make the formation of newly vessels only when needed in healing wounds and during growth [55]. Tumor cells need a blood supply for their nutrition as normal cells behave, so tumors stimulate angiogenesis. VEGF promotes angiogenesis by stimulating endothelial cells that secrete proteases, causing the degradation of the basement membrane of blood vessels, allowing cancer cells to invade other surrounding tissues. After subsequent migration and proliferation, the cells finally differentiate to form a new vessel [3, 56]. It is observed that VEGF has enhanced expression in many human cancers like breast, lung, ovarian, and rectal. Also, higher amounts were found in other diseases as malignant ascites, brain edema, rheumatoid arthritis, and synovial fluid. Hence, many previous studies prove that the blocking of over-expression of VEGF results in hindering tumor growth and inhibition of ascites [57, 58]

It is well-known that LUT has a potent anti-proliferative activity associated with suppression of metastasis and angiogenesis against different tumors

by inhibiting the binding of VEGF with its receptors. Moreover, it has a few side effects on normal cells when used in cancer treatment [32, 59-61]. Our results reveal that the VEGF expression was declined significantly in the treated groups (EAC+G), (EAC+LUT) and (EAC+G+LUT), which was in great harmony with other previous studies [53, 62]. Moreover, Ginger was found to down-regulate and inhibit genes responsible for proliferation and angiogenesis, as VEGF and IL-8. Our results also support these results, as VEGF expression was decreased after treatment with Ginger and was even more efficacious, suggesting that they could be used to prevent or treat cancer with low toxicity and few side effects [63, 64]

The histopathological examination of the liver emphasizes our finding about ginger and LUT treatment against EAC, as the hepatic lesion is minimized among the treated groups (EAC+G), (EAC+LUT) and (EAC+G+LUT).

5.CONCLUSION

Our results prove that Ginger and LUT have a potent anti-cancer activity when used together and induce apoptosis by significantly decreasing Ki-67 and Bcl-2 expression and significantly activating caspase-3, which causes inhibiting proliferation and cell cycle arrest.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

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