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How to Return the Death Programs of Cancer Cells to Work again and Cure Cancer within a Short Time?

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Abstract

Cancer is cell fleeing from death by blocking the intrinsic and extrinsic pathways of cell death programs. In the present work, the experimental formula was designed to remove these blockers. It was applied on 120 Swiss albino mice which were inoculated intraperitoneally and subcutaneously with Ehrlich Ascites Carcinoma cells; $1 \times (10^6)$ cell/mouse. The activity of the cell death programs of the tumor was detected by measuring the volume of Ascites fluid, counting the number of dead cancer cells, measuring the size of the tumor, detecting the positive reaction of caspase enzyme in cancer cells and presence of macrophages and apoptotic bodies in tumor tissue. The experimental formula succeeded in removing the blockers of the cell death program in cancer cells returning the cell death program to work again.

Keywords

Cancer, Therapeutic Strategy, Accumulation, Detoxification Enzymes, Free Radicals, Antioxidants, H_2O_2, Glutathione, NF-kB, Sulforaphane, Flavonoid, Cur Cumin

1. Introduction

Cancer is a serious problem; it is more dangerous than nuclear bombs [1]. The

World Health Organization in 2015 reported that 8.8 million people around the world died from cancer. In 2017, more than 14 million new cases were reported globally and may rise to over 21 million cases by 2030 [2] [3]. In (2018), 9.6 million deaths were recorded globally [4]. There are many types of cancer treatments like surgery, radiation, monoclonal therapy, adoptive cell transfer, target therapy, an angiogenesis inhibitor, hormone therapy, stem cell transplant and gold nanoparticles [5]. But for all types of treatment out there, they all have serious side effects and they are unable to save all cancer patients. Cancer is difficult to treat.

One of the protective mechanisms in a normal cell is its ability to flee from death; whenever there is damage to its DNA, to save its life [6]. During carcinogenesis, the cell acquires the ability to "shut down" apoptosis.

For the normal cell to become cancerous, it passes through four stages of transformation: accumulation stage, detoxification stage, free radical stage; especially superoxide free radicals and hydrogen peroxide production and nuclear factor kappa-b (NF-kB) activation stage [7]. The NF-kB is responsible for the production of six elements (muc-1, muc-4, muc-16, Bcl2, MMPs, and DcR3), which are used by cells to block the intrinsic and extrinsic programs of cell death, leading to carcinogenesis [8].

Therefore, if such blockers for cell death were removed, apoptosis pathways would be activated and cancer cells would succumb to death. To remove such blockers, we must stop the four stages of transformation until the activation of the NF-Kb stage to prevent the shutting down of the intrinsic and extrinsic programs of cell death. Shutting down one stage completely will be sufficient to stop the malignant transformation while shutting down all stages is the most comprehensive therapeutic strategy for treatment success [7].

We adopted the following strategy to remove all blockers of death programs (intrinsic and extrinsic): removing the carcinogenic substances out of the cell, regulating the levels of detoxification phase-I enzymes, scavenging the free radicals especially oxygenate origin, as superoxide free radicals and hydrogen peroxide, and keeping the NF-kB in inactive forms. The present study aimed to stimulate the death programs of cancer cells to work again and cure cancer within a short period using plants extract.

2. Materials & Methods

2.1. Experimental Formula

Broccoli extract 3.4 mg/mouse, Apple extract 14 mg/mouse, Grape fruit extract 6 mg/mouse, blue berry 4 mg/mouse, Turmeric extract 2 mg/mouse, Vitamin A 3 mg/mouse, Vitamin C 3 mg/mouse, Vitamin-E 3 mg/mouse, Glutathione 0.4 mg/mouse, cysteine 0.4 mg/mouse, N-acetyl-L-cysteine 0.8 mg/mouse, selenium 0.07 mg/mouse and aspirin 0.6 mg/mouse. All plant extracts were moisture free

All ingredients of plants were extracted by ethyl alcohol 99% for one week then alcohol was evaporated. Whole plant extracts were dissolved by Propylene

Glycol then added the water, propylene did not exceed than 5%.

2.2. Cancer Cells

- Ehrlich Ascites Carcinoma (EAC) bearing mice were obtained from the National Cancer Institute, Cairo, Egypt. Ehrlich Ascites Carcinoma cells were maintained in mice through serial intra peritoneal transplantation of 1×10^6 viable tumor cells in 0.2 ml saline.

2.3. Animals

- 120 females' healthy Swiss albino mice (±18 g) were obtained from the Laboratory Animal House, National Cancer Institute, Cairo, Egypt.
- 120 healthy live African catfish (200 - 250 g) were obtained from the local market, Ismailia, Egypt.

2.4. *In Vivo* Study (Experimental Design)

1) Mice

120 females Swiss albino mice were maintained for one week acclimation period and were kept on commercial standard diet and tap water until the end of the experiment. Mice were divided into six groups as following.

Group-1 (negative control): 20 mice were injected I.P with saline 0.9 NaCl.

Group-2 (treated only): 20 mice were given the experimental formula in drinking water 3 ml/mouse daily.

Group-3 (positive control): 20 mice were injected I.P with Ehrlich Ascites Carcinoma cells 1×10^6 cell/mouse.

Group–4 (positive treated): 20 mice were injected I.P with Ehrlich Ascites Carcinoma cells 1×10^6 cell/mouse and were treated orally by the experimental formula (3 ml/mouse daily) in drinking water.

Group-5 (positive control): 20 mice were injected sub-cut with Ehrlich Ascites Carcinoma cells 1×10^6 cell/mouse.

Group-6 (positive treated): 20 mice were injected sub-cut with Ehrlich Ascites Carcinoma cells 1×10^6 cell/mouse and were treated by the experimental formula (3 ml/mouse daily) orally in drinking water.

2) Fish

120 female and male catfish (*Clariasgariepinus*) 25 cm length and 200 - 250 g) were maintained for one week acclimation period on commercial standard diet. Fish were housed in fully prepared aquaria and will be divided into six groups as following:

Group-1 (negative control): 20 fish were injected I.P with saline 0.9 NaCl.

Group-2 (treated only): 20 fish were given experimental formula (10× mouse dosedaily) in 7.5 grams food/fish daily.

Group-3 (positive control): 20 fish were injected I.P with Ehrlich Ascites Carcinoma cells 1×10^6 cell/fish.

Group-4 (positive treated): 20 fish were injected I.P with Ehrlich Ascites

Carcinoma cells 1×10^6 cell/fish and were treated (10× mouse dose daily) in 7.5 grams foods /fish

Group-5 (positive control): 20 fish were injected sub-cut with Ehrlich Ascites Carcinoma cells 1×10^6 cell/fish.

Group-6 (positive treated): 20 fish were injected sub-cut with Ehrlich Ascites Carcinoma cells 1×10^6 cell/mouse and were treated (10× mouse dose daily) in 7.5 grams food/fish.

All procedures relating to care and maintenance of the animals and fish were in accordance with the international guiding principles for the care and the use of laboratory animals. The experimental design was approved by the Scientific Ethical Committee, Faculty of Veterinary Medicine, Suez Canal University (approval no. 2019029).

2.5. Sampling

The samples were collected from all mice groups at the 8th day after inoculation then, weekly.

2.5.1. Collection of Ehrlich Ascites Carcinoma Fluid
The ascetic fluid in peritoneal cavity of tumor injected mice was collected by puncture for Measuring the volume of the fluid, Counting the number of tumor cells, detecting apoptotic bodies and immunohistochemical detection of Caspase-3 enzyme in cell blocks; which is a marker of cell death programs.

2.5.2. Tumor Parameters Evaluation
- Tumor volume: tumor volume of ascetic fluid from each mouse was measured using a graduated cylinder.
- Tumor cell count: the ascetic fluid was taken in a WBC pipette and diluted to 100 times. Ten microliters of ascetic fluid were diluted with 0.9% saline up to 1000 µL (1:100), mixed well, then a drop of the diluted cell suspension was placed on the Neubauer's counting chamber and the numbers of cells in the 64 small squares will be counted. The cells were stained with trypan blue (0.4% in normal saline) dye. The cells that did not take up the dye were considered viable, and those that took the dye were considered nonviable.

No. of cells per ml = the average cell count per square × dilution factor × 10^4.

2.5.3. Collection of Blood Samples
Three blood samples were collected by puncture of the cardiac mice under light anesthesia.

1) First sample was freshly collected for measuring pH by pH meter.

2) The Second sample was collected in EDTA tube for determination the effects of formula on the immune system by counting RBCs and total WBCs according.

To a previous described method [9] differential leukocytes count were carried out from giemsa stained blood smears; as previously described [10], also hemoglobin content was measured; as previously described [11].

3) The third sample was left for clotting and centrifuged at 3000 round/minute

for 10 minutes for serum separation for biochemical analysis for determination liver function, by measuring the level of aspartate transaminase (AST) and alanine transaminase (ALT) and kidney function, by measuring Urea and Creatinine serum levels.

A) Determination of liver function tests

Aspartate transaminase (AST) and alanine transaminase (ALT) activities were determined by the colorimetric method using ready-made kits produced by Diamond; as previous described [12].

B) Determination of kidney function tests:

1) Determination of urea:

Urea was determined in serum samples using ready-made kits produced by Diamond; as previous described [13].

2) Determination of creatinine:

Creatinine was determined in serum samples using ready-made kits produced by Diamond; as previous described [14].

2.6. Histopathological Examination

Specimens of the heart, liver and kidney, spleen and intestine were freshly taken for histopathological examination. They were fixed in 10% formalin, then washed in running tap water, then dehydrated and cleared in different concentration gradients of alcohol and, with xylene, then embedded in paraffin wax and sliced into thin sections of 5 microns thickness. Sections were stained with H&E stain then were examined microscopically [15].

2.6.1. Immunohistochemical (IHC) Study

Immunohistochemical (IHC) staining was carried out with the streptavidin-biotin method; as previously described by[16]. Primary antibody against casapse 3 (Genemed-biotechnologies, USA, Lot-61125818) was used. This was followed by 60 min incubations with secondary antibodies (Envision + System-HRP Labelled Polymer, Dako, Glostrup, Denmark) and visualization with the Liquid DAB + Substrate Chromogen System (Dako). All slides are lightly counterstained with hematoxylin for 30 s prior to dehydration and mounting. All slides were examined by the researcher and another pathologist. Casapse 3 was considered positive when cells displayed cytoplasmic staining. The Percentage of positive cells was recorded.

2.6.2. Immunohistochemical Scoring

Immunohistochemistry scoring (Histoscore) was performed using a modified scoring method as previously reported [17] Briefly, total cells and cells with positive staining were counted and the percent positive cells in each high power field was calculated. Fields were then assigned a relative staining intensity score, in three different high power fields (400×) for each tumor section: 0 = negative, 1 = weaks, 2 = moderate, 3 = strong. The product of the percent positive cells and staining intensity was then derived to create a histoscore of 0 - 300 for each

high power field: [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)].

2.7. Statistical Analysis

Hematological, liver and kidney parameters were examined using one-way ANOVA followed by Fisher's post hoc test to detect significant differences at a probability level of 5% ($p \leq 0.05$). Other data were analyzed using Students "t" test. All data were expressed as the mean ± SE.

3. Results & Discussion

One of the important features in the process of carcinogenesis is the ability of cancer cell to flee from death by blocking the intrinsic and extrinsic programs of cell death [6]. The core of this paper is reactivating the death programs in cancer cells by inhibiting the activation of NF-kB, which is responsible for producing the six factors used by cell to shut down the intrinsic and extrinsic pathways of programs of cell death.

The strategy of our study was designed to shut down all transformation stages, by preventing new hydrogen peroxide formation and converting the present into water, and inhibiting kinase enzyme to completely inhibit the NF-kB activation.

3.1. Strategy of Experimental Formula

3.1.1. Removing the Carcinogenic Substances out of Cancer Cells

The Cell membrane consists of two layers of phospholipid; each layer is composed of lipids and phosphate groups. The Lipid layer is hydrophobic but phosphate groups are hydrophilic. As the phosphate groups are facing to cytoplasm, so the inner surface of the cell membrane is hydrophilic. The movements of the molecules in or out of the cells are controlled by the hydro-property of those molecules [17].

Carcinogenic molecule is hydrophobic, so that it can easily move into cell but so difficult to move out, due to the hydrophilic nature of the inner surface of cell membrane [18].

The hydrophobic molecules expel away from phosphate groups from all directions (the power of dissonance is equal from all directions). So, the hydrophobic molecules are kept away from inner surface of cell membrane and remain in the middle of cells, and thus remain for long time resulting in accumulation. The cell gets rid of these molecules by detoxification enzymes.

Phase-II detoxification enzymes (glutathione-S transferase, UDP Glucuronlytransferases, Sulfotransferases, Methytransferases, Arylamine N-acetyltransferases, and Amino acid conjugating) conjugate certain groups to hydrophobic molecules to increase their solubility, leading to move it from middle of the cell to the cell pole. Each hydrophobic molecule needs certain detoxification enzyme to donate it to a specific group to increase its solubility.

Cancer cell may contain several types of hydrophobic molecules, so in order to move them to the pole of cell, it needs several types of detoxification enzymes.

Thus we must activate all phase-II detoxification enzymes to move all types of carcinogenic molecules from the middle of cell to peripheral of the cell near the phosphate groups of the inner surface of the cell membrane, to prepare them for plumbing out of cell. The productions of phase-II detoxification enzymes are controlled by a protein called nuclear factor erythroid-derived 2 (Nr-F2) [19] [20]. Sulforaphane which presents in Broccoli improves the expression of the Nr-F2, which stimulates the production of the phase-II detoxification enzymes [21].

Phase-III detoxification enzymes are necessary to excrete the newly formed phase-II products out of the cell. They belong to a family of proteins called the ATP-binding cassette (ABC) transporters [22]. The molecules require chemical energy, in the form ATP, to actively pump the carcinogenic molecules through the cell membrane and out of the cell [23].

Polyphenols which present in Apples stimulate phase-III detoxification enzymes which pump the carcinogenic molecules out of the cell [24].

3.1.2. Regulating the Detoxification Phase-I Enzymes

Phase-I enzymes begin the detoxification process by chemically transforming lipid soluble compounds to water soluble compounds in preparation for phase-II. The accumulation of harmful substances leads to high levels of phase-I detoxification enzymes to metabolize them. Each molecule of the harmful substances needs one molecule of phase-I detoxification enzyme to metabolize it. The result of this process is the generation of one molecule of free radical [25]. Chemical analysis of cancer cells showed elevated levels of phase-I detoxification enzymes, as Cytochrome P450 (CYP) 1B1. It performs the bulk of phase-I reaction and serves as a source of superoxide anion and hydrogen peroxide [26] [27]. Grapefruit contains naringenin (the principal flavonoid in grape fruit), hesperetin and eriodictyol which reduce the cytochrome P450, where it represents the bulk of oxidation processing [28].

Xanthine dehydrogenase/Xanthine oxidase which is the major cytoplasmic source of superoxide radicals and hydrogen peroxide is also present in a high level in tumor cells compared to normal cell [29] [30]. Red grape & cherries, containing high flavonoid (anthocyanidins and cyanidin). Also, black grapes and blackberries are high in flavonoids epicatechin and catechin, all inhibit Xanthine enzyme and amino mono-oxidase enzymes [31].

In addition, the metabolism of polyamines by monoamine oxidase enzymes (MAO-A & MAO-B) which are found on the outer membrane of cells mitochondria-generates locally high concentrations of hydrogen peroxide [32] [33] [34]. Turmeric contains the curcumin which has a potent activating E-cadherin expression and prevents the activity of the nuclear factor Kappa B and inhibits both MAO-A and MAO-B and increases the level of Serotonin and Dopamine [35]. Morefore, Cur cumin possesses activity against cancer by activating apoptotic pathways in cancer cell and inhibiting pro-cancer processes including inflammation [36].

As a significant side effect of the phase-I detoxification process is production

of free radicals and hydrogen peroxide, for each molecule of toxin or carcinogenic substances metabolize by phase-I detoxification enzymes, one free radical molecule is generated. So, phase-I detoxification enzymes are the major source of free radicals generation [25].

3.1.3. Scavenging Free Radicals Especially Oxygenate Origin

The free radicals are molecules, ions or atoms with unpaired electrons in their outer shell of electrons. They are highly reactive due to the presence of unpaired electron(s). Free radicals formation occur continuously in the cells as a consequence of both enzymatic and non-enzymatic processes [37]. Accumulation of harmful substances in cell results in activating the detoxification phase I enzymes, resulting in excessive generation of free radicals as superoxide anion and hydrogen peroxide. Ionizing radiation also in the presence of O_2, converts superoxide, hydroxyl radical and organic radicals to hydrogen peroxide [38].

Free radicals are controlled by the antioxidant system, both enzymatic and non-enzymatic; the latter couldn't be formed by the body and are obtained from foods, such as vitamin C, A and E [39].

Vitamin–C (ascorbic acid) inhibits protease enzyme allowing collagen fibrils to be restored and accumulate in extracellular space within 11 days [40]. This leads to the proper positioning of death receptor to adhere to ligand, this facilitates the working the extrinsic program of cell death.

Beta carotene inhibits the oxidant-induced NF-kB activation and the production of interleukin IK-6 and tumor necrotic factor alpha [41] [42].

Vitamin–E reduces the accumulation of superoxide radical and decreases the generation of oxidative damage substances [43].

Enzymatic anti-oxidants include, sodium dimustase enzyme which converts superoxide anions to hydrogen peroxide and Catalase and Glutathione enzymes which convert hydrogen peroxide (activator of NF-kB) to water and oxygen and improve apoptosis [44] [45].

Other compounds that have antioxidant activity such as flavonoids and N-Acetyl-L-cysteine which increases the intracellular level of glutathione [46]. They act by donating the acetyl group to oxidized glutathione so that, it can be reduced form and works effectively. So, N-acetyl-L-cysteine serves as a co-substance to eliminate hydrogen peroxide and inhibit the activation of NF-kB [47] [48].

3.1.4. Keeping the NF-kB in Inactive Form

NF-kB is kept in inactive form by controlling both hydrogen peroxide and kinase enzyme. The excessive amount of hydrogen peroxide are produced directly by phase-I detoxification enzymes and indirectly, by sodium dimustase enzyme which converts the superoxide free radical to hydrogen peroxide.

In non-stimulated cells, both NF-kB and dynein light chain (LC8) bind with inhibitor kappa B (IKB-α) in the cytosol of the cell forming (NF-kB,-IKB-α, -LC8) complex.

LC8 inhibits the activation of NF-kB by interacting with IKB-α and thereby,

preventing its phosphorylation by kinase enzyme. When cells are exposed to H_2O_2, the LC8 forms a reversible intermolecular disulfide bond between the two CYs^2 residues, leading to a conformational change that results in dissociation of LC8 from this complex while IKB-α and NF-kB remain bound together [49]. This dissociation of LC8 from (NF-kB, IKB-α and LC8) complex allows kinase enzyme (IKKs) to phosphorylate the inhibitor kappa B (IKB-α). This phosphorylation results in dissociation of IKB-α from NF-kB. Finally, NF-kB becomes free and trans locates into the nucleus and stimulates the expression of several genes including the genes that play a direct role in the shutdown of the intrinsic and extrinsic programs of cell death as(Muc-1, Bcl-2, MMPs and Decoy-R3) and indirect role as TNF-α gene which stimulates the expression of (Muc-1, Muc-4 and Muc-16) [8]. Moreover, NF-kB responsible for the production of several cytokines which activate IKB kinase (IKKs), which phosphorylates IKB-α leading to the reactivation of NF-kB. Thus, establishing a positive auto regulation loop that can amplify the inflammatory response and increase the duration of inflammation [50] (**Figure 1**).

Therefore, H_2O_2 is the first step for activation of NF-kB and that the NF-kB needs in addition IKKs to be active. The activated NF-kB is responsible for the cell fleeing from death and its conversion to cancer cell [8].

Glutathione is the most important antioxidant for neutralization the free radicals which donates electron to H_2O_2 reduce it into $H_2O + O_2$ [46].

The ratio of the main active form of glutathione (the reduced glutathione) to depleted glutathione (Oxidized glutathione) within a cell is often used as a measure of cellular toxicity and carcinogens. Glutathione S-transferase (GST)
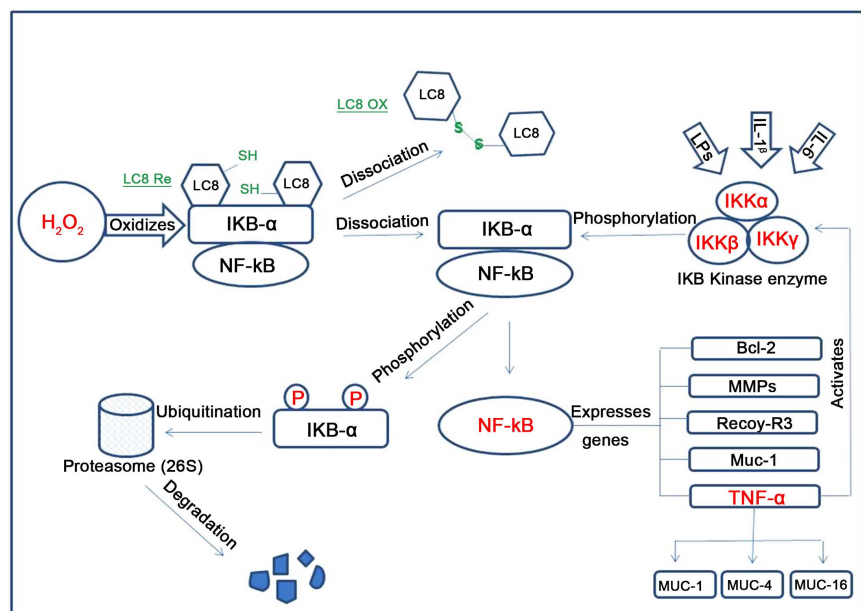

Figure 1. shows H_2O_2 oxidizes the LC8 leading in dissociate it from IKB-α, then IKKs phosphorylate the IKB-α, resulting in free NF-kB, which Trans locates into nucleus and stimulates the expression of genes which responsible for shutdown the pathway of the intrinsic and extrinsic programs of cell death. TNF-α reactive the NF-kB by stimulates lkks [8].

are family of enzymes that recycle oxidized glutathione. Conjugation of reduced glutathione to a wide range of substrates and their activity depends on adequate dietary sulfur-containing amino acids (methionine or cysteine), vitamin B6 for the conversion of methionine to cysteine, as well vitamins B2 and B3. Cysteine is the critical amino acid needed for synthesis of glutathione (GSH) [47].

Selenium is an essential trace element and co-factor for glutathione peroxidase.

Cur cumin improves the active form of glutathione (i.e., reduced glutathione), [46].

N-acetyl-L-cysteine increases the intracellular level of GSH by donating the acetyl group to oxidized glutathione, so that it can be transformed back to its reduced form and work effectively. So, N-acetyl-L-cysteine serves as a co-substance to elimination H_2O_2 and inhibits the activation of NF-kB [48].

Moreover, the anti-inflammatory effects of Aspirin and sodium salicylate results from their specific inhibition of ATP-binding to IKKβ and Thus, reducing IKKβ-dependent phosphorylation of IkB-α, preventing the activity of NF-kB pathway [51].

3.2. Sensitivity of Ehrlich Ascites Carcinoma Cells (EAC)

Cells were inoculated (I.P and sub-cut) into mice and fish. The results showed that there was no growth of EAC among the African cat fish. On the other hand, there was rapid growth of EAC among the mice which were inoculated (I.P and sub-cut).

3.3. Parameters of Death Programs Activity

3.3.1. The Volume of Ascites Fluid

At 8th day of inoculation the Ascites fluid volume of the controlled group of mice which were inoculation I.P with EAC cells was 1.63 ± 0.176 ml and continued increasing daily until reach to 5.87 ± 0.384 ml then all mice of this group dead at 21th day after inoculation. On the other side, the ascites fluid volume of treated I.P inoculation group was 1.6 ± 0.153 ml and continued decreasing gradually until reached zero ml at the 34th day of I.P inoculation of EAC (Table 1). This means

Table 1. Shows the effect of experimental formula on the ascetic fluid volume (mL) of the intraperitoneal implanted EAC (n = 3).

The collection day	Ascetic fluid volume (mL)	
	Positive control	Treated group
8th	1.63 ± 0.176	1.60 ± 0.153
14th	4.67 ± 0.393	1.37 ± 0.175**
18th	5.87 ± 0.384	1.03 ± 0.186**
21st	All dead	0.67 ± 0.120
24th	-	0.43 ± 0.088
34th	-	UD

Student's t-test was applied, where * = significantly different from control at $p \leq 0.05$ and ** = significantly different from control at $p \leq 0.01$. UD = undetected.

that the experimental formula succeeded to remove the blockers of death programs pathways of cancer cells and return the death programs to work again at 8th day of I.P inoculation of Ehrlich cells carcinoma and continued its work until disappearing cancer cells completely.

3.3.2. The Size of Solid Cancer

It was measured at 18th day in positive control sub-cut (EAC) cells inoculation group and treated sub-cut inoculated group. The result revealed the size of the solid cancer was 0.251 ± 0.033 cm^3 in positive control group, and gradually increases until reached to 2.630 ± 0.221 cm^3 at 34th day of sub cut EAC inoculation. While the size of solid cancer in treated group was 0.127 ± 0.017 cm^3 at 18th day of inoculation and gradually decrease until reached to zero at 34th day of sub-cut EAC cells inoculation (Table 2). This means that the death programs of cancer cells return to work again.

3.3.3. Counting the Number of Cancer Cells

The result revealed increasing the EAC cell number in the I.P inoculation mice group. The total number of cells in the positive control group was 217 ± 2.91 million/mL, the number of live cells was 197.33 ± 2.91 million/mL and the number of death cells was 20.33 ± 0.67 million/mL, while the total number of cells in treated group was 125.33 ± 4.67 million cells/mL, (the number of live cells was 83.67 ± 4.26 million cells/mL and the number of death cells was (41.63 ± 0.88). The death cells in treatment group represent 33.21% from all cells, (Table 3).

Table 2. Shows the effect of the experimental formula on the size (cm^3) of the subcutaneously implanted solid Ehrlich carcinoma (n = 3).

The collection day	Solid Ehrlich carcinoma size (cm^3)	
	Positive control	Treated group
18th	0.251 ± 0.033	0.127 ± 0.017*
27th	0.874 ± 0.051	0.007 ± 0.001**
34th	2.630 ± 0.221	UD

Student's t-test was applied, where * = significantly different from control at $p \leq 0.05$ and ** = significantly different from control at $p \leq 0.01$. UD = undetected.

Table 3. Shows suppressive effect of the experimental formula on the intraperitoneally implanted Ehrlich as cites carcinoma cell counts and viability at 18th days post inoculation (n = 3).

Groups	Carcinoma cell count (n × 10^6)		
	Total cells/mL	Live cells/mL	Dead cells/mL
Positive control group	217.67 ± 2.91	197.33 ± 2.91	20.33 ± 0.67
Treated group	125.33 ± 4.67**	83.67 ± 4.26**	41.63 ± 0.88**

Student's t-test was applied, where * = significantly different from control at $p \leq 0.05$ and ** = significantly different from control at $p \leq 0.01$. UD = undetected.

<u>Effect of the experimental formula on the cancer cells number</u>

Death cells number ÷ Total ascites cells (Death cells + live cells) × 100

41.63/125.33 = 33.2%.

3.4. Immunohistochemical Results

Sections examined from tumor samples showed positive reaction for caspase enzyme in tumor cells, starting from the base of tumor tissue, at the junction with the healthy tissue; it looks like an explosion of several water springs. This proves that the experimental formula succeeded in stimulating the death programs of cancer cells (**Figure 2**). Positive reaction to caspase enzyme in tumor tissue showed an extension of the reaction deep into cancerous tissue (**Figures 3-5**). Moreover, we observed the reduced density of the tumor cells near the areas of positive reaction to caspase enzyme, indicating the success of activation of the apoptotic program in eliminating tumor cells (**Figure 5**). Caspase 3 positivity was observed as moderate immunoreaction in about 15% of cells of the tumor. So a histoscore of 30% of the positivity of tumor cells is calculated.
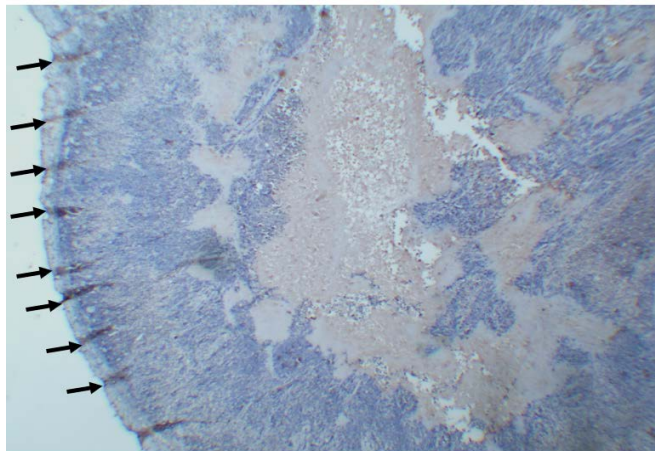
Figure 2. (IHC, ×4): A Positive reaction (black arrows) in tumor cells, beginning from base of the tumor, representing about 30% from the tissue.
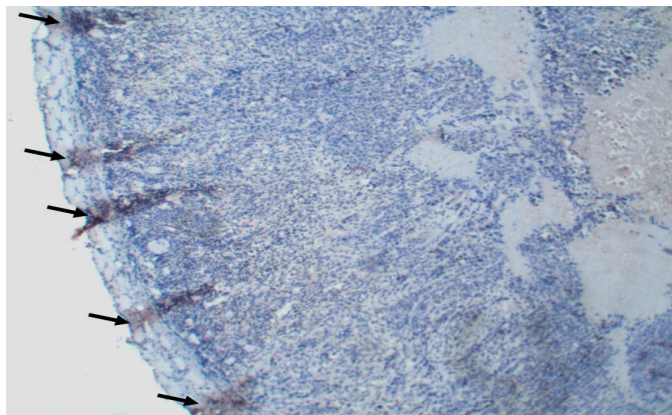

Figure 3. (IHC, ×10): A Positive reaction in tumor cells, beginning from the base of the tumor, representing about 30% from the issue.
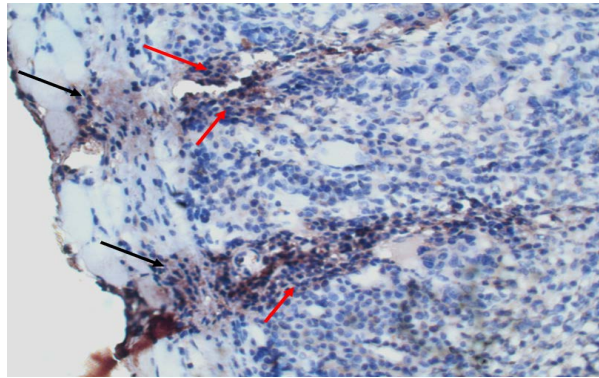
Figure 4. (IHC, ×40): Higher magnification of the previous figure showing positive cytoplasmic reaction, starting in base of tumor tissue, at its junction with normal tissue (black arrows) and extending into tumor cells (red arrows).
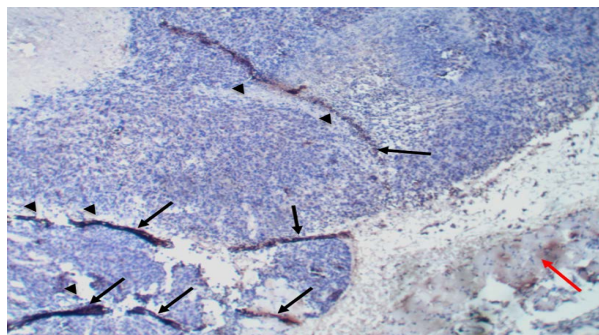

Figure 5. (IHC, ×10): Multiple areas in tumor show such positive reaction to caspase enzyme, beginning from tumor base at the junction with muscle (red arrow), and extending deep into tumor cells (black arrows). Such reactivity to caspase enzyme is seen within about 30% of the tumor tissue. In addition, there is a reduced density of the tumor cells near the areas of positive reaction to caspase enzyme (arrowheads).
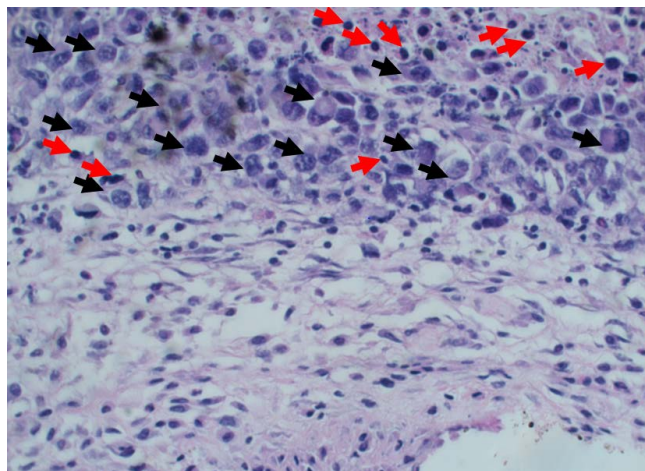

Figure 6. (H & E, ×40): There are numerous macrophages (black arrows) and apoptotic bodies (red arrows) at the base of the tumor.

In addition, histopathological examination revealed numerous macrophages and apoptotic bodies (red arrows) at the base of the tumor, indicating the suc-

cessful elimination of tumor cells (Figure 6).

The above observation of a decrease in volume of ascetic fluid, reduction in the size of tumor and the detection of caspase enzyme activity; as well as infiltration of tumor tissue by macrophages and presence of apoptotic bodies, all of these indicate the activity of death programs and prove that the death cell programs in cancer cells reworked again.

3.5. The Effects of the Experimental Formula

3.5.1. Hematological Study

Hematological studies revealed that the experimental formula regulated blood pH of cancer mice, increasing RBC count and Hb concentration. This means that the experimental formula has hematological improvement (Table 4).

The blood pH values revealed significant (P < 0.05) reduction in non-treated I.P inoculated group than negative control and other treated groups. Hemoglobin values were significantly reduced in non-treated I.P an S.C inoculated groups than the negative control and other groups. These results suggested the presence of anemia due to cancer inoculation [52]. The possible cause of such type of anemia is functional iron deficiency due to cancer progression [53]. Functional iron deficiency means a defect in iron handling instead of a lack of iron, where the total iron-binding capacity was reduced with increased iron storage [54]. The treatment of cancer inoculated mice with apple, blackberries, broccoli, grapefruit, and vitamin-C efficiently ameliorated cancer-induced anemia this could be attributed to [55] [56] [57] [58].

The total leukocytes count was significantly reduced in non-treated I.P an S.C inoculated groups when compared to the negative control. This decline in WBCs count could be illustrated by the progression of cancer that attracted leukocytes by the produced cytokines [59] thus reducing WBCs count in the circulation. These were confirmed by the presence of abundant leukocytes especially macrophages and lymphocytes around and within cancer cells, as observed in (figure [12]). Treated S.C and I.P inoculated mice revealed significant (P < 0.05)

Table 4. Shows the suppressive effect of the experimental formula on some hematological parameters at the 8th day post inoculation (n = 5).

Mice groups	pH	Hb g/dL	RBCs $\times 10^6/\mu l$	WBCs $\times 10^3/\mu l$	Platelet $\times 10^3/\mu l$
Negative control	7.36 ± 0.05^a	10.18 ± 0.63^{ab}	7.14 ± 0.48^b	11.18 ± 0.54^a	321.6 ± 21.17^c
Treated non-inoculated	7.32 ± 0.05^a	11.04 ± 0.68^a	8.88 ± 0.58^a	9.08 ± 0.77^b	310.0 ± 22.08^c
Non-treated I.P inoculated	7.20 ± 0.04^b	9.14 ± 0.55^b	4.82 ± 0.51^c	7.32 ± 0.72^{cd}	489.4 ± 28.64^a
Treated I.P inoculated	7.30 ± 0.03^a	10.90 ± 0.58^a	8.04 ± 0.66^{ab}	6.54 ± 0.63^d	387.2 ± 30.70^b
Non-treated S.C inoculated	7.35 ± 0.04^a	9.12 ± 0.51^b	5.22 ± 0.55^c	$8.32 \pm 0.61b^c$	518.8 ± 30.93^a
Treated S.C inoculated	7.34 ± 0.04^a	10.84 ± 0.57^a	7.44 ± 0.57^b	6.46 ± 0.56^d	390.8 ± 27.71^b

Data are expressed as means ± SEM, n = 5. Values having different alphabetic superscripts within the same column are significantly different at p ≤ 0.05.

reduction in WBCs count than cancer inoculated non treated group. This demonstrates the ability of glutathione in reducing cancer cells induced cytokines that capable of reducing WBCs chemotactic response to cancer area [60].

On the other side, the platelets count was significantly ($P < 0.05$) increased in S.C and I.P inoculated groups. The treatment of cancer inoculated groups with glutathione significantly ($P < 0.05$) reduced this elevation in platelet counts than inoculated non treated mice [60]. Present results are logic where previous literature confirmed the close association between cancer progression and platelets activation that was pivotal for cancer progression and activation [61] [62].

3.5.2. Biochemical Studies

Biochemical studies showed activation of the liver enzyme AST and mild activation of ALT enzyme. But the parameters of kidney were nearly at normal level see (Table 5).

The results revealed that the levels of Liver enzymes (AST and ALT) were significantly different; the level of these enzymes in the treated non-inoculation group was higher than control negative group. Also, non-treated I.P inoculated group was elevated while the level of these enzymes was decreased after treated by experimental formula but still higher than control and treated only group. But the highest levels of these enzymes were presence in sub-cut inoculation group.

The function of liver enzymes AST & ALT is responsible for transferring the amino group (NH2) between an amino acid and an α-ketoacid. They are important in the synthesis of amino acids, which form proteins [63]. The level of liver enzymes (AST & ALT) is higher in children than adults, also 25% higher than adult in strenuous exercise man [64] [65]. This means that the level of these enzymes elevate during building new cells as well as break down of cells. So we can explain the elevating of the level of enzymes in treated non-inoculated group is due to building immune cells especially lymphocytes and in non-treated inoculation I.P group due to build more cancer cells. Then the level of these enzymes decreases due to stop the cell division program and returning the death program of cancer cell to work. While the highest level in sub-cut inoculated group due to building blood vessel in tumor tissue in addition, building new Cancer cells.

Table 5. Shows the influence of experimental formula on some liver and kidney biomarkers in normal and in Ehrlich Ascites carcinoma-bearing mice.

Mice groups	AST (μ/ml)	ALT (μ/mL)	Urea (mg/dL)	Creatinine (mg/dL)
Negative control	142.2 ± 6.82[e]	64.8 ± 4.66[b]	36.1 ± 3.21[ab]	0.60 ± 0.07[b]
Treated non-inoculated	225.2 ± 11.57[d]	79.7 ± 7.66[b]	37.3 ± 3.12[ab]	0.72 ± 0.10[ab]
Non-treated I.P inoculated	272.6 ± 15.21[c]	87.8 ± 9.01[b]	34.6 ± 3.44[ab]	0.62 ± 0.09[ab]
Treated I.P inoculated	244.1 ± 15.49[cd]	77.7 ± 8.11[b]	41.2 ± 5.25[a]	0.82 ± 0.11[ab]
Non-treated S.C inoculated	958.4 ± 25.66[a]	260.2 ± 17.50[a]	32.1 ± 3.36[b]	0.76 ± 0.12[ab]
Treated S.C inoculated	770.0 ± 22.78[b]	249.2 ± 9.48[a]	40.3 ± 4.13[ab]	0.84 ± 0.13[a]

Data are expressed as means ± SEM, n = 5. Values having different alphabetic superscripts within the same column are significantly different at p ≤ 0.05.

On other sides the measuring of kidney function by measuring the level of urea and creatinine. The elevating levels means the deamination process of amino acids was increased as a product of cell break down, as evidence of the death program activity. The levels of urea and creatinine were elevated but nearly at normal level see (Table 5).

3.5.3. Histopathological Study

Histopathological examination of **the control group** revealed no significant pathological changes. **In treated only group,** examination of liver and renal tissue revealed normal tissue architecture, with minimal degenerative changes in hepatocytes and tubular epithelium of kidney. Examination of spleen and intestinal tissue revealed lymphocytic proliferation with hyperplastic lymphoid follicle in spleen and laminapropria and submucosa of intestine (Figures 7-9).

In the cancer group which received no treatment, there was the marked proliferation of cancer cells at the site of inoculation extending deeply between muscle bundles (Figure 10(a) and Figure 10(b)). Examination of the internal organs revealed an increase in degenerative changes in the hepatocyte (Figure 11).
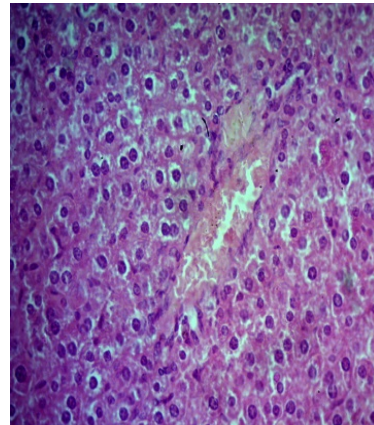

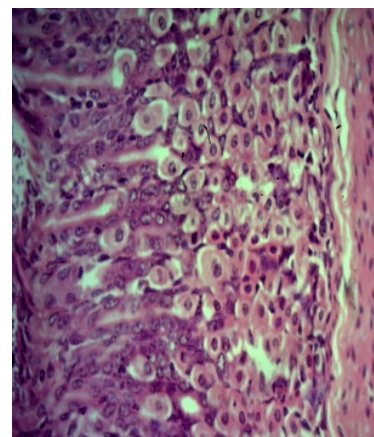Figure 7. (H & E, ×10): Mild hydropic degenerative changes in hepatocytes of the liver.

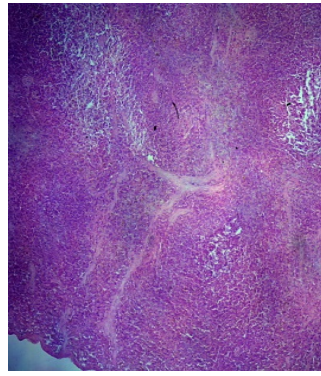Figure 8. (H & E, ×40): A proliferation of lymphocytes in lamina propria of intestine.

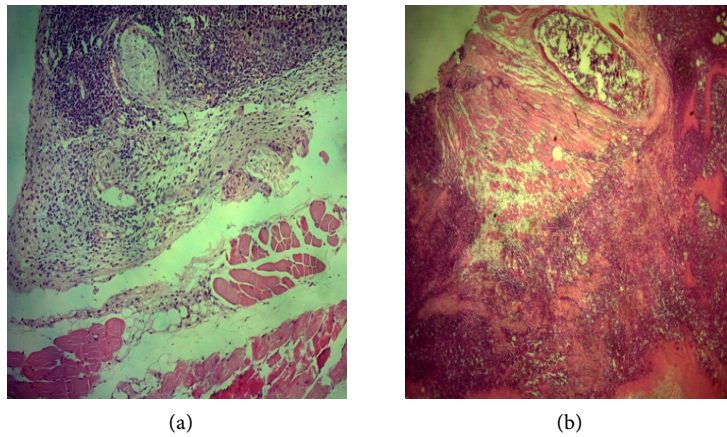**Figure 9.** (H & E, ×10): hyperplastic lymphoid follicles of spleen.

(a)   (b)

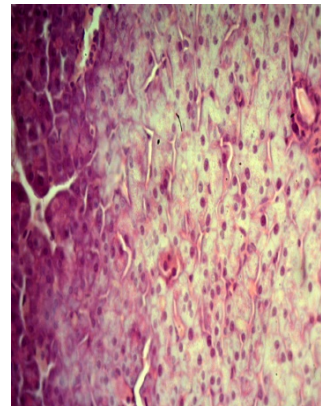**Figure 10.** (H & E, ×10): The tumor site injection showing proliferation of the cancer cells.

Figure 11. (H & E, ×40): degenerative changes in the hepatocytes.

In the cancer group which received treatment, there were numerous macrophages and apoptotic bodies at the base of the tumor, with its junction with normal tissue, indicating successful elimination of tumor cells (**Figure 6** and **Figure 12**). In addition, there was marked lymphocytic infiltration within tumor tissue. Liver cells exhibited congested sinusoids with hydropic degeneration of hepatocytes, while kidney cells show mild acute tubular injury.
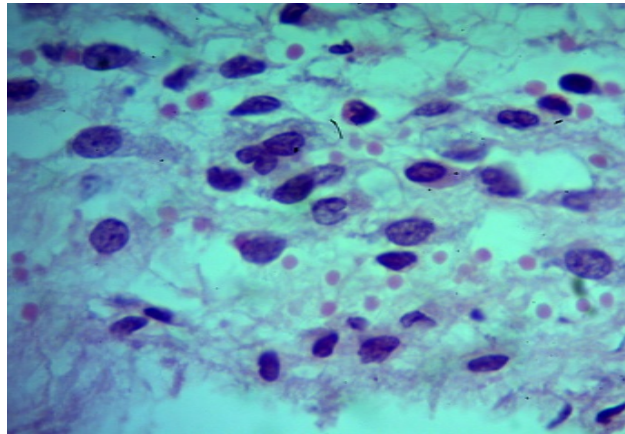
Figure 12. Tumor site showing macrophages and apoptotic bodies at the junction between tumor and healthy muscles.

3.5.4. Experimental Formula

Experimental formula succeeded in bringing the death program of cancer cells back to work again strongly leading to decrease in cancer tissue mass within 34 days. This was proved by immunohistochemical detection of caspase-3 enzymes, indicating the activation of apoptotic pathways in cancer cells. In addition, the histopathological study revealed that there no significant pathological changes in the internal organs, confirming the specificity of designed formula.

In accordance with that the hematological study revealed that the experimental formula improves the patient healthy by increasing RBC count and Hb concentration and restoring the pH of blood to normal. Moreover, the biochemical study revealed no toxic effect was observed during the use of formula.

The experimental formula contains natural plants, vitamins, glutathione, and aspirin. So, it could be given orally in a diet program or in a suspension form or in the form of capsules.

The experimental formula should be subjected to further intense research to determine the lowest effective dose, measuring LD50, as well as raising white blood cells also to modify it.

4. Conclusion

The positive reaction of caspase enzyme proved that the experimental formula succeeded in "switching on" back the death program of cancer cells and showed that such cells death stated at the base of cancer tissue. The formula was effective and led to a significant reduction in tumor mass within 34 days. It exerts its effect on cancer cells only, making it safe and with no observed side effect.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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