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Black Garlic Extract Polyphenols Constituents Adjuvant to low Doses of Gamma Irradiation Exhibit Oxidative Stress on Murine Tumor

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With precisely controlled degrees of moisture and heat, the color of raw garlic turns black. Black garlic has a strong role in preventing cancer. Also, low doses of γ-radiation exposure induce a positive impact on tumor regression and immune response. The present study evaluates the leverages of black garlic extract polyphenols (BGE) and/or low doses of γ-irradiation against Ehrlich (Eh) carcinoma-bearing mice. The in-vitro cytotoxic effect test showed that BGE (60 µg / ml) produced the death of 100% of Ehrlich Ascites Carcinoma (EAC) cells and 30% of cells appeared to burst. Subcutaneous transplantation of $2.5x10^6$ EAC cells in female mice produced a solid Eh carcinoma. Via gavages, 15 consecutive treatments of BGE (200mg/kg. b.w./day) and the subject of experimental animals to γ-radiation (0.25Gy x 2/ week) for two weeks from the 7^{th} day of tumor inoculation recording that BGE and/or γ-radiation reduced tumor size significantly and markedly amended most alterations in biochemical parameters associated with Eh carcinoma inoculation. The BGE gavages to mice bearing Eh carcinoma, exhibited an antitumor effect reflected by reducing tumor size, inhibiting systemic inflammation, and augmenting the antioxidant defense system in Eh carcinoma-bearing mice.

Keywords: Black garlic extract polyphenols, Ehrlich Carcinoma, low doses of γ-irradiation, cytotoxicity, systemic inflammation, antioxidant defense system.

Introduction

For a period of time at a high temperature under high humidity, fermentation of fresh garlic (*Allium sativum* L.) turns garlic cloves dark. Fermentation gives them a sweet taste, and alters their consistency to chewy and jelly-like (Yuan et al., 2016). The medical importance of garlic is known from ancient days in Egypt as traditional herbal medicine (Srivastava and Pathak, 2012). The most important active compounds are organosulfur compounds such as allicin, DADS, SAC, DAS, DATS, and alliin (Ushijima et al., 2018). Black garlic (black Allium sativum) has a lot of 2 medicinal actions including antitumor and, immune system enhancement (Wang et al., 2010).

The overproduction of reactive oxygen species (ROS) caused by radiation damage, disrupt lipid membranes leading to the formation of peroxide radicals (Rajapakse et al., 2007). Many reports showed that low-dose ionizing radiation from a natural source (Zdrojewicz and Belowska-Bien, 2004) or in professional radiation workers (Wall et al., 2006) may stimulate the immune system and potentiate its effector's function (Radiation Hormesis).

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The present study aims to evaluate the biological effects of black garlic extract polyphenols (BGE) adjuvant to low doses of γ-irradiation against Ehrlich (Eh) carcinoma.

Materials and Methods

Animals

In the present study, female mice (22-25g) were purchased from the breeding unit of the Egyptian Organization for Biological Products and Vaccines (Cairo). The animals were maintained on a commercial standard pellet diet and tap water *ad libitum*. According to the University Animal Ethical Committee, all procedures described were reviewed and approved. Whenever all the experimental procedures were carried out according to the principles and guidelines of the Ethics Committee of the National Research Centre, Cairo, Egypt.

Radiation facility

Whole-body γ- irradiation (0.25Gy×2/week for 2 weeks) of experimental animals was performed using a Canadian Cs^{137} Gamma Cell-40 at the National Center for Radiation Research and Technology (NCRRT), Cairo, Egypt.

Tumor Transplantation

In this study, we used a cell line of Ehrlich Ascites Carcinoma (EAC). Ehrlich Ascites Carcinoma was taken from the National Cancer Institute (NCI), Cairo University. Ehrlich Ascites Carcinoma (EAC) cells showed greater growth in female mice (Vincent and Nicholls, 1967). The tumor line was maintained in the female Swiss albino mice by weekly intraperitoneal injection of 2.5 $x10^6$ per mouse (El-Gawish, 2003). The solid form was done by inoculating 2.5×10^6 cells per mouse subcutaneously between the scapulae in the neck region. The solid form of the tumor was observed after 7 to 8 days from EAC cell inoculation.

Egyptian black garlic extract preparation (BGE)

Egyptian garlic was obtained from the local market in Egypt and used for black garlic processing. Briefly, the raw garlic bulb was incubated for 10 days at 65 to 70°C without peeling. One hundred grams of black garlic were sliced into small pieces, followed by grinding with a mortar to produce a smooth paste. Then, the garlic paste was dissolved in 100 ml of distilled water in a conical flask and filtered using a piece of gauze to obtain an aqueous solution of the extract. The filtrate was stored at 4°C till use.

Cytotoxicity of black garlic extract (BGE)

According to the method of El- Merzabani et al. (1979), the cytotoxicity effects of the black garlic extract on tumor cells were determined at concentrations of 0,10, 20, 30, 40, 60, 80 and 100 µg/ml. Under aseptic conditions using an ultraviolet laminar airflow system, the EACs of ascites fluid from the pre-inoculated mice were obtained by needle aspiration. To differentiate between dead and viable EAC cells, a trypan blue stain was used. Then the percentages of non-viable cells (NVC) were counted and calculated according to the following equations

$$\% \text{ NVC} = C/T \text{ X } 100$$

C is the total number of non-viable cells and T is the total number of viable cells.

Experimental design

All the experimental animals (50 mice) were allowed 7 days for adaptation. They were randomly distributed into 5 equal groups, 10 mice for each and were recognized as follows:

1. Group (1): Normal Control group (**N.C**): 10 mice in this group served as controls and were neither treated nor irradiated.

2. Group (2): Ehrlich Carcinoma-bearing group (Eh), 10 mice were subcutaneously injected with 0.2ml of 2.5×10^6 /ml/mouse viable EAC cells in the neck region.

3. Group (3): Ehrlich carcinoma BGE treated group (Eh+ BGE): 10 mice bearing EC were treated via gavage with 200mg/kg. b.w./day for two weeks. Black garlic extract-treated group beginning on the 7^{th} day after EAC inoculation.

4. Group (4): Ehrlich carcinoma irradiated group (Eh+IR): 10 mice bearing EC were subject to 0.25Gy x2/week for 2 weeks beginning on the 7^{th} day after EAC inoculation.

5. Group (5): Ehrlich carcinoma BGE - irradiated group (Eh+ BGE +IR): 10 mice bearing EC treated with black garlic extract and subjected to γ –radiation beginning on the 7^{th} day after EAC inoculation.

Monitoring of tumor size

To evaluate the effects of the black garlic extract on tumor growth monitoring was performed for each experimental group individually using a caliper. According to the following formula: Tumor size = length × width^2 × 0.52, tumor size was determined (Jia et al., 2005).

Samples collection

All animals in each group were sacrificed 24 hr after the last dose of black garlic extract treatment and 16-hr fasting. Samples of blood were collected and samples of tumor tissue were excised. Blood was centrifuged at 3000 rpm to obtain serum for biochemical analysis. Parts of the excised tumor tissues were used for the histopathological examination, while the other part was used for the biochemical analysis.

Biochemical assays

The activities of interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α), and Caspase-3 were assayed in serum by the standard sandwich enzyme-linked immune-sorbent (ELISA) assay technique using an ELISA kit (K0331186, KOMABIOTECH, Seoul, Korea) following the manufacturer's instructions.

In tumor tissue, lipid peroxides mainly malondialdehyde (MDA) were measured according to the reported methods of Yoshioka et al. (1979). Meanwhile, reduced glutathione (GSH) was determined according to the method of Beutler et al. (1963). On the other hand, superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were determined according to the method of Minami & Yoshikawa (1979) and Gross et al. (1967), respectively.

Histopathological Examination

Following mice sacrificing tumor tissues were rapidly dissected, excised, rinsed in saline solution and cut into suitable pieces, then fixed in neutral buffered formalin (10%) for 24 hours, following fixation, the specimens were dehydrated in an ascending series of alcohol, then tissue specimens were cleared in xylene and embedded in paraffin at 60°C. A section of 5 microns thickness was cut by a slidge microtome. The obtained tissue sections were collected on glass slides and stained with haematoxylin and an eosin stain for histopathological examination by the light microscope (Bancroft et al., 1996) for histopathological examination.

Total Phenolic Content (TPC)

By Folin- Ciocalteau assay (Sook et al., 2014) the phenol content of black garlic extract was estimated calorimetrically. 0.1 ml of the diluted extract was mixed with 0.5 ml of diluted 1:10 v/v Folin-Ciocalteau reagent and allowed to stand for 5 min. Then, at room temperature 0.4 ml, 7.5% sodium carbonate was added and allowed to stand for 2 hr in dark. The absorbance was recorded at 760 nm by a UV-Vis spectrophotometer (Shimadzu, UV mini-1240, Japan). TPC content was calculated and presented as milligrams equivalent of garlic acid per gram dry weight (mg GAE/ g DW).

Quantitative analysis of phenolic compounds by high-performance liquid chromatography (HPLC)

Polyphenol constituents of black garlic extract were determined by an HPLC-UV system with a reversed-phase column C18, Kromasil (4.6 mm x 250 mm i.d., 5 µm), Agilent 1260 series, USA and a UV detector set at 280 nm (Hewlett-Packard, Pale Alto, A). At 280 nm, polyphenols (Gallic acid, Chlorogenic acid, Catechin, Methyl gallate, Coffeic acid, Syringic acid, Pyro catechol, Rutin, Ellagic acid, Coumaric acid, Vanillin, Ferulic acid, Naringenin, Taxifolin, Cinnamic acid, and Kaempferol) were assessed quantitatively by authentic substances. Retention times and peak areas (%) were utilized to calculate the concentrations of polyphenolic compounds by the Hewlett-Packard data system.

Statistical Analysis

For statistical analysis of the results, the SPSS/PC program was used. By a one-way analysis of variance (ANOVA) followed by a Post Hoc LSD test, data was analyzed. The data were expressed as mean ± standard error. $P \leq 0.05$ was significant and $P \leq 0.01$ differences were considered highly significant.

Results

Chemosensitivity of black garlic extract on Ehrlich ascites carcinoma (EAC) cells

The tumoricidal effect of different concentrations of Black garlic extract on Ehrlich ascites carcinoma cells viability was evaluated in Table 1 and Fig. 1. The low concentration of 10 µg/ml of black garlic extract decreases the tumor cells viability by 10%.

The cytotoxicity of black garlic extract not only led to the death of Ehrlich ascites carcinoma (EAC) cells but also led to a burst of these dead cells at certain doses. Black garlic extract led to the rupture of the cells content after their death. The median lethal concentration of Black garlic extract was 30 µg/ml for EAC cells.

Table 1 and Fig. 1 show the cytotoxicity of Black garlic extract on EAC cells. A concentration of 30 µg / ml of Black garlic extract led to the death of 50% of EAC cells.

At a concentration of 60 µg / ml, black garlic extract led to the death of 100% of EAC cells and 30% of Ehrlich carcinoma cells appeared to be burst.

Role of BGE and low doses of γ- irradiation either alone or combined on tumor tissue of mice bearing Ehrlich Carcinoma (Eh)

1. Tumor (Eh) size monitoring

As shown in Fig. 2, it is clear that the inoculation of 2.5 million Ehrlich Ascites Carcinoma (EAC) cells in 2 ml physiological saline in the neck region of healthy normal female mice produced a tumor (Eh) with a mean size of 1136.4 mm^3 after 3 weeks of tumor inoculation (ATI).

Gavage of the experimental animals with black garlic extract (200 mg/kg b.wt./day) 7 days after Eh carcinoma inoculation caused a continuous delay of Eh size recorded 215.2 mm^3 on the 14^{th} day ATI and reaching 316.4 on the 21^{st} days ATI.

Exposure of the tumor-bearing animals to 0.25 Gy x2/ week for two weeks of γ-radiation begins on the 7^{th} and ends after the 21^{st} day of tumor inoculation. The tumor size exceeds 414 mm^3.

Treatments of tumor-bearing mice with BGE and γ-irradiation caused a pronounced delay in Eh progression. The tumor size was recorded at 227 mm^3 on the 21^{st} day of ATI.

2. Caspase-3 (Casp-3), and Anti-inflammatory markers

Table 2 revealed that the mean level values of the control group IL-6, TNF-α, and Casp-3 were 34.2±4.4b, 45.2±3.9, and 29.5±4, respectively. Meanwhile, in the Eh carcinoma group, they were 89.5±5.8,111.4±9.0 and 38.2±3.2 respectively recording a significant increase compared to the control group. Treatment of the experimental mice bearing Eh carcinoma with BGE produced a very highly significant decrease in the IL-6 and TNF-α levels, and a very highly significant increase in the Casp-3 level against the Eh carcinoma-bearing group. Whole-body γ-irradiation of experimental mice bearing Eh carcinoma revealed a highly significant increase in the Casp-3, and a significant decrease in the IL-6 and TNF-α levels compared to the Eh carcinoma-bearing group. Treatment of experimental mice bearing Eh carcinoma with BGE and exposed to 0.25 Gy twice a week x 2 weeks a very highly significant decrease in the IL-6 and TNF-α levels and a very highly significant increase in the Caspase-3 level compared to Eh carcinoma–bearing group observed.

3. Tumor TBARS levels and Antioxidant Status

The results presented in Table 3 showed that the mean value of tumor tissue MDA, GSH, SOD levels, and the GSH-Px activity were 5.7±0.6, 19.5±3.1, 34.2±4.8, 45.2±3.6, respectively. The results revealed that the treatment of EC-bearing mice with BGE and/or exposure to low doses of gamma radiation caused a significant increase in MDA levels. While the activity of tumor SOD, GSH and GSH-Px decreased upon treatment with BGE and/or exposure to the low doses of γ-radiation and showed a significant decrease in Eh+ BGE, Eh+IR, Eh+IR+ BGE groups when compared to the tumor group.

4. Ehrlich carcinoma tumor tissue

Under a light microscope, a histopathological examination of the Ehrlich carcinoma tumor showed compactness and aggregation of the tumor tissue cells spread subcutaneously within the soft tissues in the neck region. Groups of large, round and polygonal cells, with pleomorphic shapes, hyperchromatic nuclei, and binucleation were observed in the Ehrlich carcinoma tumor tissue section. Also, in Fig. 3 (A&B) several degrees of cellular and nuclear pleomorphism were noticed.

Black garlic extract (BGE) treatment of female mice bearing Eh carcinoma tumor recorded great destruction of tumor tissue [dead, remnants, and fat vacuolations (↑)] (Fig. 3 C&D). Exposure to fractionated low doses of γ-radiation of female mice bearing Ehrlich carcinoma showed the presence of necrotic cells and remnant tumor cells encircled by the fat soft tissue (↑) (Fig. 3E). Whenever combined treatment of female mice bearing Ehrlich carcinoma by BGE and low doses of γ-irradiation represented great destruction of tumor tissue and the presence of remnant tumor cells (→) (Fig. 3 F).

Chemical composition analysis of black garlic

The chemical composition analysis of black garlic is shown in Table 4. The highest contents were moisture and crude carbohydrates content (51.36 ± 3 %, 35.34 content (35.34 ± 2.3 %). Also, crude protein, crude ash, crude fiber, and crude oil were 7.5 ± 0.3, 5.89 ± 0.2, 0.8 ± 0.001 and 2.11 ± 0.11, respectively. Several scientists have investigated the gross chemical composition of black garlic. For instance, the moisture, ash, crude proteins, crude oil, and carbohydrates content of black garlic were 67.5 ± 0.12, 0.51 ± 0.01, 6.38 ±0.05, 0.41 ± 0.01, and 22.91 ±0.03, respectively. The data published in the literature is difficult to compare with the present work since there are many factors affecting, i.e., location, variety, climate, etc.

TABLE 1. The effect of Black garlic extract on the viability of EAC cells.

Black garlic extract			
Black garlic extract concentration (µg/mL)	**% of viable cells**	**% of dead cells**	**% of burst cells**
0	99	1	-
10	90	10	-
20	80	20	-
30	50	50	-
40	20	80	10
60	0	100	30
80	0	100	40
100	0	100	50

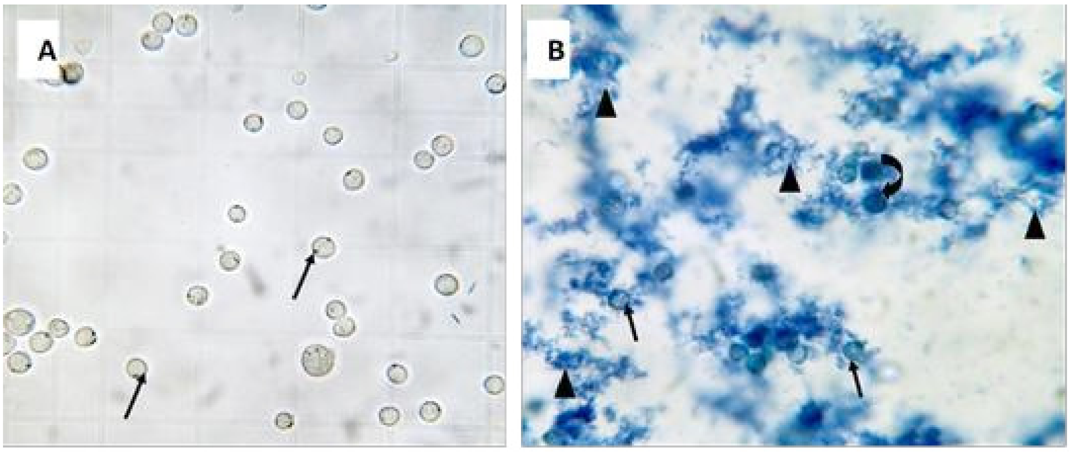

Fig. 1. Photomicrograph of Ehrlich carcinoma cells line representing 100 % of viable cells (↑) in A. The effect of BGE on the viability of Ehrlich ascites carcinoma cells. In B Photomicrograph of Ehrlich carcinoma cells line as affected by BGE.

TABLE 2. Effect of BGE and/or low doses of γ-irradiation on serum IL-6 (Pg/ml serum), TNF-a level(pg/ml), and Casp-3level(ng/ml) in different groups of mice bearing Eh carcinoma.

Groups \ parameters	IL-6	TNF-α	Caspase-3
Control	34.2±4.4 [b]	45.2±3.9 [b]	29.5±4 [b]
Eh	89.5±5.8 [a]	111.4±9.0 [a]	38.2±3.2 [a]
Eh+ BGE	46.2±6.8 [a b]	56±4.3 [a b]	58.4±5.5 [a b]
Eh+IR	54.2±5.6 [a]	94.2±3.2 [a b]	44.2±3.9 [a b]
Eh+IR+ BGE	68.4±6.6 [a b]	73.2±3.5 [ab]	73.2±5.4 [ab]

Each value represents the mean of 10 records ± SE.
a Significant difference versus the normal control group at $P< 0.05$.
b Significant differences versus Ehrlich carcinoma (Eh) bearing animals group at $P< 0.05$.

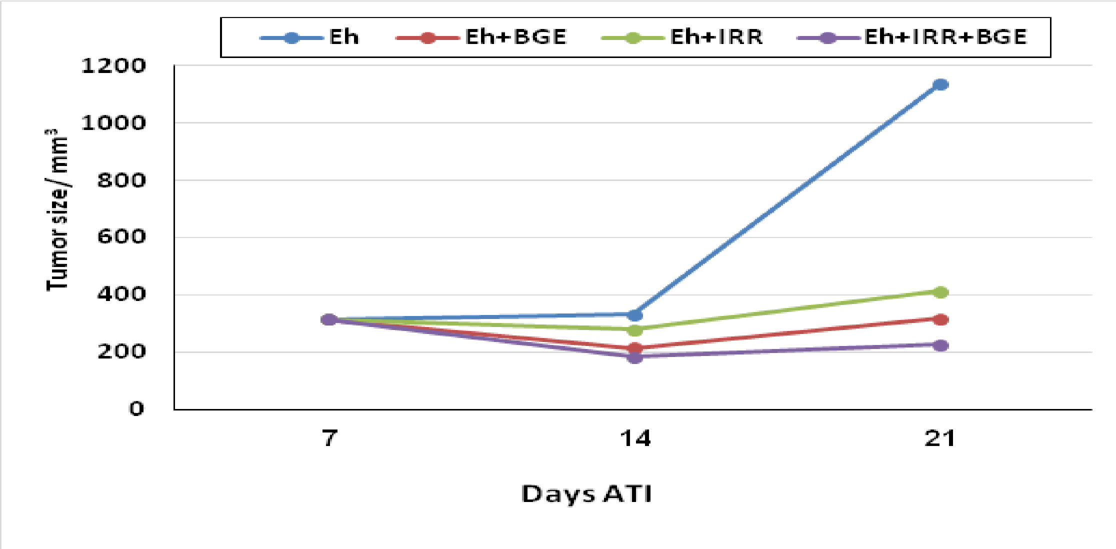

Fig. 2. Effect of BGE and/or low doses of γ-irradiation on tumor size of Eh.

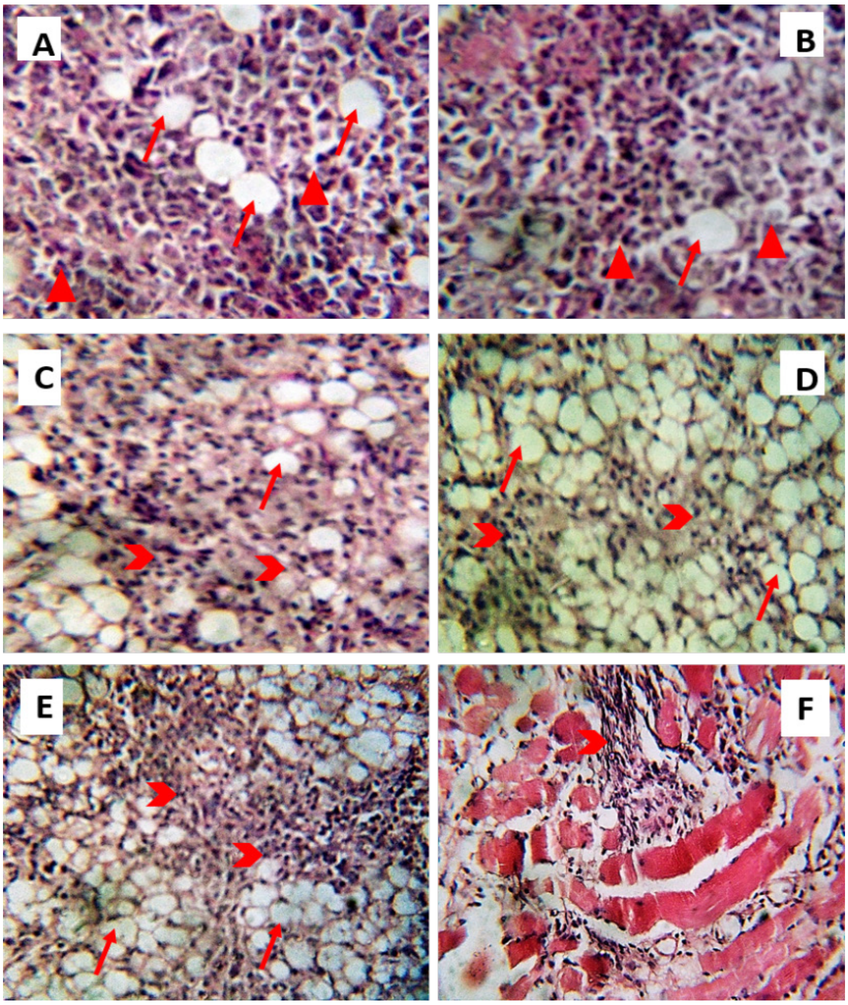

Fig. 3. Photomicrograph of Ehrlich carcinoma-bearing mice represents (A&B): control Ehrlich carcinoma in mice. (C&D): treated with BGE. E: exposed to low doses of γ- radiation. F: treated with BGE and exposed to low doses of γ- radiation. (H & E x 400).

TABLE 3. Effect of BGE and/or low doses of γ-irradiation on Tumor MDA levels and antioxidant status in different groups in mice bearing Eh carcinoma.

Groups \ Parameters	MDA mmol/gm	SOD µ/mg	GSH GSH/gm	GSH-PX gm GSH/min/gm
Eh	5.7±0.6	19.5±3.1	34.2±4.8	45.2±3.6
Eh+ BGE	11.4±0.7^{b}	12.3±1.7^{b}	15.6±2.7^{b}	20.6±4.8^{b}
Eh+IR	23±0.8^{b}	9.7±0.97^{b}	25.2±0.8^{b}	28.4±0.5^{b}
Eh+IR+ BGE	15.3±0.7^{b}	14.6±1.7^{b}	11.3±2.8^{b}	34.2±3.5^{b}

Legends are as in Table 2

TABLE 4. Gross chemical composition (%) of black garlic " Allium sativum".

Components	**g/100 g DW**
Moisture	51.36 ± 3
Total carbohydrates	35.34 ± 2.3
crude proteins	7.5 ± 0.3
crude oils	0.8 ± 0.001
crude fiber	2.11 ± 0.11
Ash	5.89 ± 0.2

All values were mean ± standard division.

Quantitative analysis of phenolic compounds in black garlic by high-performance liquid chromatography (HPLC) (Fig. 4 and 5)

Table 5 showed the characterization and quantification of phenolic components of black garlic extract by HPLC. The highest concentrations of phenolic compounds were gallic acid (60.64 µg/ g) and chlorogenic acid (20.8 µg/g). The concentrations of caffeic acid and naringenin were 1.16 and 3.34 µg/ g, respectively. the concentrations of Catechin, methyl gallate, syringic acid, pyrocatechol, ellagic acid, coumaric acid, vanillin, taxifolin, and cinnamic acids were less than 1 µg/ g. these results demonstrated that there were great differences between the concentrations of phenolic ingredients of black garlic extract. Najman et. al, (2020) and Najman et. al., (2021) reported that the fermentation process of garlic to produce black garlic has a significant effect on total polyphenols and phenolic acids which are separated by HPLC. These results may be due to Millard's reaction after processing raw garlic to produce black garlic. The higher content of phenolic bioactive ingredients influences their higher antioxidant potential.

TABLE 5. Quantitative analysis (ppm) of polyphenolic compounds of black garlic.

Phenolic compound	Conc. (µg/ml=µg/g)
Gallic acid	60.64
Chlorogenic acid	20.80
Catechin	0.44
Methyl gallate	0.20
Coffeic acid	1.16
Syringic acid	0.11
Pyro catechol	0.44
Rutin	0.00
Ellagic acid	0.19
Coumaric acid	0.44
Vanillin	0.57
Ferulic acid	0.00
Naringenin	3.34
Taxifolin	0.14
Cinnamic acid	0.07
Kaempferol	0.00

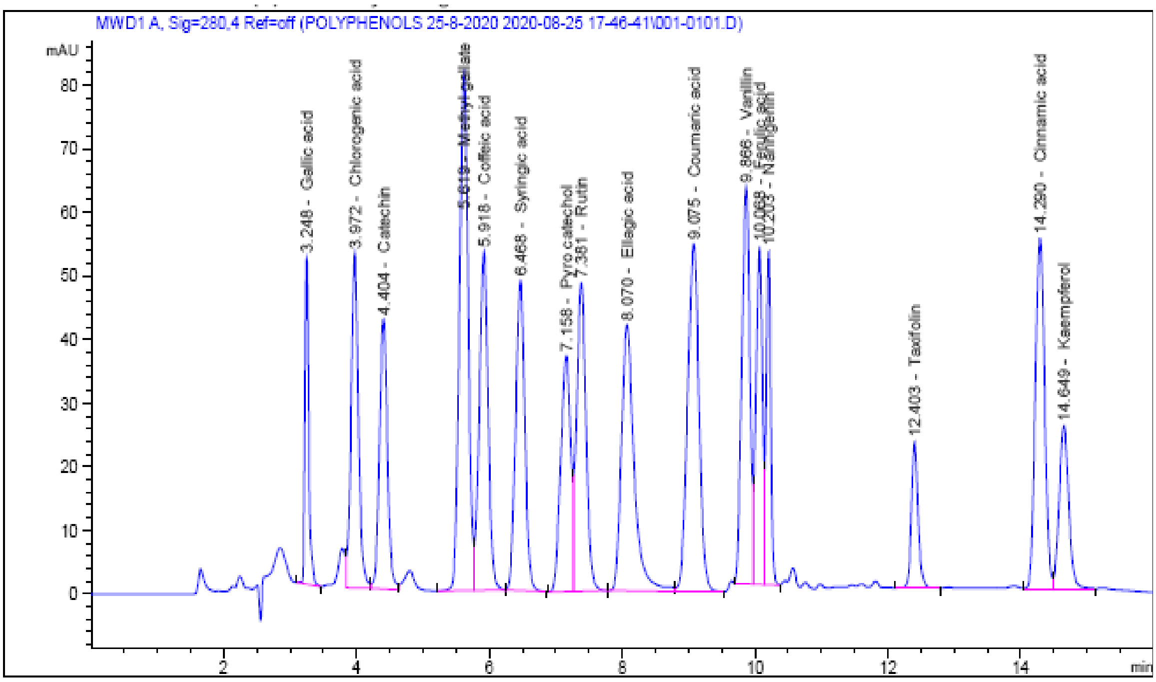

Fig. 4. HPLC- chromatogram of standard phenolic compounds.

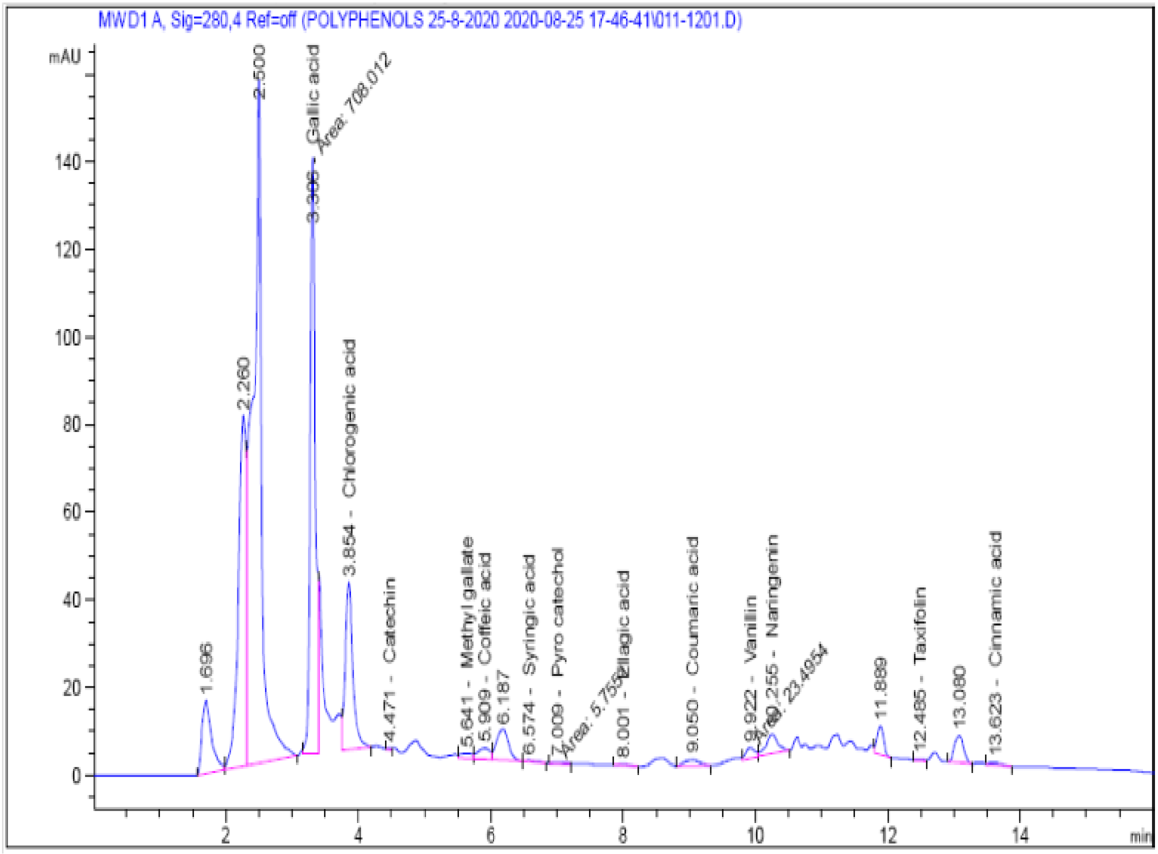

Fig. 5. HPLC -chromatogram of phenolic compounds in black garlic extract.

Discussion

The effects of chemotherapeutics on normal cells are described as the side effects of therapy. In addition, some chemotherapy drugs may have a direct effect on organ(s) in the body and cause toxicity over time. In parallel with the widespread cellular/tissue damage that results in site-specific side effects, the child undergoing chemotherapy may also experience generalized effects, such as fatigue, anorexia, taste changes, nausea and vomiting, and pain (Selwood, 2008). .

Despite advanced therapeutic strategies and approaches for cancer, it remains the most devastating disease that ultimately leads to death (de Mesquita et al., 2009). Garlic (Allium sativum) is rich in sulfur compounds which are responsible for garlic odor, and flavor and were used for medicinal purposes as an antihypertensive, antibiotic, and anticancer agent (Ross et al., 2006). Garlic also can be used as a preventive agent in some cancers such as gastric, pancreatic, intestinal, and breast cancer (González el al., 2006).

Numerous in vivo and in vitro studies demonstrated fermented garlic (Black garlic) extract (BGE) polyphenols possess strong antioxidant and anticancer properties and may inhibit the proliferation of a variety of tumor cell lines by altering the cell cycle and inducing apoptosis (Cha et al., 2012; Shirzad et al., 2011; Kaschula et al., 2012).

In the present study, the in-vitro effect of different concentrations of BGE on Ehrlich ascites carcinoma cells viability not only led to the death of Ehrlich ascites carcinoma (EAC) cells but also led to a burst of these dead cells at certain doses. black garlic extract led to the rupture of cells content after their death. At a concentration of 60 µg / ml, BGE led to the death of 100% of EAC cells and 30% of Ehrlich carcinoma cells appeared to be burst.

On the other hand, the in vivo study showed the gavages of the experimental animals with BGE (200 mg/kg b.wt./day) 7 days after Eh carcinoma inoculation caused a continuous delay of Eh carcinoma size and reached 316.4 on the 21st day ATI. Histopathologically, Eh carcinoma tumor tissue recorded great destruction represented by the appearance of dead, remnants, and fat vacuolations.

The cellular antioxidant activities of BGE polyphenolic compounds were consistent with the results of in vitro experimental antioxidant properties (Xiaoming et al., 2016). On the other hand, the anticarcinogenic benefits of garlic and associated allyl sulfur compounds are not limited to carcinogen formation and bioactivation, but also appear to be related to changes in the rate of cellular proliferation and apoptosis (Sundaram and Milner, 1996a; Sundaram and Milner, 1996b).

In several cancers, black garlic shows chemopreventive effects either in vitro or in vivo. In SGC-7901 human gastric cancer cells, it inhibits cell proliferation and induces apoptosis. Also, in Kunming mice inoculated with murine fore-gastric carcinoma cell lines, it inhibits the growth of inoculated tumors (Wang et al., 2012). Meanwhile, black garlic reduces cell motility, invasiveness, and activities of matrix metalloproteinase-2 (MMP-2) and MMP-9 in AGS human gastric cancer cells (Shin et al., 2010). The extract of black garlic induces caspase-dependent apoptosis through both intrinsic and extrinsic pathways in U937 leukemic cells ((Park and kim, 2010) and inhibits HT29 colon cancer cell growth via the phosphatidylinositol 3-kinase/ protein kinase B (PI3K/Akt) signaling pathway (Dong et al., 2014).

Treatment for malignant tumors characterized by uncontrolled growth and the capability of invading neighboring tissues and metastasizing through local Radiotherapy (Barcellos-Hoff et al., 2005).

Studies have considered the impacts of low-dose radiation, given as a single dose or fractionated or prolonged over time, with regard to altering cancer progression or resolution through their impacts on the systemic immune system (Nowosielska et al., 2011). It was found that low-dose radiation increases cellular antioxidant activity; facilitates DNA damage repair; reduces malignant transformation and mutagenesis, and stimulates immune surveillance (Rigaud and Moustacchi, 1996).

To several stressors, the cell nucleus and DNA are susceptible (Rittich et al., 2004; Kratochvílová et al., 2017; Freneau et al., 2018) and can be greatly harmed with comparatively low doses of ionizing radiation (Falk et al., 2010; Hausmann et al., 2017). As adverse effects of ionizing radiation on cancer cells are mainly mediated by the fragmentation of nuclear chromatin via inserting double-strand breaks (DSBs) into the DNA molecule (Schipler and Iliakis, 2013).

The effect of ROS production, as a result of tumor growth, on other organs in the body can be explained by the fact that ROS causes activation in nuclear factor-κB (NF-κB) and phosphorylation of its inhibitor (IκB). Thus, they enable NF-κB to translocate to the cell nucleus in which it binds to DNA and regulates the transcription of various target genes (i.e., inducible nitric oxide synthase, cyclooxygenase II, cytokines, etc.), which contribute to cell damage (Aschner et a., 2007). Interestingly, in tumor cells, cytokines activate NF-κB which protects the tumor cells from TNF-α induced apoptotic cell death (Pöppelmann et al., 2005). NF-κB activation in cancer cells regulates the transcription of genes involved in cell proliferation, anti-apoptosis, and invasion. Thus, activation of NF-κB induces tumor growth and metastasis and reduces cytokines-induced apoptosis (Takada et al., 2006).

The results obtained in the present study showed that the exposure of the tumor-bearing animals to the low dose of γ-radiation produced tumor size regression compared to tumor-bearing animals. Meanwhile, the data obtained also revealed that the administration of BGE to Eh carcinoma-bearing mice exposed to γ-radiation separately or in combination produced a significant reduction in tumor size represented by the appearance of dead, remnants and necrotic cells that pronounced a synergistic effect of such combined treatment.

Due to radiation oxidative stress induction which enhanced levels of peroxidative damage and DNA fragmentation the exposure to a low dose of γ-radiation caused a continuous delay of tumor size recorded in Eh carcinoma-bearing mice (Agrawal et al.,2001). The decrease in tumor size might result from the fact that Eh carcinoma is an undifferentiated malignancy and is sensitive to radiation exposure (Jagetia and Venkatesha, 2005). Much evidence recorded that low-dose radiation stimulates immune functions and can induce some degree of tumor regression and metastases suppression. Previous mechanistic studies have revealed that low-dose radiation enhances immune responses such as mitogenic response, IL-1 production, IL-2 response, and plaque-forming cell reaction (Kojima et al., 2004).

Inflammation; is part of the normal host response to infection and injury. To various inflammatory reactions several cytokines, such as TNF-a, IL-1b, and IL-6, are related. There is a considerable directory supporting the key role of proinflammatory cytokines, especially, TNF-α and IL-6 (Tilg, 2010). two pathways can be thought of as consisting of the connection between inflammation and cancer: An extrinsic mechanism and an intrinsic mechanism. An extrinsic mechanism where a constant inflammatory state contributes to increased cancer risk (such as inflammatory bowel disease). An intrinsic mechanism, where acquired genetic alterations (such as activation of oncogenes) trigger tumor development. Both precancerous inflammation and inflammation produced from genetic alteration can cause cell transformation and promote tumor progression. The cancer development process depends on the specific interactions between genetic/epigenetic factors and environmental factors (Shikhar et al., 2010). Many studies indicate a significant correlation between IL 6 overexpression and cancer development. It may be used as a tumor marker for cancer diagnosis (Vinocha et al., 2018). Tumor necrosis factor-alpha (TNF-α) is a 17 kilodalton cytokine that is synthesized by monocytes/macrophages, natural killer cells/ large granular lymphocytes, and T lymphocytes subsets (Goeddel et al., 1986) and implicated in the promotion or inhibition of tumor development. It is produced by tumor cells or inflammatory cells in the tumor microenvironment. The function of TNF-α in chronic inflammatory diseases and tumor-promoting effects is well known (Mocellin et al., 2005) and it is an essential factor implicated in the initiation, proliferation, angiogenesis, and metastasis of different types of cancers.

In the present study, female Swiss albino mice were accompanied by systemic inflammation as manifested biochemically by a high concentration of TNF-α serum level. The increase of proinflammatory cytokine in the present study might be attributed to the fact that Eh carcinoma can cause a series of deleterious side effects, including oxidative stress that may trigger lipid peroxidation which in turn initiates the release of malondialdehyde and combines with hepatocyte proteins starting a potentially dangerous immune response and excites neutrophil chemotaxis or stimulates transcriptional factor NF-κB which in turn intensifies the production of proinflammatory cytokines (Duvnjak et al., 2007)..

Previous research showed that black garlic had an antioxidant ability Imai et al., 1994; Lee et al., 2009). Dr. Yoon's group had been investigating the effect of different extraction methods of ABG in the TNF-α stimulated human umbilical vein endothelial cell (HUVEC) model. Extract of ABG was pretreated in TNF-a-stimulated HUVECs. This ABG extract inhibited reactive oxygen species formation and mRNA expression of vascular cell adhesion molecule-1 (VCAM-1) and reduced THP-1 monocyte adhesion to TNF-α-stimulated HUVECs. Extract of ABG also inhibited the activation of nuclear factor kappa B (NF-kB) transcription factor in TNF-α-stimulated HUVECs (Lee et al., 2011).

However, the exposure of female mice bearing EC to low doses of **γ-** radiation showed a significant decrease in TNF-α serum level compared to the Eh-bearing group for the enhancement of the immune response. Due to its cytotoxic effect on tumor cells, radiation exposure can supply a source of antigen that is suitable for cross-presentation by the host antigen-presenting cells (*i.e*., dendritic cell) which in turn can produce an antigen-specific immune response. Furthermore, other immuno-potentiating features of radiation therapy may be detected by its impact on the tumor microenvironment to increase cell trafficking to tumor sites, its impact on altering changing antigen presentation itself, and its direct impacts on the immune effector cells (Eriksson and Stigbrand, 2010; Verheij and Bartelink, 2000).

The present study shows that the treatment of the experimental mice-bearing Eh carcinoma with black garlic extract either alone or with γ-radiation exposure induced a very highly significant decrease in TNF- α serum level against the Eh carcinoma-bearing group. The promulgation of Jin-ichi et al. (2007) showed that black garlic has an anti-tumor potency. It's strengthened and its curative rate in the mouse model reached 50%. On the other hand, Min et al. (2014) suggest that aged garlic extract (AGE) is a more promising nutraceutical or medicinal agent to prevent or cure inflammation-related diseases for safety aspects. Also, Previous mechanistic studies have revealed that low-dose radiation enhances immune responses such as mitogenic response, IL-1 production, IL-2 response, and plaque-forming cell reaction (Kojimaet al., 2004).

Meanwhile, the present results revealed that the treatment of EC-bearing mice with BGE and/or exposure to low doses of gamma radiation caused a significant increase in MDA levels and a significant decrease in the activity of tumor SOD, GSH, and GSH-Px in Eh+ BGE, Eh+IR, Eh+IR+ BGE groups when compared to the Eh- carcinoma group.

Oxidative stress in tumor tissue manifested by a significant increase in LPO level concomitant with a significant decrease in GSH content and GSH-Px activity upon treatment with black garlic extract and/or γ-irradiation. These findings were in harmony with other studies which reported that EC induces excessive production of intracellular free radicals resulting in oxidative stress causing antioxidant disturbances, acceleration in lipid peroxide, and a decrease in GSH-Px activity (Sabiba et al., 2013; Wang et al., 2014).

Malondialdehyde, the end product of LPO, due to its high cytotoxicity and inhibitory action on defensive enzymes, is indicated to act as a tumor promoter and a cocarcinogenic agent (Wang et al., 2014; Kang, 2002). In the present work, increased levels of LPO level in tumor tissue might be attributed to the deficiency of antioxidant defense mechanisms or probably due to the generation of ROS and altered redox statuses which are common biochemical aspects in tumor cells. ROS can act on the polyunsaturated fatty acids of lipid membranes and lead to lipid peroxidation (Manju et al., 2002).

The present study shows that the treatment of the experimental mice-bearing EC with black garlic extract alone or with γ-radiation exposure induced a significant decrease in GSH content and GSH-Px activity and a significant increase in the LPO level in tumor tissue compared to Eh carcinoma-bearing group recording the antitumor activity of black garlic polyphenolic extract either alone or combined with low doses of γ-radiation exposure.

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