Original Article

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An Evaluation the Effect of Glycyrrhetinic and Glycyrrhizic Acids Derived from Licorice Extract on Gastric Cancer Cell Lines

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ABSTRACT

BACKGROUND AND OBJECTIVE: Gastric cancer is the second most prevalent carcinogenic disease and surgery, chemotherapy and radiation are its principal treatment modalities. However, in most cases, poor response to treatment and adverse side effects are observed regarding these modalities. Given the lack of response to treatment and growing rates of gastric cancer, researchers are trying to come up with more efficient treatments with fewer side effects. In the traditional medicine, licorice has been suggested as a cancer treatment considering its high antioxidant properties and few side effects. This study aimed to evaluate the effect of licorice extract on gastric cancer cell lines.

METHODS: In this experimental study, adenocarcinoma gastric cell lines were prepared from cell bank and were cultured. After passage, the cells were transferred into a 96-well plate. In each well, approximately 2,000 cells in RPMI-1640 culture medium with FBS (10%) were placed. The cells were repeatedly exposed to different concentrations of Glycyrrhetinic acid (0, 1, 10 and100.1 µM) and Glycyrrhizic acid (10, 1, 100 and 0.1 µM) for 24 and 48 hours. Finally, the obtained results of the experimental and control groups were compared with each other.

FINDINGS: According to our results, the toxic effect of Glycyrrhetinic and *Glycyrrhizic acids* is dose and time dependent. In 24 hours, the mean optical density (MOD) in 100 µM concentration of Glycyrrhetinic acid was 0.41±0.02 and 0.79±0.04 in the experimental and control groups, respectively (p=0.0002). After 48 hours, MOD was 0.16±0.004 and 1.749±0.24 in the experimental and control groups, respectively (p=0.0003). Moreover, the MOD of 100 µM concentration of Glycyrrhizic acid was 0.78±0.53 and 2.09±0.49 in the experimental and control groups in 48 hours. There was a significant difference between the experimental and control groups (p=0.035).

CONCLUSION: The results of this study demonstrated that the licorice compounds have a toxic effect on carcinogenic cells. Therefore, it is recommended to perform more study on both Glycyrrhizic and Glycyrrhizic acids as effective compounds on gastric cancer treatment.

KEY WORDS: Gastric Cancer, Glycyrrhetinic Acid, Glycyrrhizic Acid, Licorice.

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Introduction

Gastric cancer is the second most prevalent carcinogenic disease. Epidemiological studies have demonstrated that gastric cancer is the second leading cause of death (due to cancer) in the world

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following lung cancer (1). In 2014, the number of patients with gastric cancer in the USA was reported to be 1665540, out of which 585720 cases died (2). Gastric cancer is often asymptomatic in the early stages and the lack of clinical symptoms in these patients leads to delayed diagnosis. Almost 80-90% of these patients refer to hospitals in the advanced stages of the disease with adjacent metastasis. According to epidemiological studies, chance of survival in the patients with advanced stages of gastric cancer with metastasis is very low despite undergoing chemotherapy and aggressive treatments such as surgery for five years (3). Current studies have focused much attention on the use of natural products for the treatment of different diseases and have reached significant results in this regard. Many studies showed that the use of natural products could be useful in numerous areas such as strengthening the immune system (4, 5), treating incurable diseases such as Alzheimer's (6, 7), atherosclerosis (8,9), diabetes (10, 11), gastrointestinal diseases (12, 13) and cancer (14, 15). In fact, it has created high hopes for prevention and treatment of several diseases. Licorice is one of effective plants in the treatment of numerous diseases. Flavonoids derived from licorice root are also applied for treatment of many diseases including microbial infections causing hepatitis C, stomach ulcer, skin and respiratory infections; it is also used as a carcinogenic treatment (16). Licorice is from the Fabaceae family (also known as Glycyrrhiza glabra). In European and Asian traditional medicine, it was used for treatment of gastritis, peptic ulcers and respiratory infections (16). This plant is grown in many countries and is known as an anti-inflammatory, antitumor and antioxidant substance. Anti-tumor activities of the licorice compounds include cell cycle arrest, apoptosis inducing and antioxidant effects. According to the studies conducted on the lab rats, some licorice compounds inhibit PI3-K, MKK4, MKK7, JNK1, mTOR, and Cdk2 activities through reducing carcinogenesis in several cells (4). Additionally, licorice is used for the treatment of viral hepatitis and cytomegalovirus (CMV) infection. Moreover, Glycyrrhizic acid derived from licorice prevents the growth of viruses and viral components (5, 6). Glycyrrhetinic acid and Glycyrrhizic acid are two compounds isolated from licorice. Glycyrrhetinic acid is a pentacyclic triterpenoid derivative of the Glycyrrhizic acid (formula: $C_{30}H_{46}O_4$) which is obtained from licorice. Glycyrrhizic acid is a natural substance comprising of Glycyrrhetinic acid and two molecules of Glucuronic acid (7). Glycyrrhizic acid and Glycyrrhetinic acid can effect on the androgen steroids' activity through acting on glucocorticoid receptors and preventing the conversion of cortisol to cortisone in liver and kidney. In addition, compounds in licorice extract can cause lipoxygenase and cyclooxygenase disruption as well as arachidonic acid reduction. Glycyrrhetinic acid is used as an analgesic and anti-inflammatory. It is also used for treatment of allergy, gastric ulcer, hepatitis, liver toxicity and tumors. Glycyrrhetinic acid is an active pharmaceutical ingredient. It helps with reducing lipids through elevating the level of corticosterone (8, 9). In rats, Glycyrrhetinic acid plays a key role in prevention of development and progression of tumors by means of DMBA (7, 12-DiMethyl Benz (a) Anthracene) (trans-7, 12-dimethyl benzene) and TPA (12-o-Tetradecanoylphorbol-13-Acetate) (10), while Glycyrrhizic acid prevents the formation of tumors only by means of DMBA and TPA has no role (11). Given the efficacy of licorice compounds in treatment of various diseases with few side effects, this study aimed to determine the effects of Glycyrrhetinic acid and Glycyrrhizic acid derived licorice extract on gastric carcinogenic cell line.

Methods

Cell Culture: In our study, the components of Glycyrrhetinic acid and Glycyrrhizic acid were purchased from the Sigma Company and AGS carcinogenic cell line (adenocarcinoma gastric cell line) was purchased from Pasteur Institute (Tehran, Iran). The cells were cultured in 25 ml flasks containing 5 ml culture medium (the culture conditions: RPMI-1640 containing FBS (10%) (Gibco, Manchester, UK). These cells were

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incubated at 37 °C containing 5% CO_2. The culture medium was changed every 24 hours. Three or four days following the primary culture, cell density was evaluated under an inverted microscope. When the cells were grown to 80%, the passage was performed.

Cell viability: Approximately 2,000 cells in 50 µL RPMI-1640 culture medium containing 10% Fetal Bovine Serum (FBS) were added to each well in a 96-well plate and were kept in the CO_2 incubator overnight. Subsequently, the cells were repeatedly exposed to different concentrations of Glycyrrhetinic acid (0.1, 1, 10 and100 µM) and Glycyrrhizic acid (0.1, 1, 10 and100 µM), which were dissolved in dimethyl sulfoxide. A control group was considered for each concentration of Glycyrrhetinic and Glycyrrhizic acids. The control group was only located in a RPMI-1640 culture medium with FBS (10%). The cells were incubated with different amounts of the Glycyrrhetinic acid and Glycyrrhizic acid for 24 and 48 hours. After the incubation time, 10 ml of XTT solution (salt, 2, 3-bis, (2-methoxy-4-nitrobenzonitrile-5 sulfosuccinates) 2 tetrazolium-5 carboxyaniline) was added to each well in a 96-well plate in order to evaluate cell viability. After a 4-hour period, supernatant reading was evaluated by dint of ELISA (Stat Fax, Palm City, FL) and mean optical density (MOD) at 540 nm in each well was performed (12). The optical density of cells determined by ELISA was converted into percentage of cell viability using the following formula:

$$\text{Percentage viability} = \frac{\text{average OD of cell control}}{\text{test OD}} \times 100$$

All the tests were repeated three times. The cells were repeatedly exposed to various concentrations of Glycyrrhetinic acid and Glycyrrhizic acid for 24 and 48 hours, then, MOD was calculated. Eventually, statistical analysis was performed.

Statistical analysis: In this study, SPSS was used to analyze the data and independent Kruskal-Wallis and Dunn's post hoc test were performed. $p<0.05$ was considered significant in all the analysis.

Result

The incubation of AGS cells with different concentrations of Glycyrrhetinic acid: The obtained results showed that the lowest percentage of cell survival was observed in 100 µM concentrations among different concentrations (0.1, 1, 10 and100 µM) of Glycyrrhetinic acid after 24 hours of incubation. The MOD in 100 µM concentrations of Glycyrrhetinic acid was 0.41±0.02 and 0.79±0.04 in the experimental and control groups, respectively. There was a significant difference between the two groups (p=0.0002, CI95%) (fig 1. A). In addition, 48-hours incubation of AGS cells demonstrated the lowest percentage of cell survival in 100 µM concentrations of Glycyrrhetinic acid. The MOD in 100 µM concentrations was 0.16±0.004 and 1.749±0.24 in the experimental and control groups, respectively. The comparison between the two groups showed that there was a significant difference between the experimental and control groups (p=0.0003, CI95%) (fig 1. B).

The incubation of AGS cells with different concentrations of Glycyrrhizic acid: After 24 hours of incubation, the lowest percentage of cell survival was observed in 100 µM concentrations of Glycyrrhizic acid. The MOD in 100 µM concentrations was 0.33±0.29 and 0.81±0.08 in the experimental and control groups, respectively. The comparison between the experimental and control groups demonstrated that there were no significant differences between the two groups (fig 2. A). What's more, in 48-hours incubation of AGS cells, the lowest percentage of cell survival was observed in 100 µM concentrations of Glycyrrhizic acid. The MOD in 100 µM concentrations of Glycyrrhizic acid was 0.78±0.53 and 2.09±0.49 in the experimental and control groups, respectively. There was a significant difference between the two groups (p=0.035, CI95%) (fig 2. B). In our study, AGS cells were treated for 24 and 48 hours with various concentrations of glycyrrhetinic acid and glycyrrhizic acid. The increased cell death was observed at high doses of Glycyrrhetinic acid and Glycyrrhizic acid. Therefore, the effectiveness of these herbs is dose dependent, i.e., the rate of cell death increases by increasing the dose of these herbal medicines (fig 3).

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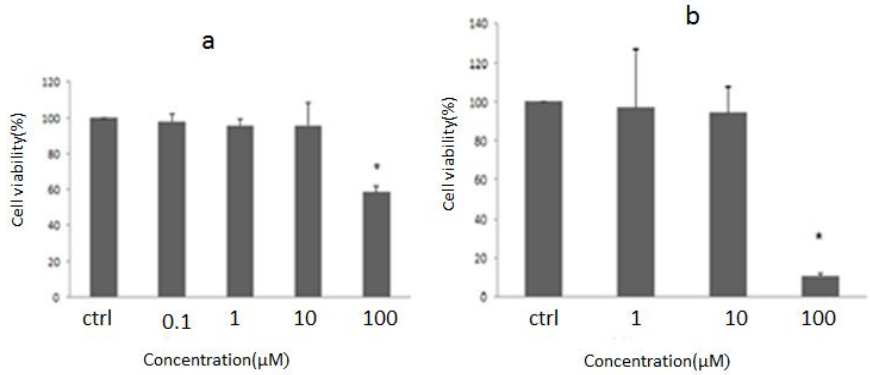

Figure 1. The percentage of cell survival in AGS cells after 24 (a) and 48 (b) hours of exposure to different concentrations of Glycyrrhetinic acid

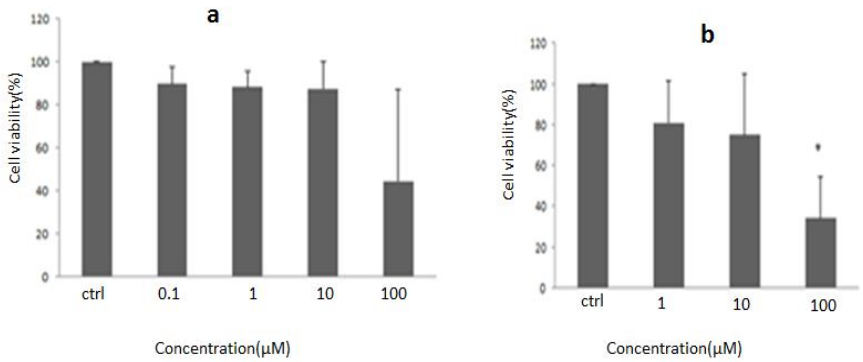

Figure 2. The percentage of cell survival in AGS cells after 24 (a) and 48 (b) hours of exposure to different concentrations of Glycyrrhizic acid

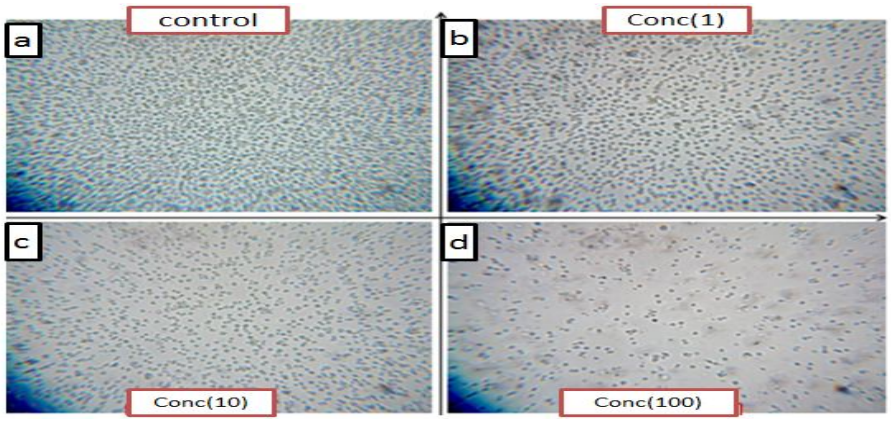

Figure 3. The number of AGS cells decrease by increasing the dose of Glycyrrhetinic **acid and Glycyrrhizic acid(a-d). (The greatest amount of cell death in 100 µM concentration(d))**

Discussion

According the obtained results, two compounds derived from licorice (Glycyrrhetinic acid and Glycyrrhizic acid) increased the rate of carcinogenic cells death following 24 and 48 hours of incubation. A study performed to determine the effect of Glycyrrhetinic acid on NCI-H460 NSCLC cells showed that inducing apoptosis and cell cycle arrest occurred in G1 phase. This might be due to an increase in the PARP (Poly ADP-ribose polymerase) and caspase-3 expression and a decrease in Bcl-xl, BCL2, cyclin D1 and cyclin E expression. Moreover, Glycyrrhetinic acid leads to

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decreased amount of protein kinase C-beta (PKC-beta) phosphorylates and extracellular signal-regulated kinase (ERK) phosphorylates and increased phosphorylation of protein kinase Cδ (PKCδ) and c-Jun N-terminal kinases (JNKs), both of which can affect cell death (13). The aforementioned processes are the possible mechanisms involved in gastric cancer treatment. In an investigation of the anti-carcinogenic effects of licorice extract components on gastric cancer cells and immortalized gastric epithelial cell lines, it was found that Licochalcone A, which is isolated from root of Glycyrrhiza glabra (licorice), is the most toxic component of the licorice that has the greatest impact on cell cycle arrest and inducing apoptosis. Some researchers have indicated that licorice extract Licochalcone A prevents the growth of carcinogenic cells in a dose-dependent manner. Moreover, they reported that the Licochalcone A component of licorice extract induced lowered expression of cyclin A, cyclin B and murine double minute 2 homolog (MDM2) oncogene. Additionally, it leads to increased expression of retinoblastoma protein (Rb) in the carcinogenic cells. Furthermore, Licochalcone A component of licorice extract causes apoptosis through affecting on the expression of Bcl-2, BaX and caspase 3. These findings suggest that licorice extract is effective in treatment of gastric cancer (14). Another study pointed out that the licorice extract induces elevated expression of BaX and decreased expression of Bcl2, MKN-28 (significantly), AGS (moderately), and MKN-45 cells (slightly). Therefore, the increase in the ratio of Bax/Bcl-2 leads to release of cytochrome C from mitochondria to the cytosol where cytochrome C can bind to apoptotic protease, activating factor1 that is also known as APAF-1. This causes inducing apoptosis in cells and caspase-3 and poly (ADP-ribose) polymerase (PARP) activation (15). The obtained results of our study showed two compounds derived from licorice (Glycyrrhetinic acid and Glycyrrhizic acid) had a toxic effect on carcinogenic cells. According to our study, Glycyrrhetinic acid is more effect than Glycyrrhizic acid in treatment of gastric cancer. Therefore, Glycyrrhetinic acid as an effective combination treatment can be employed for treatment of gastric cancer. Although the combination of Glycyrrhizic acid prevents the growth of carcinogenic cells, its influence is more gradual than Glycyrrhetinic acid. These differences might lead to various neurological pathways to prevent the growth of carcinogenic cells and could induce different effects on the AGS cell line. Impacts of oxidative stress on numerous diseases such as cancer (16,17) and also the effects of antioxidants on various diseases, especially the diseases associated with free radicals and oxidative stress such as cancer (19, 20), diabetes (21-23), infection (24,25) and atherosclerosis (26) (27) have been determined. In other words, the antioxidant effects of Glycyrrhetinic acid and Glycyrrhizic acid might result in carcinogenic cells' death. There are numerous plants with antioxidant properties (25, 28-31), the anti-carcinogenic effects of which must be examined.

According to our study, Glycyrrhetinic acid had a more significant impact on treatment of gastric cancer in a shorter time as compared to Glycyrrhizic acid. Thus, it can be applied for gastric cancer treatment. Further in-vivo studies are required to determine the positive effects of these components on gastric cancer treatment

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