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The Anticancer Effect of *Arctium lappa* and *Glycyrrhiza glabra* on HT-29 Colon Cancer and MCF-7 Breast Cancer Cell Lines

Shiva Alizadeh Nazmi[1], Alireza Nourazarian[2*], Reza Bahhaj[3], Fatemeh Khakikhatibi[4,2]

Abstract

Objectives: Breast and colon cancer are the most common malignancy in the world and the common causes of mortality in Western societies. The aim of the current study was to examine the effectiveness of *Glycyrrhiza glabra* and *Arctium* extracts on breast cancer (MCF7) and gastrointestinal cancer (HT 29) cell lines, with regard to their naturalness, low cost and easy public access.

Materials and Methods: In this study, dose and time were considered as variables in order to determine the optimal effects of the drug. Two MCF7- and HT29 cell lines were cultured on a single layer in RPMI 1640 culture medium and MTT method and flow cytometric method were used to examine apoptosis.

Results: The study of cell survival using MTT showed that the aqueous extract of *G. glabra* at a dose of 2000 µg/mL and incubation for 24 hours had the most inhibitory effect on the MCF7- cell line. In addition, a dose of 2000 µg/mL of *G. glabra* aqueous extract with -24hour incubation represents apoptosis by flow cytometric method.

Conclusions: The results of this study confirm the effectualness of this herbal supplement for the treatment of breast cancer, and the inclusion of this herbal supplement in the diet may be effective in treating breast cancer, given the naturalness of the product, its low cost and public availability.

Keywords: *Arctium, Glycyrrhiza*, Colonic Neoplasm, Breast Neoplasm

Introduction

Breast cancer as the most prevalent cancer among women worldwide (1) includes 3% of all cancers and 15% of malignancy-related deaths among women are related to it (2). Breast cancer accounts for 41.24% of all reported cases of cancer in Iran (3). Despite important advances in its diagnosis and management, it is a common disorder in women.

Surgery and complementary therapies such as medication, chemotherapy, and radiotherapy are the methods used to treat breast cancer (4). Although the use of effective antitumor drugs such as tamoxifen and raloxifene or anastrozole accompanied by radiation therapy or therapeutic adjuvants has saved the lives of many women over the past 30 years (5), several disadvantages have limited the use of these therapeutic systems, including recurrence of the disease caused by metastasis in a few months after treatment, the effects of taking medications on the metabolism of glucocorticoids, and the risks of cancer after chemotherapy for breast cancer (6,7).

Gastrointestinal cancers, especially colon cancer, are among the most common causes of mortality in Western societies. These cancers are also increasing because of the changes in the pattern of nutrition in developing societies so that it is now the third most prevalent malignancy in men and women. Different methods have been proposed for the treatment of colon cancer such as surgery, chemotherapy, and immunotherapy but they did not have any significant success, and most of these methods were effective only in delaying its growth (8).

Recently, the effects of medicinal plants have been widely addressed by researchers, and a number of studies have reported the anticancer functions of *Glycyrrhiza glabra* (9). The licorice plant with the scientific name of *G. glabra* is a perennial herb from the Fabaceae family that is used in pharmaceutical, nutritional, and even tobacco compounds (10). Licorice can be found as a weed in the fields of cultivating vegetables, wheat, cotton, potatoes, sugar beet, and forage, including clover; it prevents

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[1]Department of Toxicology, Ahar Branch, Islamic Azad University, Ahar, Iran. [2]Department of Biochemistry and Clinical Laboratories, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran. [3]Department of Parasitology and Mycology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran. [4]Drug Applied Research Center, Tabriz University of Medical Sciences, Tabriz, Iran.
*Corresponding Author: Alireza Nourazarian, Tel: +984133364666, Fax: +984133364666, Email: alinour65@gmail.com

the growth of the products in fields and gardens due to the large spread of its roots (12). *G. glabra*, which is the scientific name of this plant, is a Greek name derived from the two words: glycosis (sweet) and rhiza (root). *Shajar al sus* and *Araq sus* are the Arabic names of this plant. The meaning of glabra is smooth and uncurled; it is named so because of the lack of cracks on its fruit (12,13).

The main substance in the root of this plant is glycyrrhizin, which is at least 50 times sweeter than sugar. This substance is involved in the treatment of peptic ulcer disease and helps to reduce gastric acid by inhibiting the *Helicobacter pylori* (12-14).

The amount of glycyrrhizin increases with the increase of the age of the plant so the licorice root has the highest glycyrrhizin content in its last years (16,17).

Other substances include sugar, starch, asparagine, and resin, which have anti-inflammatory properties and prevent the metabolism of cortisol in the body (18,19). In addition, other substances including flavonoids present, which are important for pharmaceutical purposes. Flavonoids have a major effect on reducing melanin production, which reduces hyperpigmentation. Licorice has many different properties, including the licorice root, which is useful for the treatment of tooth decay and its antimicrobial properties are used as an anti-decay substance in mouthwashes and toothpastes. The most important property of licorice is its usefulness in the treatment of peptic ulcer and gastric cancer that has been discovered in recent years. The licorice plant has a laxative effect and is useful for reducing abdominal bloating. Licorice is used in traditional Asian and European medicine to treat gastritis, respiratory infections, and peptic ulcers (20,21).

In addition, it is used in the traditional medicine of Iran to treat stomach ulcers and prevent coughing (22).

Arctium also called the burdock belongs to the Asteraceae family, which is a plant of dicotyledons. It is wildly grown in the temperate regions of Asia and some parts of Iran, such as the Alborz, Khorasan, Kerman, and Rudbar areas. The root of the plant is spindly and long and has a brownish skin with a white content, and sweet flavor. This plant contains various compounds including inulin, carbonate, potassium nitrate, various resins, and glucoside (1,23).

The aim of the current study was to examine the effects of *A. lappa* and *G. glabra* on the growth of colon cancer cell line (HT-29) and breast cancer cell line (MCF-7).

Materials and Methods

Extraction of *Arctium lappa* and *Glycyrrhiza glabra*

The present research was carried out in the Faculty of Advanced Medical Sciences, Tabriz University of Medical Sciences, the extraction laboratory of Islamic Azad University, Marand Branch and Tabriz Pharmaceutical Sciences Research Laboratory. The plant materials were obtained from the Research Center of the Agricultural and Natural Resources of East Azerbaijan, Iran.

Some of the roots of the *G. glabra* were cut into smaller pieces and then were placed in an oven at 45°C for 24 hours until the final moisture content of the samples reached less than 5%. Then they were transferred to a desiccator; the samples were powdered by a mill after cooling down and then passed through a laboratory sieve with a mesh of 50 µm to achieve the same size gradation. We could not use the Clevenger apparatus in large quantities to obtain the aqueous extract of *Arctium lappa*, due to the presence of inulin. For this reason, a specified amount of the root powder was mixed with distilled water and treated in a Bain-Marie at 65°C for 6 hours.

The obtained extracts were strained under vacuum using a Whatman 40 paper filter and a Buchner funnel and then were subjected to filtration by centrifugation at 4500 rpm for 10 minutes at room temperature. We then filtered these solutions with a 0.22 mL filter to remove microorganisms.

The whole extract was filtered after collection. The filtered extract was then vaporized at low pressure. The product was dried by freezing and the resulted powder was kept frozen (-70°C).

Cell Culture and Treatment

For this study, the MCF-7 and HT29 cell lines were purchased from the cell bank of Pasteur Institute (Tehran, Iran). The tissue culture medium was prepared by mixing with medium RPMI-1640 (Sigma) as minimal essential tissue culture, 10% heat-inactivated FBS (fetal bovine serum: Gibco-Life technologies), 100 units/mL of penicillin, and 100 µg/mL of streptomycin (Sigma). Cells were grown at 37°C in a humidified 5% CO2 atmosphere and the medium exchange.

The cultured cells were treated with 20, 200, and 2000 µg/mL of *A. lappa* and *G. glabra* extracts for various periods of time (24, 48, and 72 hours). The treated cells were tested for viability using control cells and were reported as a percentage (9).

MTT Assay for Cell Viability

The effect of *A. lappa* and G. glabra extracts on preventing the proliferation of the HT29 and MCF-7 cell lines was assayed by the MTT assay. The cultured cells were seeded at a density of 15 000 cells/well in 96-well tissue culture plates. Then, the incubation was done at 37°C in an incubator that was humidified by 5% CO2. The cultured cells were then treated with different concentrations of these extracts (20–2000 µg/mL) until 60%-70% confluence. For the MTT assay, MTT solution (2 mg/mL) was poured to each well and waited in the incubator at 37°C for 3 hours. The medium was poured out. The crystallized blue formazans were then solvated in a mixture of 200 µL DMSO and 25 µL Sorenson buffer. The absorbance of the mixture was read at 570 nm in a microplate reader (BioTek, model ELx808) (9).

Flow Cytometry

The flow cytometry approach was used to measure apoptotic rate. The HT-29 and MCF-7 cultured cells were plated for 24 hours in 6 plates with a proportion of 1 × 10^6 cells/wells. The cells were then treated with 20 µg/mL and 2000 µg/mL of *A. lappa* and *G. glabra* at 37°C and 5% humidified CO2 for additional 24 hours. The cells of various time groups along with their time-matched controls were then collected and centrifuged at 1200 rpm for 10 minutes. The cell pellets were washed with PBS buffer 2 times. Thereafter, the cells were resuspended in a 500 µL binding buffer (Ref No: 00-0055-56, eBioscience) for additional 15 minutes. Incubation at room temperature was done for 15 minutes and then with 100 µL binding buffer that contained 5 µL of FITC-conjugated Annexin V (Ref No: 11-8005-74, eBioscience). Another wash was carried out for 15 minutes by binding buffer and exposing them to 5 µL of PI solution (Ref No: 00-6990-42, eBioscience). Finally, they were examined by a flow cytometer with an excitation wavelength of 488 nm and detection at 515 nm for Annexin V.

Statistics

The data were entered into SPSS version 19.0 for the statistical analysis. To compare the groups, the statistical procedures of ANOVA and Tukey-Kramer multiple comparisons were carried out. The significance level was $P<0.05$.

Results

The Effects of *Arctium lappa* and *Glycyrrhiza glabra* Extracts on the Growth of Colon Cancer Cell Line HT-29

To examine the effects of *A. lappa* and *Glycyrrhiza* extracts on the growth and proliferation of the HT-29 colon cancer cell line and MCF-7 breast cancer cell line incubation with different doses of *A. lappa* and *G. glabra* extracts followed the MTT assay for 24, 48, and 72 hours was carried out.

The results showed that the cytotoxic influence of *G. glabra* on the cells treated with it were dependent on time and dose. A significant reduction was observed for the viability of the treated cells incubated for 24, 48, and 72 hours. The findings also indicated that the *G. glabra* extract prevented HT-29 and MCF-7 cell proliferation in 200 and 2000 µg/mL dosages after 24 hours of incubation (Figures 1 and 2).

There were not any cytotoxic effects of *A. lappa* on the viability of the treated cells incubated for 24, 48, and 72 hours in the HT-29 and MCF-7 cell lines (Figures 3 and 4).

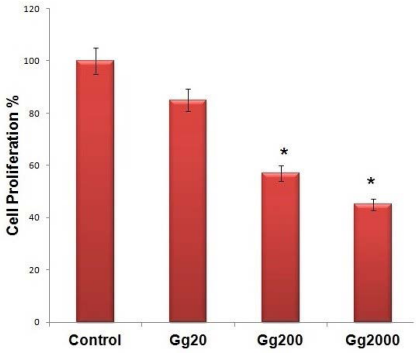

Figure 1. Effects of *Glycyrrhiza glabra* (Gg) Extract on Proliferation of Colon Cancer Cell Line HT-29.
The cell viability was expressed as the percentage of control over 24 hours. Study groups: Control, Gg20 (20 µg/mL of *Glycyrrhiza glabra*), Gg200 (200 µg/mL *of Glycyrrhiza glabra*), Gg2000 (2000 µg/mL *Glycyrrhiza glabra*).

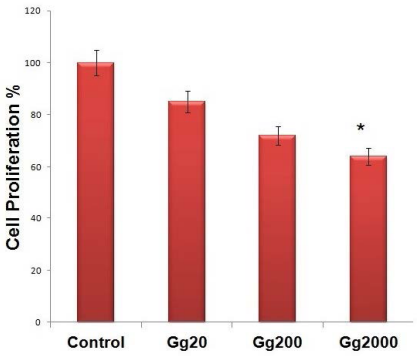

Figure 2. Effects of *Glycyrrhiza glabra* (Gg) Extract on Proliferation of Breast Cancer Cell Line MCF7.
The cell viability was expressed as the percentage of control over 24 h. Study groups: Control, Gg20 (20 µg/mL of *Glycyrrhiza glabra*), Gg200 (200 µg/mL *Glycyrrhiza glabra*), Gg2000 (2000 µg/mL *Glycyrrhiza glabra*)

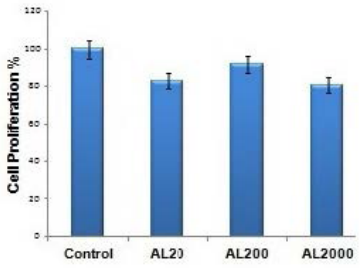

Figure 3. Effects of *Arctium Lappa* Extract on Proliferation of Colon Cancer Cell Line HT-29.
The cell viability was expressed as the percentage of control over 24 h. Study groups: Control, AL 20 (20 µg/mL of *Arctium lappa*), AL 200 (200 µg/mL of *Arctium lappa*), AL2000 (2000 µg/mL of *Arctium lappa*).

Flow cytometry

According to the results, in 52.7% and 68.3 % of the HT-29 cells treated with 200 µg/mL and 2000 µg/mL of *G. glabra* extract, there was significant occurrence of apoptosis ($P<0.05$), compared to the control group, in which the apoptosis was 21.3%, (Figure 5a). In addition, in 40.8% and 53.3% of the MCF-7 cells treated with 200 µg/mL and 2000 µg/mL of *G. glabra* extract, apoptosis was significantly higher than that in the control group (18.2%) (Figure 5b).

Apoptosis of human colon cancer cell line HT-29 (expressed by percentage) incubated with 200 µg/mL and 2000 µg/mL *G. glabra* extract differed meaningfully from

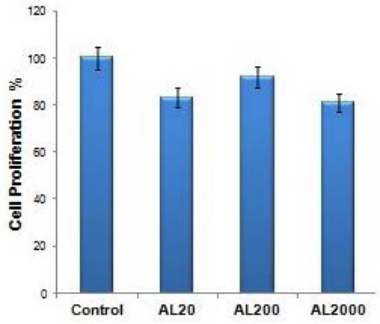

Figure 4. Effects of *Arctium Lappa* Extract on the Growth of MCF7 Breast Cancer Cell Lines.
The cell viability was expressed as the percentage of control over 24 h. Study groups: Control, AL 20 (20 µg/mL of Arctium lappa), AL 200 (200 µg/mL of *Arctium lappa*), AL2000 (2000 µg/mL of *Arctium lappa*).

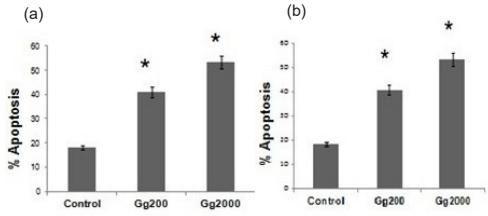

Figure 5. Effects of *Glycyrrhiza glabra* Extract on Apoptosis Rate in Colon Cancer Cell Line HT29 (**a**) and Breast Cancer Cell Line MCF7 (**b**) After 24 hours.
Study groups: Control, Gg200 (200 µg/mL of *Glycyrrhiza glabra*), Gg2000 (2000 µg/mL of *Glycyrrhiza glabra*).
* The difference between the treated cells and control was significant ($P<0.05$).

treated cells and controls ($P<0.05$).

Discussion

Cancer as one of the most complex health problems imposes high costs on individual and the community. Breast cancer is the most prevalent type of malignancy in women. It is also the second leading cause of death after lung cancer in them. The prevalence of death because of breast cancer in the United States in 2014 was around 40 000 women (24). Hence, many studies were carried out in most parts of the world in order to find ways to cure this health problem.

One of the therapeutic approaches used by experts is chemotherapy with drugs that inhibit the growth of cancer cells. Tamoxifen is one of the drugs that is commonly used as first-line treatment for breast cancer. This drug is pharmacologically related to a family of selective estrogen receptor modulators (SERMs) that selectively binds to estrogen receptors in eligible cells and exerts its antagonistic effects on estradiol (25). Despite the massive use of tamoxifen and other drugs from this family, there are problems and barriers to the use of this drug. First, it has been shown that these drugs can have side effects such as increased weight gain, increased risk of endometrial cancer, venous thrombosis, and stroke. Women who use this medication also complain of increased temperature and hot flashes (26). Therefore, many efforts have been made to find alternative compounds with the appropriate therapeutic and non-adverse effects. Phytoesterogenic compounds in plants have been introduced as viable ingredients. These compounds have a structure similar to that of endogenous estrogens and can mimic its effects to a degree. In addition, they will have fewer side effects, since they are naturally occurring compounds.

Extracts of *G. glabra* were used for treating a number of diseases in Iranian traditional medicine since a long time ago. Reactive oxygen species cause great damage to various parts of tissue cells and have numerous pathological side effects. DNA damage, peroxidation of proteins and lipids, as well as cellular degeneration, are some of the main pathophysiological side effects of diseases like cardiovascular disorders, inflammatory diseases, aging, malignancy, and some other disorders (9). Since *G. glabra* is a potent antioxidant and has the distinctive capability of scavenging free radicals, Nagaraj et al proposed that its extract could bring about protective effects against ischemic injuries. (28). The present study showed similar results, supporting that the extract of *G. glabra* could have pro-apoptotic and anti-proliferative effects on HT-29 as well as MCF-7 cell lines. The results also indicated that *G. glabra* could potentially serve as a cancer chemoprotective agent, but making firm claims on its use as a supplementary anticancer agent requires more thorough studies. The aqueous extract of *G. glabra* has been involved in inhibiting the growth of breast cancer and colon cancer. Researchers should pay special attention to these two plants and take a step towards reducing the pain and problems of these types of patients by conducting research and production of anti-cancer drugs with fewer complications. It is necessary to carry out in vitro experiments using various solvents and cancer cell lines. Isolation and characterization of the chemical structure(s) of *G. glabra* and showing its significant in vitro activities may lead to the conduction of in vivo studies.

Conflict of Interests

No competing financial interest exists.

Ethical Issues

Working with cell lines in the current research was in accordance with standard condition and guidelines of Islamic Azad University of Ahar (number: 2201/0603921013), Ahar, Iran.

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