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Targeting Aerobic Glycolysis: Gallic Acid as Promising Anticancer Drug

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Abstract

It has been noticed that Gallic acid can interfere with substantial pathways in different types of cancer, thus it was suggested to be a candidate for anticancer treatment. In this research, a new thought was tested to observe if Gallic acid would interfere to inhibit the glycolysis pathway. Inhibition of lactate dehydrogenase (LDH) by Gallic acid was tested using the normal human serum. The concentration of Gallic acid was ranged between 10^{-1} to 10^{-7} M, results indicated that the lowest and the highest concertation of Gallic acid were able to induce activity inhibition percentage between half and three quarters respectively. Furthermore, the anti-proliferation activity of Gallic acid against different types of cancer cells was tested *in vitro* using the MTT assay. these were the brain tumor glioblastoma (AMGM), skeletal muscle tumor rhabdomyosarcoma (RD), cervical cancer (HeLa), mice mammary gland adenocarcinoma cancer (AMN3) with two human breast cancers (AMJ13 and MCF7), and human ovarian cancer (SK-OV-3). Three concentrations were tested 10^{-1} 10^{-2} and 10^{-3} M for three incubation times 24hr, 48hr, 72hr. The results indicated a profound growth inhibition activity with DNA fragmentation as an end event of apoptosis.

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Keywords: Gallic acid, cancer cells , Aerobic Glycolysis inhibition ,LDH enzyme inhibition **.**

Introduction

Cancer can be defined at the cellular level as the type of disease that involves genetic disorders in cellular growth machinery. Cells grow uncontrolled, spread over surrounding cells in the tissues, and may move to other nearby or distant tissues(1). Different types of cancers can infect and affect different tissues in the body. When cells divide uncontrollably, they form a disorganized mass of tissues known as tumors. These growing tumors can interfere substantially with the organ function leading to a major disturbance in the whole body, especially if it reached many organs, and that may cause death (**2).**

malignant cells need to accommodate their metabolic functions to survive and multiply in the scuppered stipulates imposed by the tumor microenvironment. Cellular bioenergetics modifications are the signature hallmark of cancer. Cells manipulate their vital activities to maintain unregulated proliferation, this shift leaves them dependent on an exotic maneuvers to gain nutrients and energy. They alter their metabolism to support their rapid proliferation and expansion across the body. One of the unusual metabolic beavers that malignant cell undertake to scoop up its growth was known as Warburg effect, in which Otto Heinrich Warburg assumed that cancer cells rely on the pathway of glycolysis to gain most of its metabolic energy in alternative to oxidative phosphorylation in the presence of aerobic conditions (3). The cancer cells do not depend on protein and fat as food (4).

Several mechanisms have been suggested to affect energy metabolism and may contribute to the Warburg effect. These mechanisms include mitochondrial defects, adaptation to the hypoxic environment in cancer tissues, oncogenic signals, and abnormal expression of certain metabolic enzymes(5).

The molecular and biochemical events that lead to the increment of aerobic glycolysis in malignant cells are complex to substantial level and cannot be assigned to a single rather than multiple factors besides those mentioned above. The malignant cells seems to become addictive to metabolize sugar through glycolysis and generate ATP almost predominantly through this pathway. The generation of ATP via glycolysis is way less economical (two ATP per one glucose molecule) comparing to

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oxidative phosphorylation (36 ATP per one glucose molecule). In that sense, cancer cells presumably would consume more glucose than normal cells in order to maintain ATP required for their essential actives and proliferation. In parallel with that, elevated glycolytic activity is essential for cancer cells to survive and grow. Featuring this metabolic abnormality led to the following hypotheses: if the glycolysis could be inhibited radically that may consequently reduce ATP generation in malignant cells and thus may preferentially eliminate them (6)(7)(8).

When glycolysis is inhibited, the intact mitochondria in normal cells enable them to use alternative energy sources such as fatty acids and amino acids to produce metabolic intermediates channeled to the TCA cycle for ATP production through respiration. As such, cells with normal mitochondria are expected to be less sensitive to agents that inhibit glycolysis (9).Current insight revealed that aerobic glycolysis supports various biosynthetic pathways and, consequently, the metabolic requirements of cancer cells for proliferation(10)(11)(12).

After these discoveries of the altered cancer cell metabolism, tremendous studies have focused there aspects toward cancer cell metabolism with a defined goal, to find new ways for effectively eliminating tumor cells by targeting their energy metabolism. Glycolytic pathway inhibitors such as a dichloroacetic acid (DCA) and 2-deoxy-d-glucose (2DG) are classical examples of many cancer therapies had been proposed to target the metabolism of a tumor cell, now being used in clinical studies as potential anticancer agents (13)(14).

lactate dehydrogenase enzyme (LDH) is one of the glycolytic pathway enzymes consist of a tetramer of A and B subunits encoded by two separate genes. This enzyme catalysis the conversion of pyruvate to lactate coupled with oxidation of NADH to NAD+, which is essential for the glycolytic pathway (15). This enzyme has considerable importance in this manner because of the nature of the environment cancer cells present in. Hypoxia is a significant signature of tumors microenvironment in this microenvironment LDH activity gain a vital role to maintain glycolysis (16). Besides that, it is well known that tumors have higher levels of LDH than normal tissues(17), for that, this enzyme level in serum of cancer patients was considered as a prognostic marker (18).

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Gallic acid is one of the phenolic compounds(19) that exists in many natural herbal plants and widely in plant foods (20), fruits, nuts and leafy vegetables (21), such as walnuts, sumac, grapes, green tea leaves, strawberries, lemon, banana, pineapple, apple peel and berries(22). Gallic acid is considered to stimulate many pharmacological and biochemical pathways and is a factor determinant of antioxidant properties (23), anti-inflammatory, anti-allergic and anti-cancer activities (24).

In this research, we have asked if Gallic acid can modulate the activity of LDH *de novo* and test if Gallic acid can inhibit cancer cell proliferation *in vitro* using different types of cancer cell lines of different origins that hadn't been tested before. We have employed the brain tumor glioblastoma (AMGM), skeletal muscle tumor rhabdomyosarcoma (RD), cervical cancer (HeLa), mice mammary gland adenocarcinoma cancer (AMN3) with two human breast cancers (AMJ13 and MCF7), and human ovarian cancer (SK-OV-3). We test also if the late event of apoptosis can be induced by Gallic acid using DNA fragmentation assay.

Materials and methods

Cell lines and cultivation

All cell lines used in this study were provided from the cell bank unit in the department of experimental therapy of the Iraqi center for cancer and medical genetics research, University of Mustansiriyah, Baghdad. Different types of cell lines were used collectively they were, brain tumor glioblastoma (AMGM), skeletal muscle tumor rhabdomyosarcoma (RD), cervical cancer (HeLa), mice mammary gland adenocarcinoma cancer (AMN3) with two human breast cancers (AMJ13 and MCF7), and human ovarian cancer (SK-OV-3). Cells were grown either in RPMI-1640 (USbiological life science, USA) supplemented with 10% fetal calf serum (Santa Cruse biotechnology Inc. Dallas, Texas) with 1% streptomycin and penicillin (State Company for drugs industry and medicals, Samarra, Iraq), all cell lines were grown at 37^{o}C in atmosphere of 5% CO2 and 95% humidified incubator.

Cells viability assay

From a confluent monolayer tissue culture vessel (Santa Cruse biotechnology Inc. Dallas, Texas), cells were suspended by trypsin-versen (USbiological life science, USA), 1× 10^{6} cell/mL were distributed in 200 µL volumes to 96 well flat bottom

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tissue culture plate (Santa Cruse biotechnology Inc. Dallas, Texas). Plates were incubated for 24 hr at 37 °C to make cells adheres to the wells. Serial dilutions of Gallic acid (Sigma Aldrich, St. Louis, Missouri, USA.) (10, 100, and 1000 µM) were prepared in serum-free RPMI-1640 media, 200 µL of each dilution was distributed over the adherent cells and serum-free media was added to control untreated well, four replicates were used for each dilution of Gallic acid and control wells in two separated plates at each run. Plates were incubated for 24, 48, and 72 hours at 37°C in the atmosphere of 5% CO2 and 95% humidified incubator. After incubation time ended 100 µL of media containing Gallic acid was removed and 100 µL MTT solution (bioWORLD, 4150 Tuller Rd., Ste 228, Dublin, Ohio (OH) 43017, USA.) (0.4 mg/mL dissolved in PBS) was added to each well and plates were re-incubated for another 4 hrs at the same incubation conditions. After that plates centrifugation were centrifuged at 1000 rpm at room temperature (Hettich GmbH & Co. KG Föhrenstr, Tuttlingen, Germany) and a 100 µL form each well was aspirated and 100 µL of DMSO (bioWORLD, 4150 Tuller Rd., Ste 228, Dublin, Ohio (OH) 43017, USA.) was added and plates were re-incubated for 2 hrs to let crystals of formazan to dissolve. Absorbance was measured with a microtiter plate reader (Human Co. GmbH, Germany) at 570 nm. Cell growth inhibition was calculated using the following equation

Percent of growth inhibition = absorbance of control untreated wells − absorbance of treated wells/absorbance of control untreated wells

The experiment was repeated three times on different days.

Changes in cells morphology

To visualize the effect of Gallic acid on the morphology of treated and control untreated cells, we used crystal violate (bioWORLD, 4150 Tuller Rd., Ste 228, Dublin, Ohio (OH) 43017, USA.) to stain the cells. After exposing cells to serial dilutions of Gallic acid in 96 well flat-bottom plates for the indicated time of incubation as previously mentioned, media was decanted from the plates and 50 µL of crystal violate stain (0.5 mg/ mL dissolved in solution composed of 20% methanol, 10% formaldehyde, and 5% acetic acid) was added to each well. Plates incubated for 5 min at 37 °C, then stain solution was decanted and plates washed 3 times with distilled water and dried at 40 °C in the air-forced incubator. Cells were imaged with

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a CCD camera (Leica Microsystems Wetzlar, Germany) mounted on an inverted microscope (Leica Microsystems Wetzlar, Germany).

Nuclear fragmentation detection

The nuclear of the cells exposed to Gallic acid and those of control untreated were observed using propidium iodide fluoresces dye (bioWORLD, 4150 Tuller Rd., Ste 228, Dublin, Ohio (OH) 43017, USA.). Cells were cultured in 96 well plates, exposed to Gallic acid exactly as described in the previous sections for 1000 µM and 24 hr. time of incubation. After the end of incubation time, plates were centrifuged for 10 min using a micro-titer-plate centrifuge at 1000 Xg and 4 ^{o}C. The media was aspirated carefully in order not to lose the non-adherent cells. Cells were fixed in the plates with -20 ^{o}C cold methanol for 5 min and 50 µL of 0.4 mg/ mL propidium iodide solution was added for each well, plates were incubated for 5 min at 37 ^{o}C and plates were washed with distilled water for three times. Stained cells were imaged using CCD camera (Leica Microsystems Wetzlar, Germany). mounted upon inverted fluorescence microscope (Leica Microsystems Wetzlar, Germany) using the green filter. Condensed and fragmented nuclear of the cells was counted using Image J software.

Results and Discussion:

LDH activity:

This work investigated the effect of Gallic acid on LDH enzyme activity in normal human serum. The biochemical tests illustrated an inhibitory effect on LDH enzyme activity. Table (1) shows the activity of LDH without and with Gallic acid and the enzyme activity inhibition percentage. Although LDH activity inhibition was concentration-independent, it's obvious that the lowest concentration of Gallic acid used was able to induce activity inhibition percentage between half and three quarters.

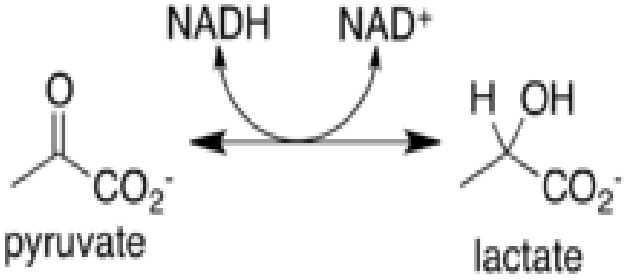

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Table (1): LDH activity without and with inhibitors and the inhibition% .

Gallic acid Conc. (M)	LDH Activity (U/L)	Inhibition%
0	32.38	-
10^{-7}	18.62	42.50
10^{-6}	26.71	17.51
10^{-5}	24.29	24.98
10^{-4}	18.62	42.50
10^{-3}	21.86	32.49
10^{-2}	24.29	24.98
10^{-1}	8.10	75.98

Figure (1) shows the type of Gallic acid effect on LDH enzyme activity in human serum which revealed an inhibitory effect. The results showed that any increase in Gallic acid concentration caused an increase in the inhibition percentage of the enzyme activity.

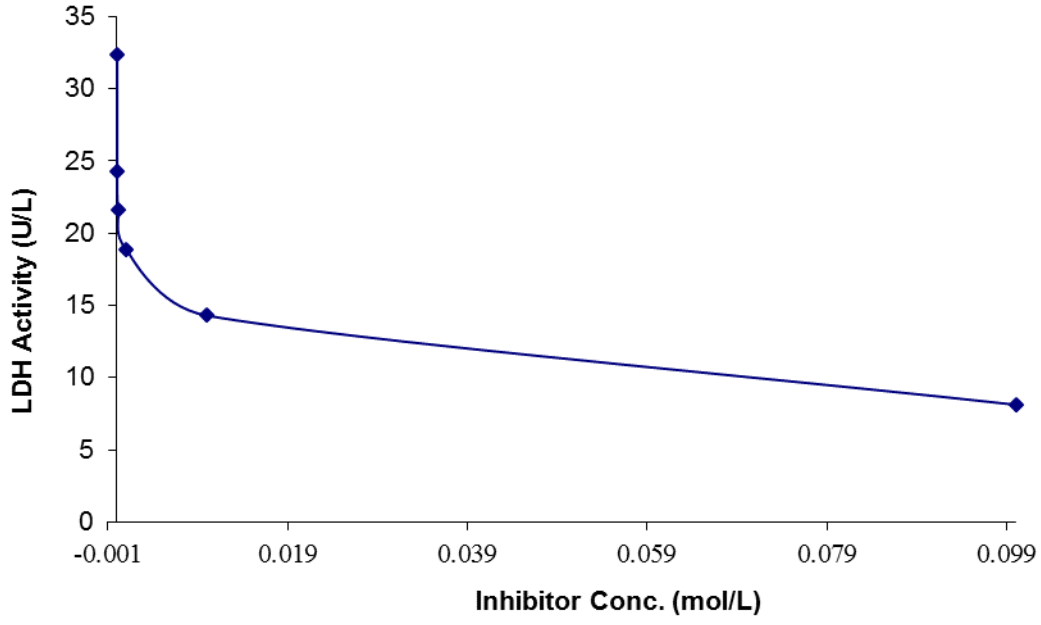

Figure (1): The relation between Gallic acid concentration and LDH enzyme activity .

Three sets of rate determination experiments –in which enzyme concentration was held constant- were carried out. In the first experiment, the velocity of the enzyme

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without inhibitor was achieved while in the second and third experiment the constant concentration of the inhibitor (10^{-5}, 10^{-1} M) were included in each enzyme assay. The type of inhibition using the Lineweaver-burg plot was non-competitive inhibition that changed V_{max} value but not K_m as shown in Table (2) and Figure (2) (25).

Table (1): Kinetic Properties of LDH without and with Gallic acid as an inhibitor

Gallic acid Conc. (M)	K_m (M)	V_{max} (U/L)
0	5	64.94
10^{-5}	5	50.00
10^{-1}	5	41.15

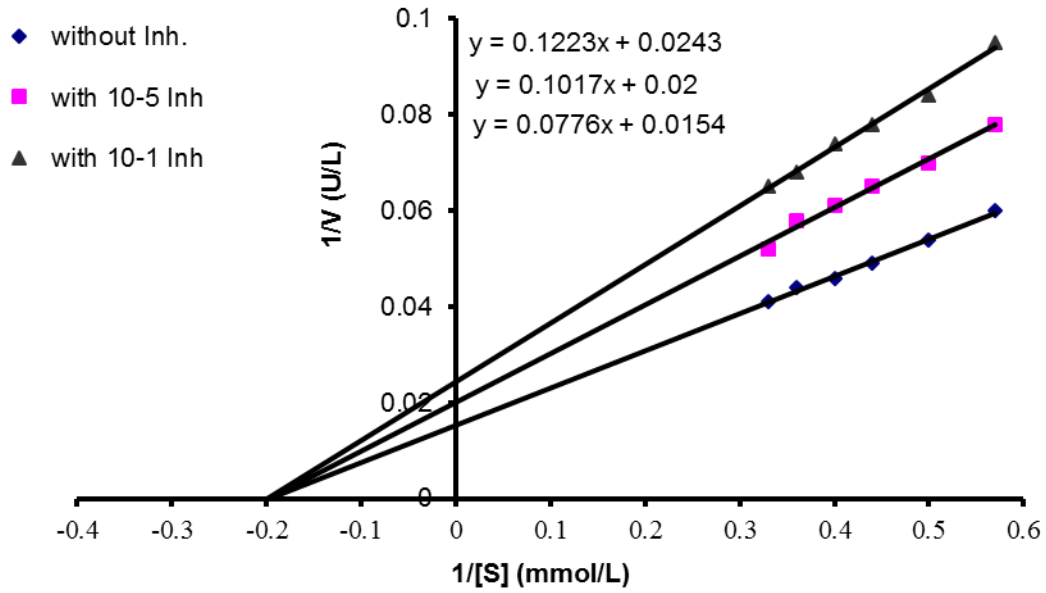

Figure (2): Lineweaver-Burg Plot for Gallic acid effect on LDH activity .

Cell Lines proliferation and apoptosis:

According to our knowledge, this is the first time that a comprehensive study included different types of cell lines that tested the anticancer activity of Gallic acid using diluted concentrations. The cell lines used represent a brain tumor glioblastoma (AMGM), skeletal muscle tumor rhabdomyosarcoma (RD), cervical cancer (HeLa), mice mammary gland adenocarcinoma cancer (AMN3) with two human breast cancers (AMJ13 and MCF7), and human ovarian cancer (SK-OV-3). The three

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highest concentrations used in LDH inhibition activity assay were used for that purpose. The dilutions used were able to induce growth inhibition in all cell lines tested with different percentages (Fig. 3, 4, 5, 6, 7, 8, and 9). The cyto-pathological prominent attribute of Gallic acid toward the cell lines under investigation indicated in various features. They include nuclear condensation, cytoplasmic shrinkage and lose of adhesiveness as a result of cell membrane disintegration (figures 10, 11, 12, 13, 14, 15, and 16). The induction of apoptosis via exposure to Gallic acid followed with propidium iodide staining (Fig. 17), the exposed cells suffered from obvious nuclear condensation and fragmentation as well as cell membrane blebbing.

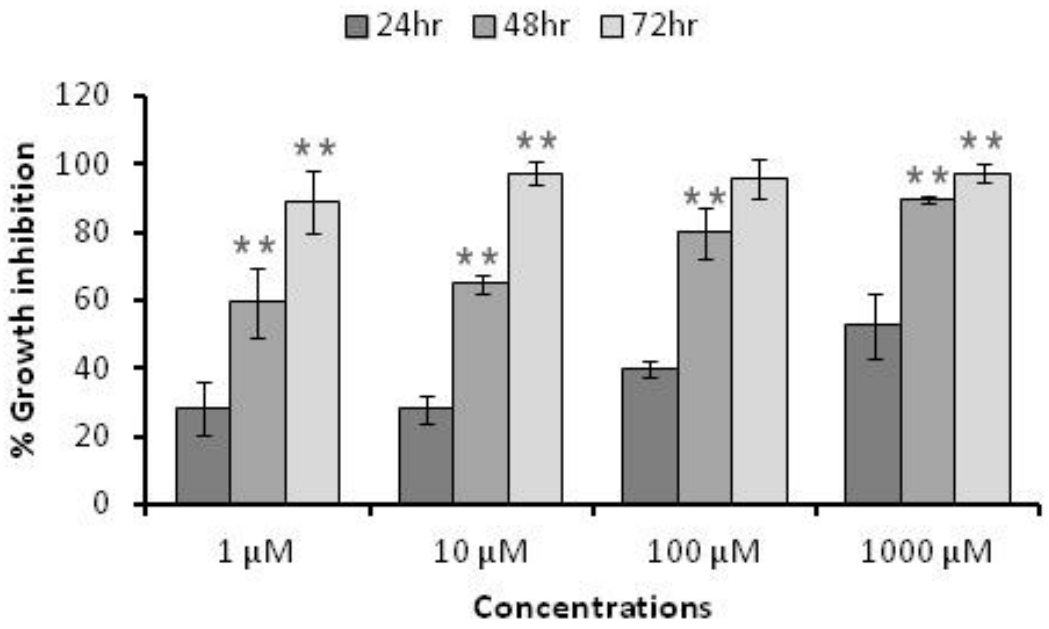

Figure 3:- growth inhibition of glioblastoma cells (AMGM) exposed to different concentrations of Gallic acid for three different times, whiskers represent SE±, stars represent P < 0.01 among different incubation times for one concentration

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used.

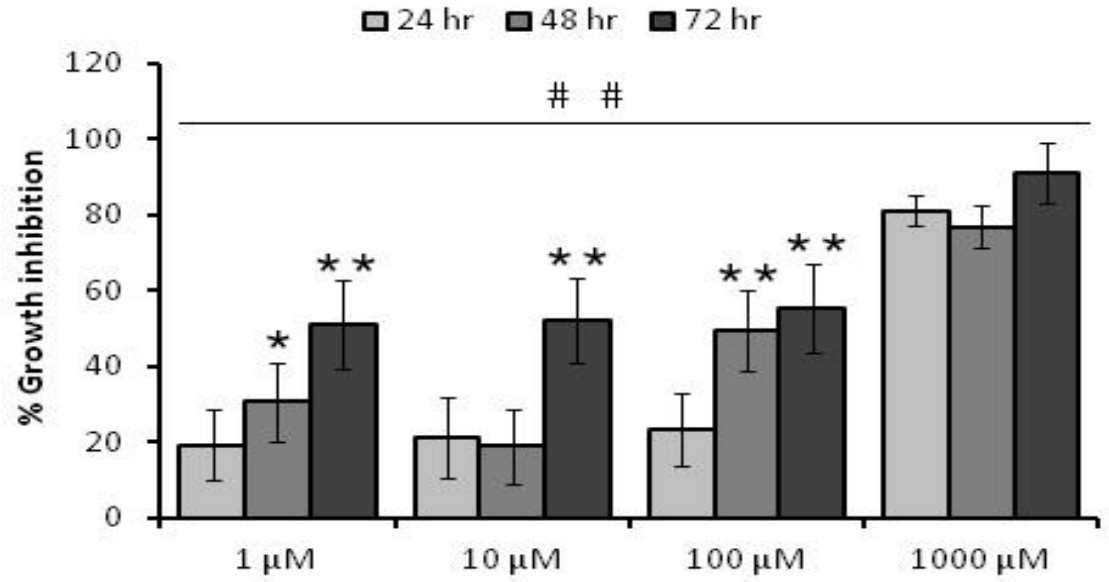

Figure 4:- growth inhibition of rhabdomyosarcoma cells (RD) exposed to different concentration of gallic acid for three different times, whiskers represent SE±, one-star represent P < 0.05., two stars represents P < 0.01 among different incubation times for one concentration used, ## represents P < 0.01 among different concentrations.

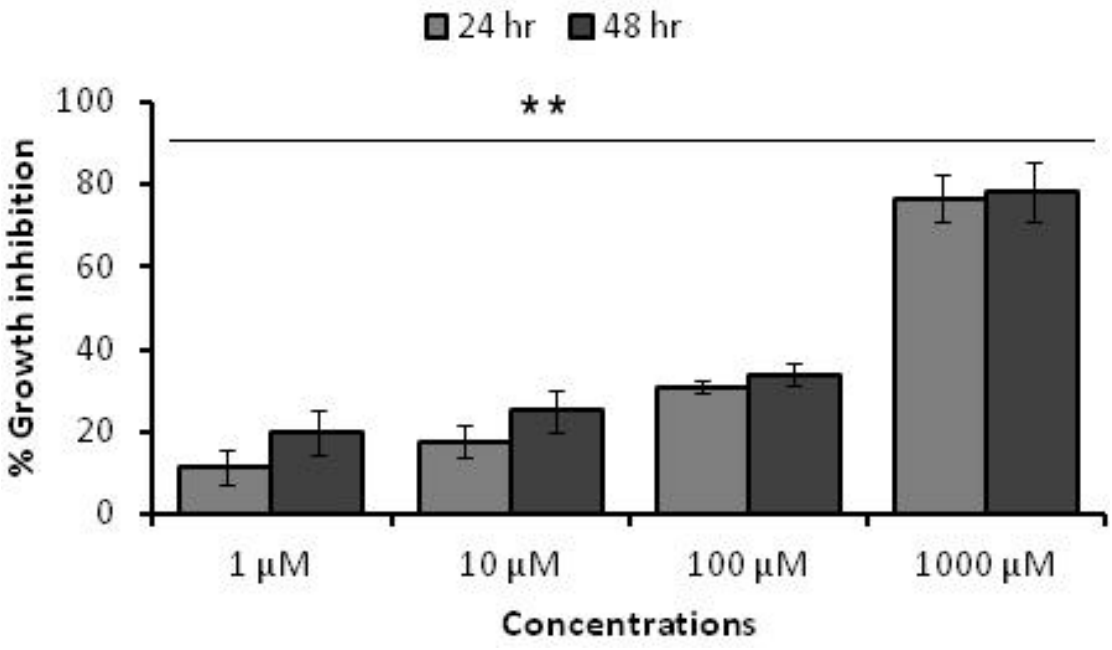

Figure 5:- growth inhibition of cervical carcinoma cells (HeLa) exposed to different concentrations of gallic acid for three different times, whiskers represent SE±, stars represent P<0.01 among different concentrations.

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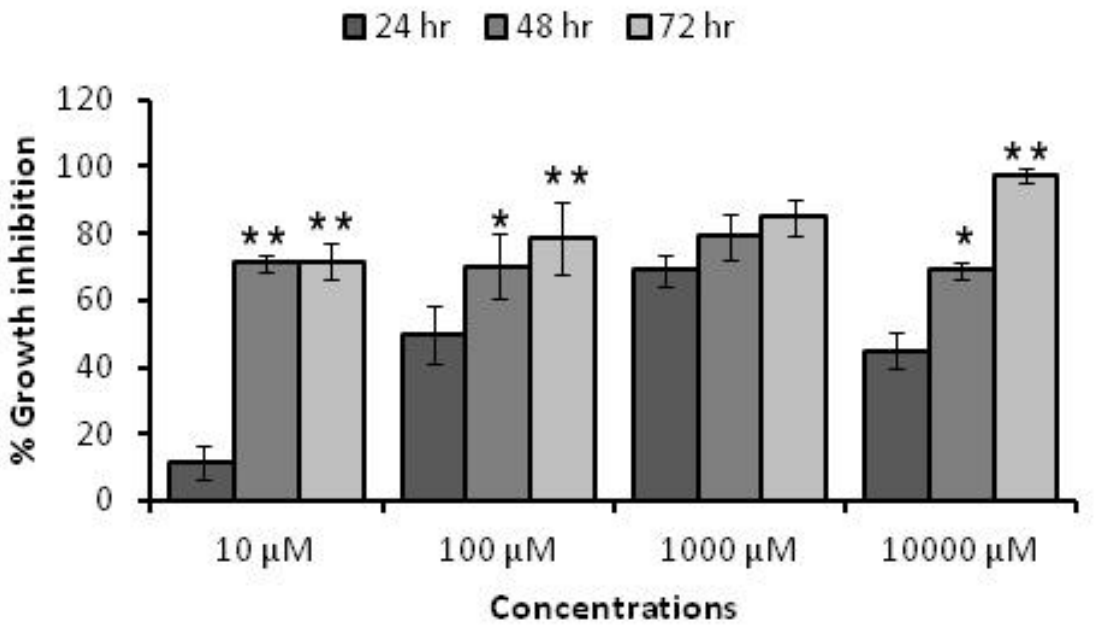

Figure 6:- growth inhibition of mice mammary adenocarcinoma cells (AMN3) exposed to different concentration of gallic acid for three different times, whiskers represent SE±, one-star represent p<0.05, two stars represents p<0.01 among different incubation time for the same concentration used.

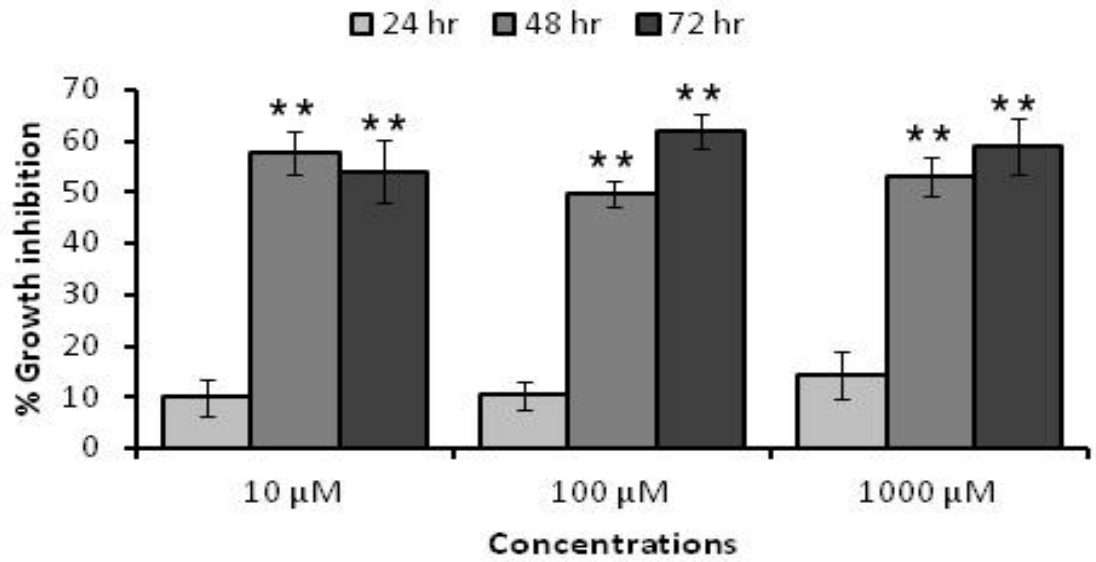

Figure 7:- Growth inhibition of human mammary adenocarcinoma cells (AMJ13) exposed to different concentrations of gallic acid for three different times, whiskers represent SE±, two stars represent p<0.01 among different times of incubation for the same concentration.

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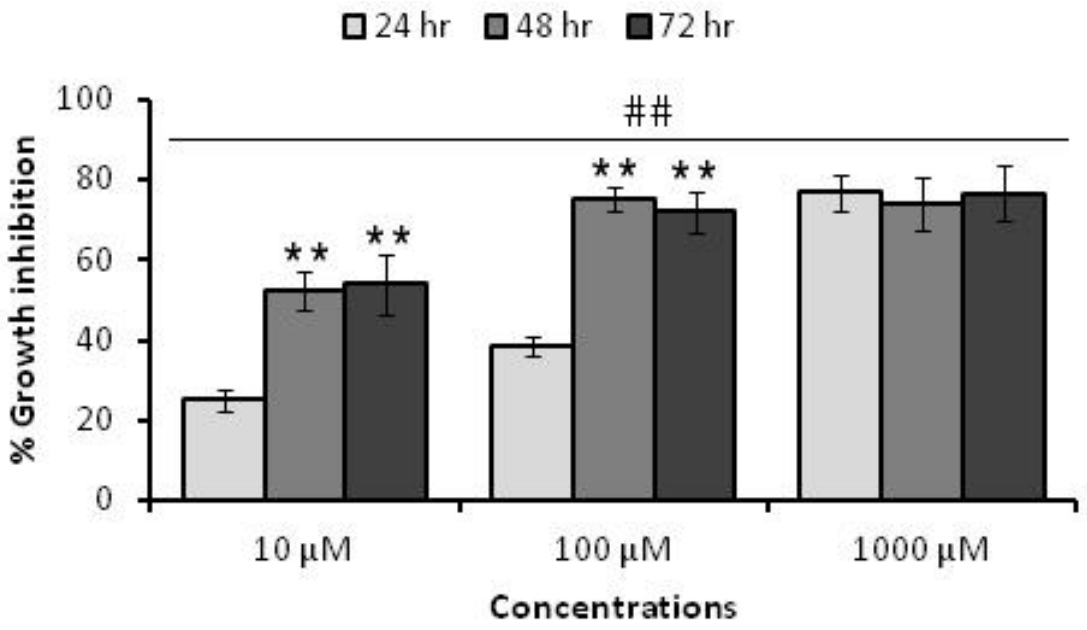

Figure 8:- Growth inhibition of human breast adenocarcinoma cells (MCF-7) exposed to different concentration of gallic acid for three different times, whiskers represent SE±, two stars represent P<0.01 among different times of incubation for the same concentration used, ## represents P < 0.01 among different concentrations for the same incubation time.

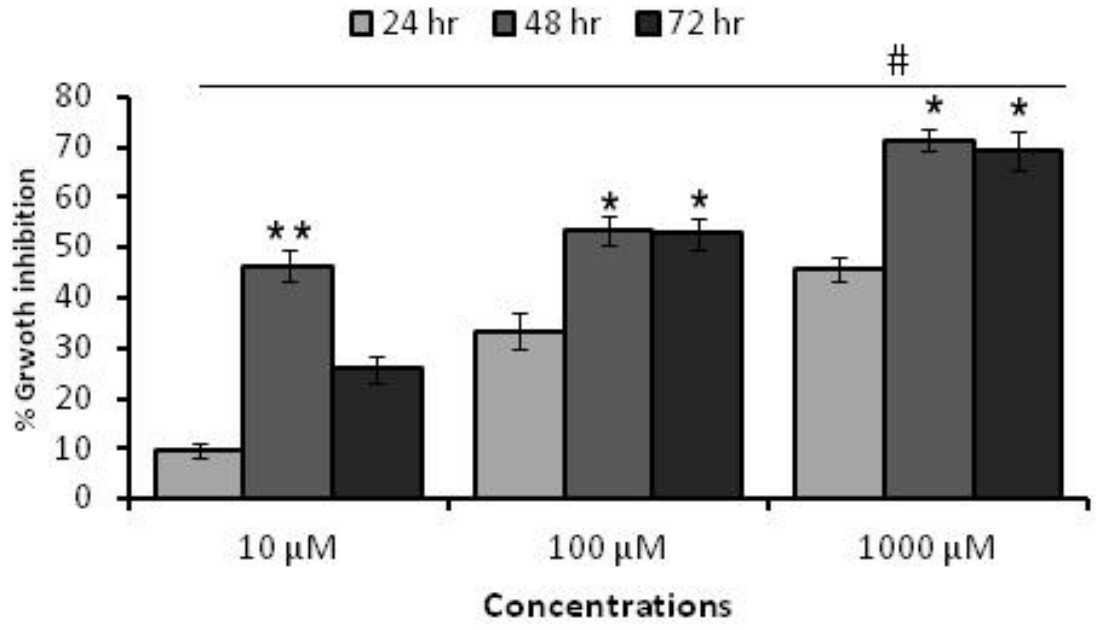

Figure 9:- Growth inhibition of human ovarian cancer cells (SK-OV-3) exposed to different concentration of gallic acid for three different times, whiskers represent SE±, one-star represent p<0.05, two stars represent p<0.01 among

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different incubation times for the same concentration used, ## represents P<0.01 among different concentrations for the same incubation time used.

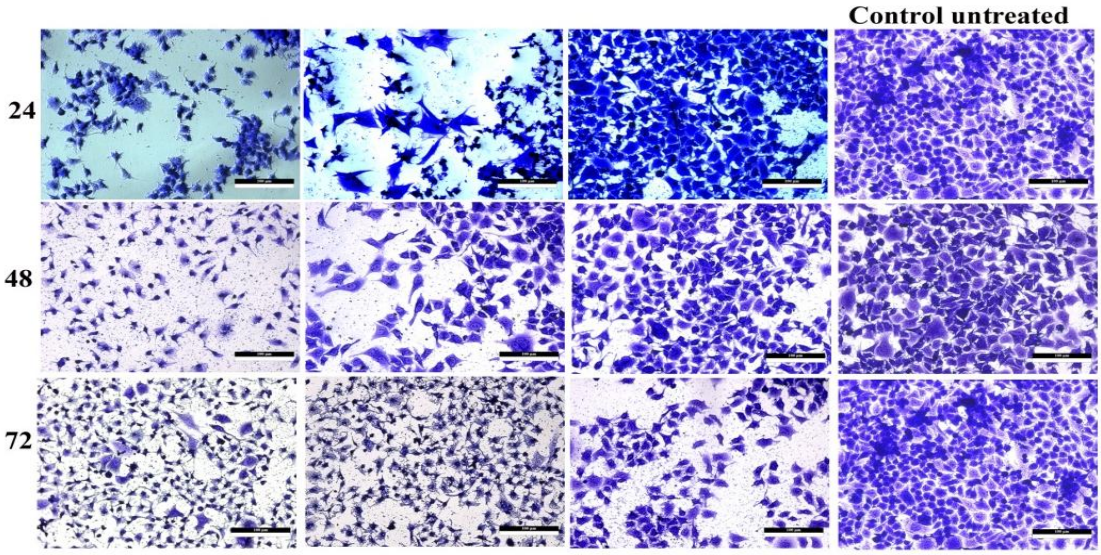

Figure 10:- Representative microscopic images for the growth inhibition activity of glioblastoma cells (AMGM) exposed to different concentrations of gallic acid for three different times (24 hr, 48 hr, and 72 hr). From left, columns of the images represent gallic acid concentrations 1000, 100, 10 µM, and control untreated cells.

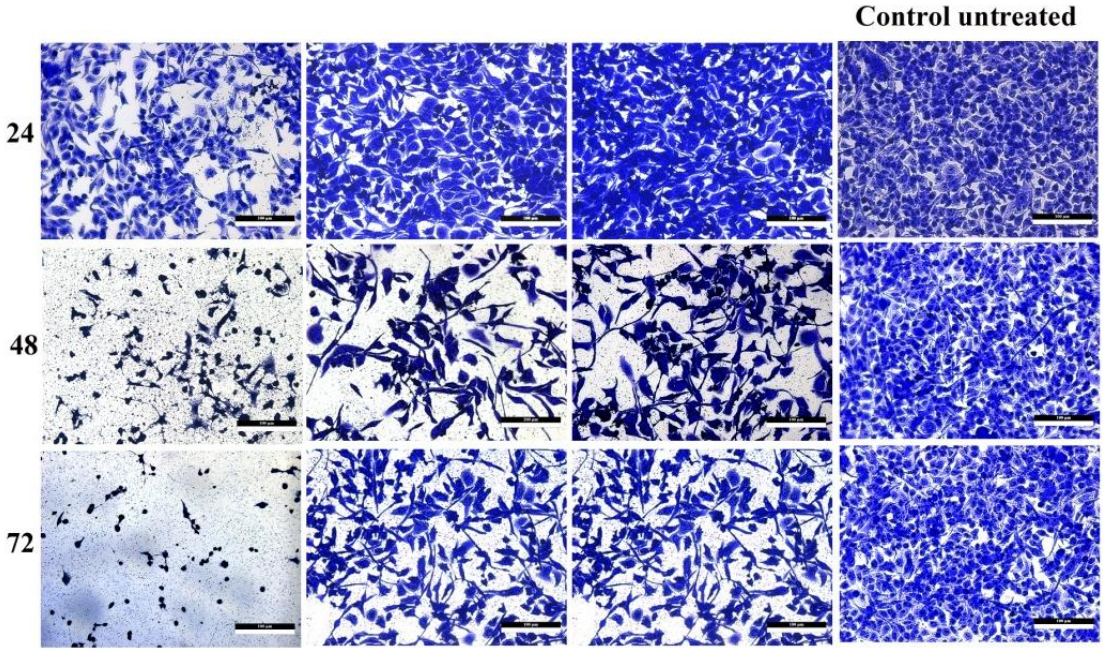

Figure 11:- Representative microscopic images for the growth inhibition activity of rhabdomyosarcoma cells (RD) exposed to different concentrations of gallic acid for three different times (24 hr, 48 hr, and 72 hr). From left, columns of the

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images represent gallic acid concentrations 1000, 100, 10 µM, and control untreated cells.

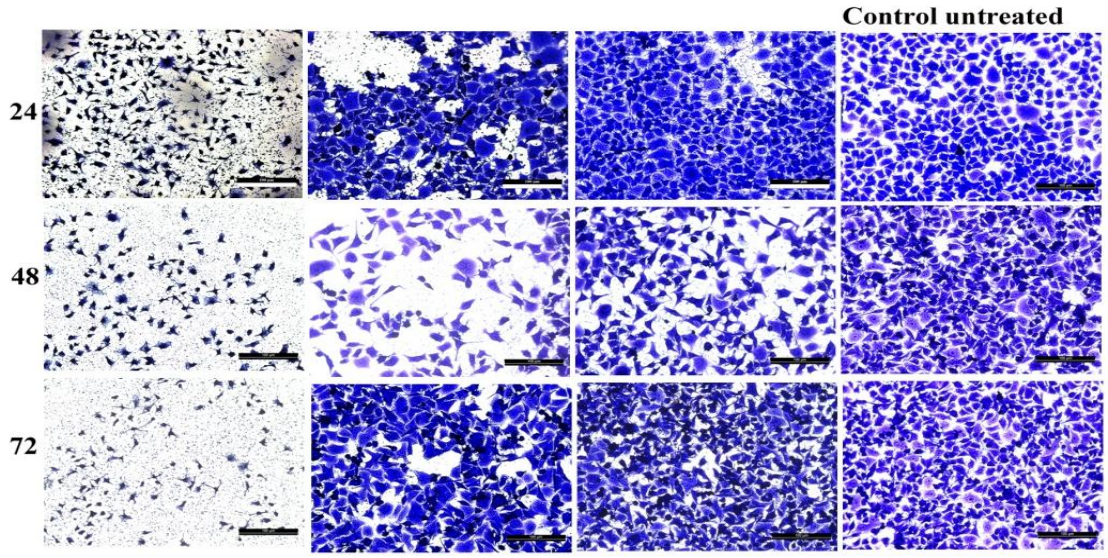

Figure 12:- Representative microscopic images for the growth inhibition activity of cervical carcinoma cells (HeLa) exposed to different concentrations of gallic acid for three different times (24 hr, 48 hr, and 72 hr). From left, columns of the images represent gallic acid concentration 1000, 100, 10 µM, and control untreated cells.

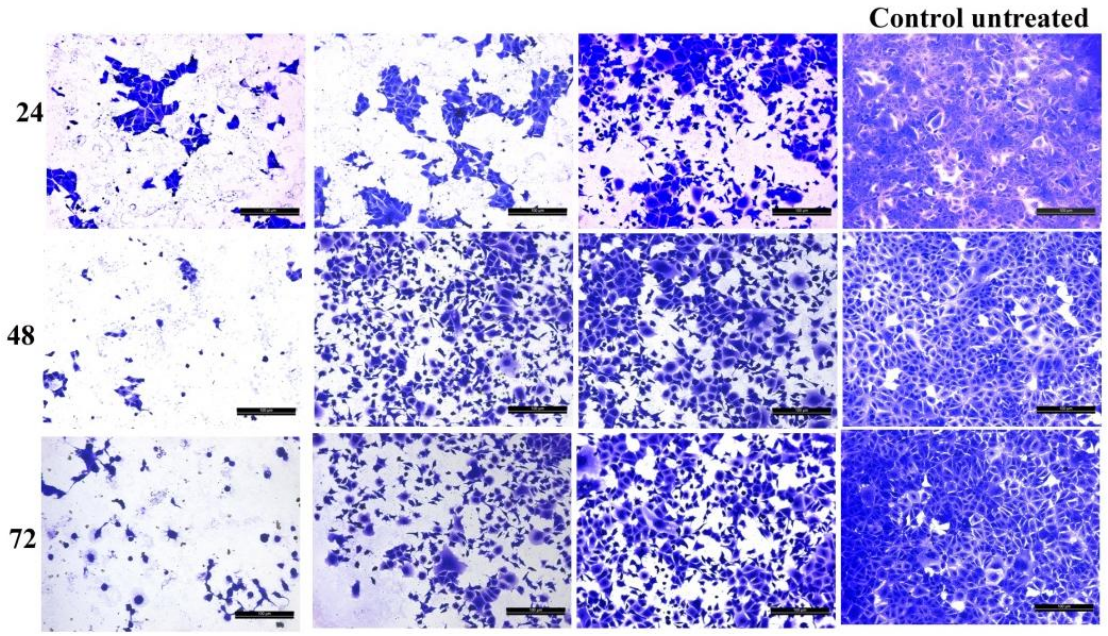

Figure 13:- Representative microscopic images for the growth inhibition activity of mice mammary adenocarcinoma a cells (AMN3) exposed to different concentrations of gallic acid for three different times (24 hr, 48 hr, and 72 hr).

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From left, columns of the images represent gallic acid concentration 1000, 100, 10 µM, and control untreated cells.

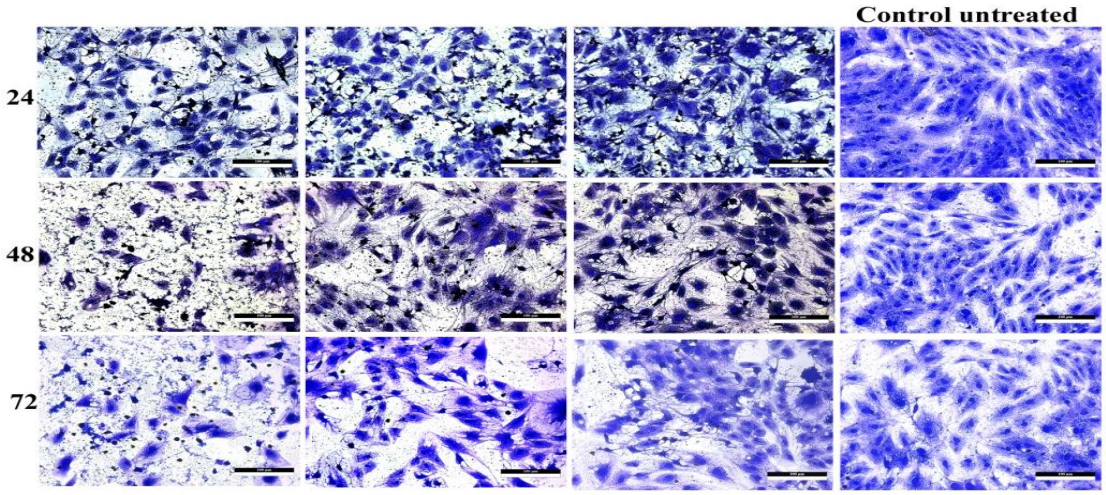

Figure 14:- Representative microscopic images for the growth inhibition activity of human mammary adenocarcinoma a cells (AMJ13) exposed to different concentrations of gallic acid for three different times (24 hr, 48 hr, and 72 hr). From left, columns of the images represent gallic acid concentration 1000, 100, 10 µM, and control untreated cells.

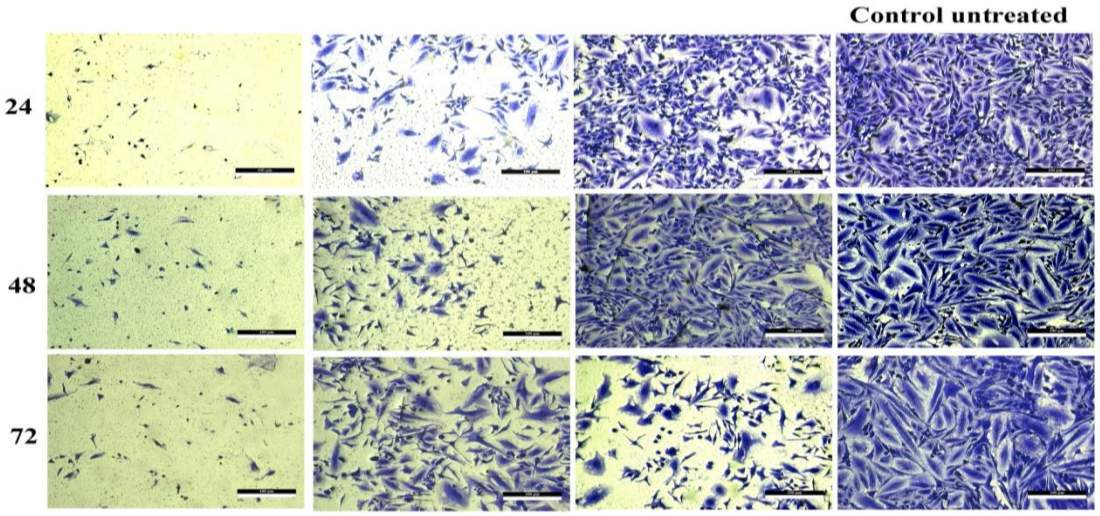

Figure 15:- Representative microscopic images for the growth inhibition activity of human breast adenocarcinoma cells (MCF-7) exposed to different concentrations of gallic acid for three different times (24 hr, 48 hr, and 72 hr). From left, columns of the images represent gallic acid concentration 1000, 100, 10 µM, and control untreated cells.

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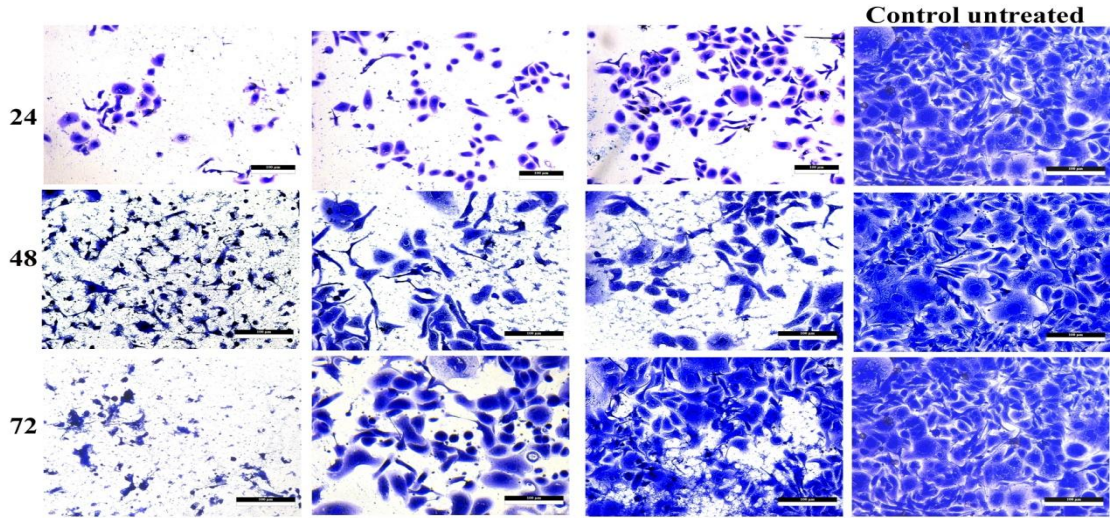

Figure 16:- Representative microscopic images for the growth inhibition activity of human ovarian cancer cells (SK-OV-3) exposed to different concentrations of gallic acid for three different times (24 hr, 48 hr, and 72 hr). From left, columns of the images represent gallic acid concentration 1000, 100, 10 µM, and control untreated cells.

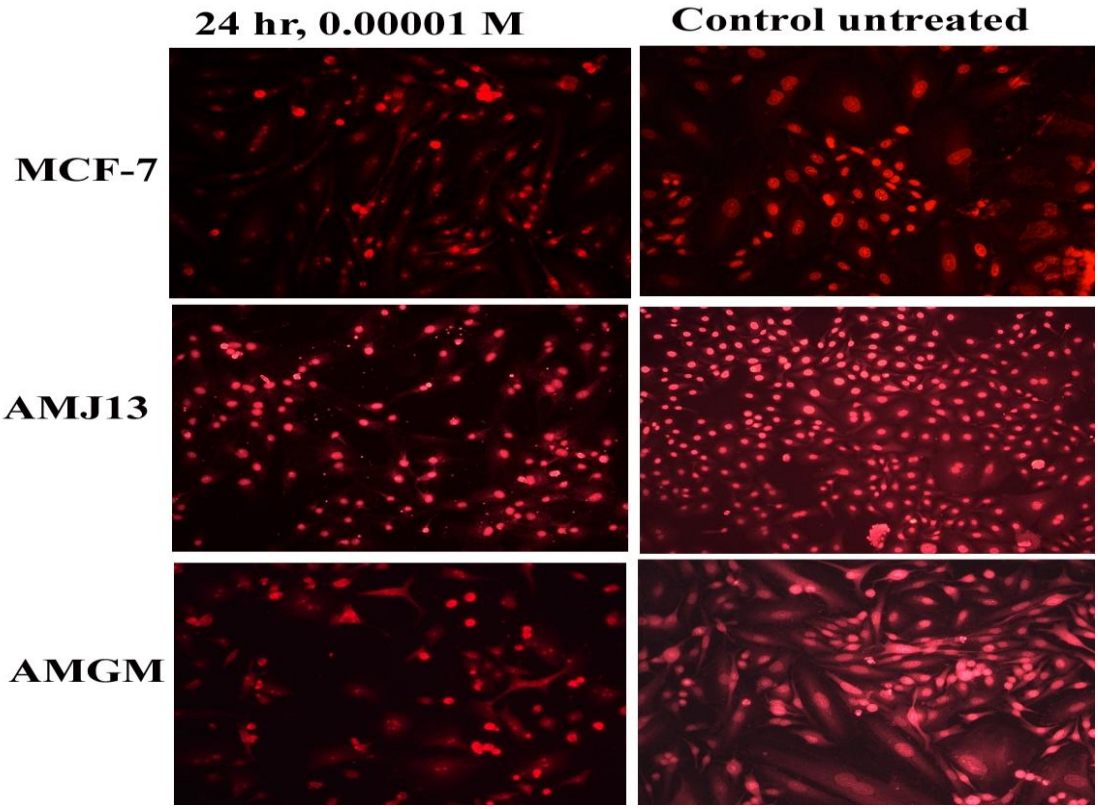

Figure 20:- Representative fluorescence microscopic images for the nuclei fragmentation activity induced in MCF-7, AMJ13, and AMGM cells exposed to 1000 µM gallic acid for 24 hr.

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Discussion

In previous studies, the anticancer activity of Gallic acid was studded indifferent cancer cell lines such as breast cancer (26) and cervical carcinoma (27) (28) and bladder cancer cells (29), However, this activity did not test against glioblastomas, rhabdomyosarcomas, or ovarian carcinomas cells yet. The results presented here indicated that this phytochemical was able to inhibit the growth of these cells (glioblastomas, rhabdomyosarcomas, or ovarian carcinomas) alongside the cells which were studded before.

Many cellular mechanisms were investigated to determine the actual effector pathway that Gallic acid could interfere with to induce apoptosis in cancer cells. Among these pathways, those that related to cell cycle, metastasis, angiogenesis, and kinase enzymes signaling. Throughout these vital pathways, Gallic acid can be a sword of two edges, preventing cancer initiation from one side and inhibit cancer cell proliferation from the other side (30). Results presented here also claim another effector mechanism of Gallic acid that has never tested before, it is the capability of interfering with the most important energy metabolism pathway for a cancer cell, glycolysis. This phytochemical can inhibit or reduce the activity of an essential enzyme of glycolysis, which is lactate dehydrogenase (LDH). The role of LDH is essential in the glycolysis, it is maintain the continuity of the cycle via producing NAD+, and not only in normal cells (31)but also in cancer cells, where the need for energy to maintain the cellular proliferation is at most regency (32).

The Warburg effect in cancer cells results in increasing the lactic acid accumulation in the tumor microenvironment thus making this environment more stable for cancer cells proliferation as well as inhibiting the anti-tumor immunity to take its rule in attacking the tumor cells, furthermore this acidity inhibit or attenuate the anti-tumor chemotherapy effectiveness (33). from this point, any possible tool to inhibit the taking place of Warburg effect in the tumors will enhance the effective eradication of these tumors. In this research an attempt to use gallic acid as anticancer agent and assess its role in inhibiting the activity of an essential enzyme (lactate dehydrogenase) of the glycolysis was attempted. Results were encouraging, the ability of gallic acid to reduce the LDH activity up to more than 70% was approved via using the normal LDH enzyme. Moreover this compound was capable of reduce the

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proliferation of different cancer cell and induce its apoptosis in concentration and time dependent manner. Therefore we can suggest using this compound in further studies to establish and confirm its mechanistic role in the interfering with the glycolysis via inhibiting the Warburg effect in cancer cells and eradicate all the burdens that this effect impose against cancer treatment.

conclusion

The use of gallic acid in the concentrations between 1 to 1000 µM can inhibit the growth and proliferation of the cancer cells under investigation. Its presumably can inhibit the activity of an important glycolysis enzyme Lactate dehydrogenase. With this inhibition, the availability of NAD and NADH will be terminated during glycolysis and that will lead to terminate glucose consumption and energy production, as a result ending the cellular proliferation process.

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