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INVESTIGATION OF THE TOXIC EFFECTS OF GALBANIC ACID WITH IONIZING RADIATION ON HUMAN COLON CANCER HT-29 CELLS

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Colorectal cancer is the third most common malignancy and the fourth leading cause of death globally. The primary treatments for this cancer are surgery and radiotherapy that is used to prevent recurrence of the disease. However, local invasions and metastases are leading causes of failure of the colon cancer treatment. This is related to a complex effect of radiation on the tumor cells and tissue and the activation of signaling pathways that lead to radioresistance. Therefore, it is possible to overcome radioresistance of the tumor by using factors that cause radiosensitivity and improve the output of radiotherapy. Today, herbal remedies are used to prevent and even treat cancer. One of these herbal remedies is galbanic acid (GBA). Due to the anti-tumor properties of GBA on various cancer cell lines, it is expected that GBA causes radiosensitivity in HT-29 cells. We have used the Alamar Blue test to evaluate the toxic effects of GBA on HT-29 cells. The cells were cultured in 96-well plates and treated with various concentrations (5, 10, 20, and 40 μg/mL GBA for 24, 48, and 72 h. In addition, the clonogenic assay was used to evaluate the effect of GBA on the radiation sensitivity of HT-29 cells. In this test, the cells were treated with only one drug concentration (10 μg/mL) at radiation doses of 2, 4, and 6 Gy. The cells were seeded in a 6-well culture plate, and after eight days, the colonies were formed. These colonies were then fixed with methanol and stained with Giemsa stain. Based on these findings, it is established that GBA inhibits the growth of HT-29 cells. Moreover, it was found that the survival rate of cells decreases with increasing concentration of medication. However, the effect did not depend on the treatment duration. The IC50 values for 24, 48, and 72 h exposures were 17.13, 23.58, and 19.49 μg/mL. According to the clonogenic assay results, there was no significant difference between the survival fraction of cells treated with radiation and combined therapy (radiation and medicine). Despite the observed toxicity of GBA to HT-29 cells, it did not influence the radiation sensitivity.

Keywords: colon cancer; HT-29; galbanic acid; radioresistance.

1. INTRODUCTION

Colorectal cancer is the third most common malignancy and the fourth leading cause of death globally. Moreover, its global rate is expected to increase by 60% by 2030, including 2.2 million new cases and 1.1 million deaths [1]. The Primary treatment for this cancer is surgery, and radiotherapy is used to prevent recurrence [2]. However, local invasions and metastases are the leading causes of the failure of colon cancer treatment [3]. This is due to the complex effects of radiation on tumor cells and tissue and the activation of signaling pathways that lead to radioresistance [4].

For example, protein kinase B (AKT) and extracellular-signal-regulated kinase (ERK) in the phosphoinositide 3-kinase (PI3K) and Ras-Raf-ERK pathways are central proteins in the epidermal growth factor (EGFR) signaling pathway. Moreover, AKT and ERK play critical roles in the cellular response to radiation or other therapeutic agents. The AKT is a serine/threonine kinase that plays an essential role in protecting cells from apoptotic death; therefore, it is a determinant of radioresistance and chemoresistance [5]. The ERK is found in the cytoplasm and nuclei and directly affects the phosphorylation of transcription factors associated with

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increased radioresistance [6]. Consequently, by using elements that cause radiosensitivity and can improve the radiotherapy output, it is possible to overcome radioresistance of the tumor cells.

Today, herbal remedies are widely used to prevent and even treat cancer due to their low toxicity and impact on many cancers. One of these herbal remedies is galbanic acid (GBA) which belongs to the category of sesquiterpene coumarin (Fig. 1) [7] and is isolated from the asafoetida plant (Umbelliferae family), especially *Ferula szowitsiana* and *Ferula asafoetida* [8]. GBA has antitumor effect on human umbilical vein endothelial cells (HUVECs) [9], ovarian cancer cells (OCAR-3) [10], and H460 non-small cell lung carcinoma (NSCLC) [11], inhibits the PI3K/AKT signaling pathway [9] and degrades EGFR protein [10]. Therefore, GBA is expected to cause radiosensitivity in HT-29 cells. In this regard, the present study was aimed to evaluate the radiosensitivity induced by GBA in HT-29 cells.

2. MATERIALS AND METHODS

2.1. Medication and Alamar Blue Test

Dr. Mehrdad Iranshahi provided the GBA (Product Brand: Golexir Pars; purity: greater than 92 percent) provided for this study from the Faculty of Pharmacy, Mashhad University of Medical Sciences, Mashhad, Iran). To prepare a stock solution with a concentration of 40 μg/mL, 2 mg of GBA was dissolved in 100 μL of dimethyl sulfoxide and this solution was stored at -20°C. In addition, A*lamar Blue* (Product Brand: Sigma, purity: 80 percent) was obtained from Dr. Hashemi in the Faculty of Pharmacy, Mashhad University of Medical Sciences.

Cell culture. The HT-29 cell lone was obtained from the Bouali Research Institute, Mashhad, Iran. The cells were cultured in RPMI1640 medium (Beta cell, Iran). This medium contained 10% fetal bovine serum (manufactured in Gibco, Brazil) and 1% penicillin and streptomycin (manufactured in Dena Zist, Iran). These cells were stored in an incubator at 37°C in a humid environment containing 5% CO_2.

Cytotoxicity assay. The *Alamar Blue* test was used to evaluate the toxic effects of GBA on HT-29 cells. For this purpose, 8000 cells were cultured in each well of a 96-well plate. After the cells adhered to the bottom of the wells, they were treated with a medium that contained medication. The drug concentrations were 5, 10, 20, and 40 μg/mL and the durations of cell treatment were 24, 48, and 72 h. After the treatment, 20 μL (10% of the volume of each well) resazurin was added to each well, and the culture plates were kept in an incubator for 4 h. When resazurin enters the cell and is converted to resorufin, red color is produced. Then, the absorbance was measured at 600 nm using a microplate reader (BioTek, USA). The survival rate at each concentration was determined three times and calculated using the following equation:

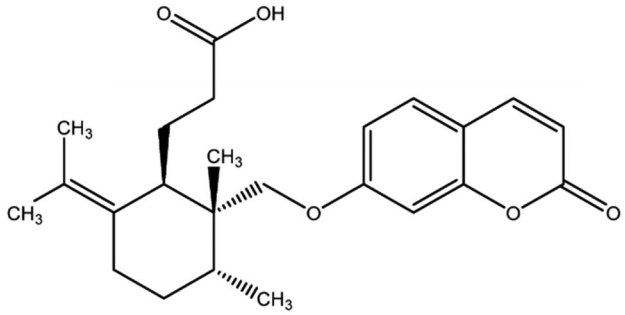

Fig. 1. Chemical structure of Galbanic acid.

$$\text{Cells Viability} = 1 - \frac{AT - AU}{AB - AU} \quad (1)$$

where AT, AU and AB are the absorbances of treated cells, untreated cells and blank control, respectively. The blank control was a well that contained the culture medium without cells [12].

Clonogenic assay. First, 2×10^5 cells were cultured in eight T25 flasks, representing the control group and the groups treated with medication, radiation, and combination therapy (radiation and medicine). Afterward, four flasks were treated for 24 h with GBA with a concentration of 10 μg/mL. After changing the culture medium, six flasks with doses of 2, 4, and 6 Gy were irradiated with an x-ray machine (Phillips, Germany). Each radiation dose was provided for two flasks, one of which represented the radiation-treated group and the other represented the combination therapy group. The flasks were stored in an incubator for 24 h.

After preparing the suspension solution from each flask and counting cells, the serial dilution process was performed for each suspension solution. Subsequently, the appropriate number of cells was seeded in each well of a six-well culture plate (manufactured in SPL Life Sciences, Korea). 600, 600, 1000, 1800, and 2800 cells were seeded for the control, drug-treated, and radiation-treated groups at doses of 2, 4, and 6 Gy, respectively. Two hundred more cells were seeded for the combination therapy group than for the radiation group. Moreover, three replicates were considered for each group. The six-well culture plates were kept in the incubator for eight days. The colonies were fixed using methanol and stained using Giemsa stain diluted with water withn 1- to 10-fold [13].

The plating efficiency (PE) and survival fraction (SF) were calculated using the following equations:

$$\text{PE} = \frac{\text{number of colonies formed}}{\text{number of cells seeded}} \times 100\%, \quad (2)$$

$$\text{SF} = \frac{\text{number of colonies formed after treatment}}{\text{number of cells seeded} \times \text{PE}} \times 100\%. \quad (3)$$

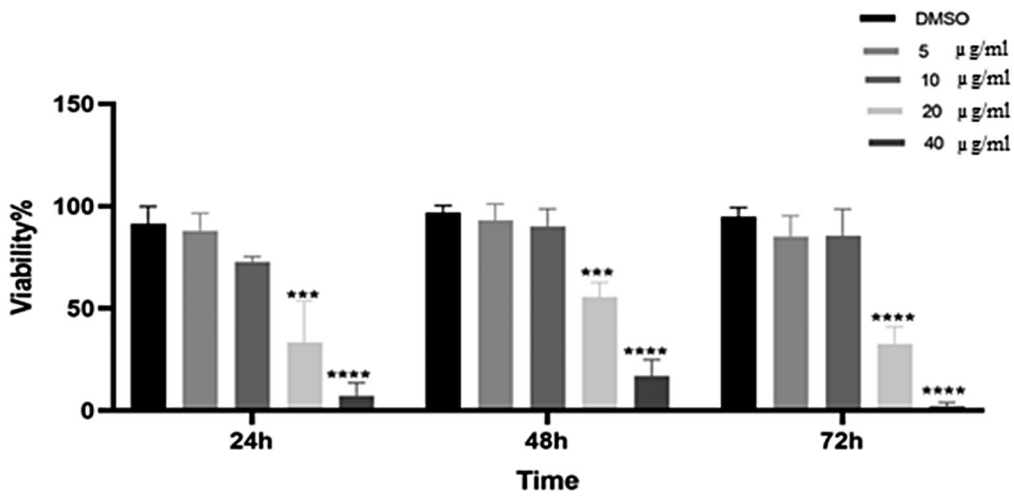

Fig. 2. Effects of Galbanic acid on the survival of HT-29 cell. The cells were treated with different concentrations for 24, 48, 72 h. The survival rate was determined by *alamarBlue* test. The obtained data are based on three replications and are reported through the mean value. *** $P < 0.001$ and **** $P < 0.0001$, which were compared with the control group.

The SF diagram was drawn according to a linear-quadratic model using the following equation [14, 15]:

$$SF = e^{-\alpha D - \beta D^2} \quad (4)$$

and for exploring the effective combination therapy, the following equation was used:

$$\text{Synergism} = \frac{\text{survival fraction for the Radiation group}}{\text{survival fraction for the combination group}} \quad (5)$$

where the synergism level for successful therapy must be greater than 1.

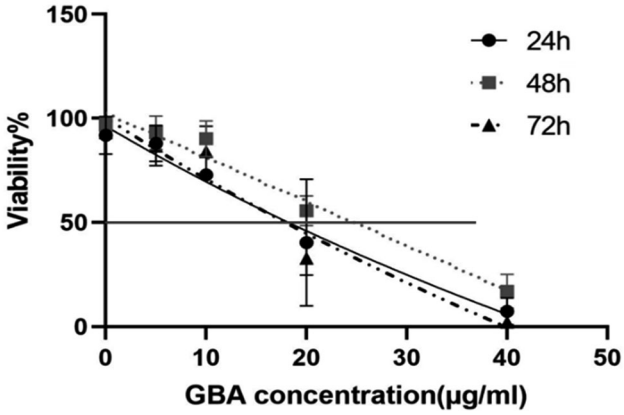

Fig. 3. The survival rate of cells decreases with the increase of the medication concentration; however, it is not associated with the treatment duration.

2.2. Statistical Data Analysis

All data were obtained in three replications, and the mean values were calculated in the SPSS software (Version 16). In addition, significant differences between the groups were determined using one-way ANOVA and Tukey's test. It should be noted that a P-value of less than 0.05 was considered statistically significant in all tests.

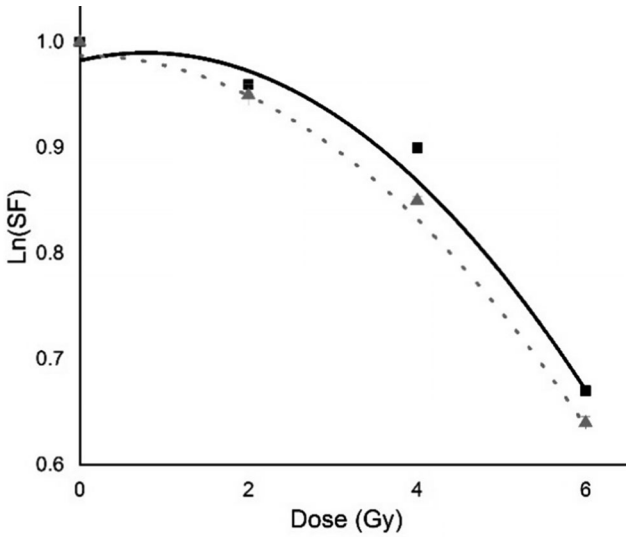

Fig. 4. Survival fraction curves for radiation-treated and combination therapy groups of HT-29 cells. Each data is the obtained mean value of 3 replicates. The cells were treated with different doses (2,4, and 6 Gy). The error bars indicate the standard deviations. The graphs are normalized to the plating efficiency of the control group, and the linear-quadratic model was used to fit the curve.

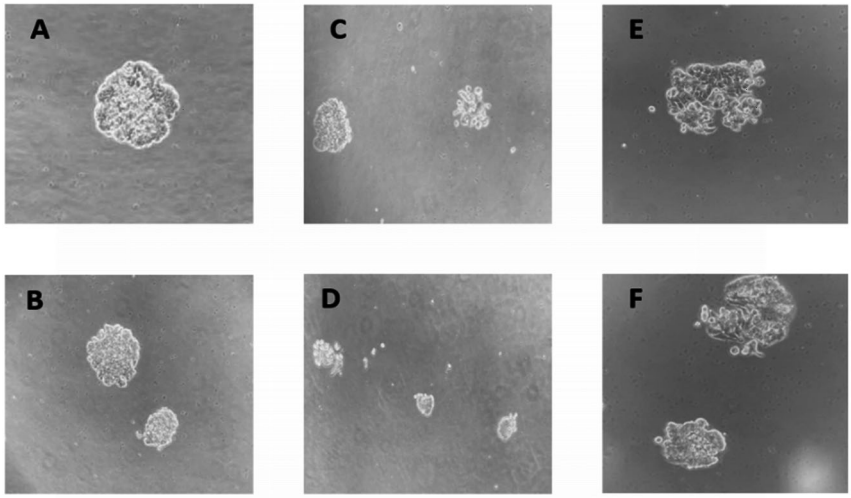

Fig. 5. Colonies formed in groups A (control), B (medication-treated), C (radiation treated at a dose of 2Gy), D (combination therapy at a dose of 2Gy), E (radiation-treated at a dose of 4Gy), and F (combination therapy at a dose of 4Gy).

3. RESULTS

According to the obtained results, GBA inhibits the growth of HT-29 cells. To investigate the toxic effects on HT-29 cells, the GBA concentrations of 5, 10, 20, and 40 μg/mL of were used for 24, 48, and 72 h. Eventually, the survival fractions of the cells were assessed by the *alamar Blue* test. Compared to the control group, statistically significant differences in cytotoxicity were observed only at the 20 and 40 μg/mL concentrations of GBA. The toxic effect of GBA on HT-29 cells is shown in Fig. 2.

Furthermore, data indicated that GBA acted in a time-independent manner as there were no statistically significant differences in cytotoxicity between treatment time frames of 24, 48, and 72 h ($P > 0.05$). The IC_{50} values for 24, 48, and 72 h were 17.13, 23.58, and 19.49 μg/mL as shown in Fig. 3.

The clonogenic assay was used to evaluate the effect of GBA on the radiosensitivity of HT-29 cells. Results of the Alamar Blue test revealed that the concentration of 10 μg/mL had toxic effects on HT-29 cells. However, there were no statistically significant differences between the survival fraction of the control group and the survival fraction of this concentration. So, to examine the consequences of combination therapy clearly, only a concentration of 10 μg/mL of GBA was used because a high concentration of GBA may interfere with the results of combination therapy.

For each radiation dose, the clonogenic assay test was performed independently and PE values were calculated according to Eq. (2). The PE (%) values for 2, 4, and 6 Gy were 52.99 ± 0.28, 49.28 ± 0.35, and 58.44 ± 1.36 %, respectively. The survival fraction curves of cells for the radiation-treated and combination therapy groups were calculated by Eq. (3). These values are presented in Table 2, which shows that there is no significant difference between the survival fraction of cells treated with radiation and combination therapy ($P > 0.05$). The synergism values for 2, 4, and 6 Gy were 0.71, 0.99, and 0.86, which shows that no synergism was manifested in combination therapy. In addition, survival fraction curves are shown in Fig. 4. The obtained data regarding curve fitting are also demonstrated in Table 1.

4. DISCUSSION

In this study, the *Alamar Blue* test was used to evaluate the toxic effects of GBA on HT-29 cells. These cells were treated with different concentrations of GBA for 24, 48, and 72 h. The results indicated that the toxicity of GBA on HT-29 cells depends on the medication concentration, while it is not associated with the treatment duration (Fig. 5).

TABLE 1. Values of α and β were obtained from the fitting of radiation and combined treatment curves using the linear-quadratic model.

Treatment	α value	β value
Radiation	0.016	–0.011
Combination therapy	0.003	–0.01

TABLE 2. SF values (%) for cells treated with irradiation and GBA, alone and in combination, at different doses (2, 4, and 6 Gy).

Dose of radiation(Gy)	2	4	6
SF(Radiation)	80.95 ± 1.04	61.62 ± 1.03	21.43 ± 0.5
SF(Radiation+GBA)	81.14 ± 4.1	50.16 ± 1.01	19.08 ± 0.55

The observed toxic effects can be due to apoptotic deaths and cessation of cell growth since the apoptotic and anti-growth properties of GBA have been observed in other cancer cell lines, including HUVCECs [9], OVCAR-3 [10], and H460 non-small cell lung carcinoma (NSCLC) [11]. For example, the apoptotic death of GBA-treated H460 and non-small cell lung carcinoma (NSCLC) occurs by activating Caspase9 and the Bax apoptotic protein and reducing the expression of anti-apoptotic proteins, such as Bcl-2 and Bcl-xL [11]. GBA can also inhibit the cyclin/CDK4/6/RB/E2F signal pathway and arrest the cell cycle in the G1 phase [12]The Clonogenic assay was used to evaluate the effect of GBA on the radiosensitivity of HT-29 cells. In this test, the cells were treated with only one medication concentration (10 mg/mL) at doses of 2, 4, and 6 Gy. Based on the obtained results, it is concluded that the use of Galbanic does not cause radiosensitivity in HT-29 cells.

The conflict between the death and survival signaling pathways determines the cell's fate in response to radiation. The PI3K/AKT and RAS-RAF-MEK-ERK1/2 signaling pathways affect cell survival and growth [13]. The AKT and ERK in the PI3K and Ras-Raf-ERK pathways are central proteins in the EGFR signaling pathway and play a vital role in the cell's response to radiation or other therapeutic agents [5]. Based on the results of previous studies, there is a link between the activation of the AKT and ERK1/2 signaling pathways to protect cells from toxic stresses that lead to radiation and medication resistance [14, 15]; therefore, inhibition of these proteins causes radiosensitivity.

GBA can inhibit the PI3K/AKT pathway by reducing the AKT expression level [9]. Therefore, the effect of GBA and radiation on HT-29 cells were predicted to be synergistic since radiation activates the PI3K/AKT pathway in HT-29 cells, and GBA increases apoptosis through the inhibition of AKT kinase. However, this effect was not observed in the present study. The expression level of EGFR in cancer is directly related to resistance to conventional toxic medications and radiotherapy, leading to a very poor prognosis [16, 17]. The activation of EGFR by ligand or radiation causes signaling events from the three major pathways of PI3K, Ras-Raf-Erk, and signal transducer and activator of transcription (STAT) [18, 19]. Therefore, this receptor and its associated kinases are essential targets for cancer treatment. For this reason, the radiosensitivity was expected to increase due to the inhibitory role of GBA in EGFR protein [10] and the involvement of most survival pathways; however, the EGFR expression had no association with AKT and ERK proteins in HT-29 cells [20]. The expression of AKT and ERK proteins can occur through other signaling pathways, and targeting these pathways can cause radiosensitivity. This requires further identification of the signaling pathways that activate these kinases, and more specific drugs may be needed for effective treatment.

The HT-29 cell has an Eph receptor A4 (EPHA4) responsible for survival, growth, and metastasis [21]. This receptor can cause the expression of ERK protein and radioresistance; moreover, the increase and expression of this receptor in various cancerous tumors, such as the colon, leads to aggressive properties and metastasis [22]. Although the PI3K/AKT and ERK1/2 pathways belong to the EphA4 receptor, only the activation of ERK1/2 directly depends on EphA4 [23]. Despite the effect of GBA on AKT expression level and inhibition of EGFR, the failure of combination and medication therapy could be due to its lack of specificity in involving signaling pathways responsible for growth, invasion, and metastasis in HT-29 cells. Despite the observed toxicity of GBA on HT-29 cells, it does not affect the radiation sensitivity.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Shokouhozaman Soleymanifard, Mojtaba Farahi and Raziyeh Hashemi. The first draft of the manuscript was written by Mojtaba Farahi and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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