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EXAMINING THE EXPRESSION CHANGES OF GENES RELATED TO INFLAMMATORY CYTOKINES IL-6 AND IL-4 IN CACO-2 CELL LINE TREATED WITH GLYCYRRHIZIN

***Ali Sami Atshan (Corresponding Author)**
M.Sc. in Molecular Biology Islamic Azad University, Science and Research Branch, Tehran, Iran
E-mail: alisamibio79@gmail.com

Kawther Chasib Bardan
College of Science, Al-Muthanna University, Iraq
E-mail: Kawther.allinone@gmail.com

Haider Ali Hussain
College of Science, Al-Muthanna University, Iraq
Email: alialiali333336@gmail.com

Haneen Mohammed Karim
College of Science, Al-Muthanna University, Iraq
Email : anhaneenmohammad@gmail.com

Abstract:

Colorectal cancer is one of the most common types of fatal cancer worldwide. In recent years, extensive studies have been conducted on medicinal plants for their anti-cancer effects, including licorice. The aim of this research was to investigate the expression changes of genes related to inflammatory cytokine IL-6 and IL-4 in CaCO-2 cell line treated with licorice extract (glyceryzin). Licorice plants were collected from different regions and then their dry roots were ground into powder and finally extracted. CaCO2 cell line was purchased from Pasteur Institute of Iran and DMEM medium was used for cultivation. Cells were cultured with different concentrations of the extract (determined by Bradford) and after 24 hours, the lethal effects of licorice extract (glycyrrhizin) were investigated by MTT colorimetric method. Then the cells were cultured with a concentration of 59.90 µg/mL and the RNA of the cells was extracted using the kit. After cDNA synthesis, the expression of IL-4 and IL-6 genes compared to control samples was evaluated by Real Time PCR technique. Licorice extract (glycyrrhizin) significantly ($P<0.05$) decreased the viability of treated cancer cells compared to untreated cells, which was concentration-dependent. The concentration of IC50, equivalent to 59.90 µg/mL, was observed. The relative expression of IL-4 and IL-6 cytokine genes in the samples treated with glycyrrhizin extract was reduced by 2.097 and 2.094 times, respectively, compared to untreated cells. Licorice extract (glycyrrhizin) has anti-cancer properties

against CaCO-2 cells and it can probably be used as an anti-cancer drug or agent with clinical studies.
Key words: CaCO2 cell line, licorice extract (glycyrrhizin), IL-4 gene, IL-6 gene.

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1. Introduction

According to new and updated research from the World Health Organization, in 2023, the incidence and mortality rate of cancer in the United States was 1,958,310 new cancer cases and 609,820 deaths (Siegel et al., 2023).

Cancer occurs as a result of the accumulation of various genetic mutations that cause uncontrolled cell division and tumor formation. Tumor growth is a stage that changes in terms of tissue shape, which is accompanied by morphological, molecular and genetic changes). Aseel Shomar et al., 2022)

Statement of the problem

Colorectal cancer is the third most common cancer (after lung cancer and breast cancer) worldwide, and is more common in men (10.1%) than in women (9.2%). The total number of deaths from colorectal cancer is 693,933 (8.5%) (Morini et al., 2017).

Ten human Caco-2 cells are an experimental model of the intestinal epithelial barrier. Caco-2 cells are derived from colon adenocarcinoma and reproduce the morphological and biochemical characteristics of polarized enterocytes in the small intestine. Caco-2 cells are mainly used to evaluate the bioavailability of experimental drugs, but are also used to study lipid and cholesterol homeostasis, including cholesterol absorption and clearance. (Hipple et al., 2020).

Research objectives

To study the change in genes associated with the inflammatory cytokines IL6 and IL4 in the CaCO-2 cell line treated with glycyrrhizin Importance and necessity of research Colorectal cancer (CRC) is the second most deadly cancer. Global incidence and mortality rates are likely to rise and fall in the coming decades. Although the mortality rate associated with CRC is high in high-income countries, the incidence and mortality rate associated with CRC are also increasing in developing countries. Early diagnosis of CRC can be completely cured with surgery and subsequent medications. However, the recurrence rate of the disease is high and drug resistance of cancer increases the rate of treatment failure. Access to early diagnosis and treatment for CRC for survival is somewhat possible in developed countries. However, these facilities are rarely available in developing countries. Given that conventional methods such as chemotherapy have side effects today, researchers are looking to develop new methods that can replace these classical methods or can successfully work as a complement to the aforementioned methods. Therefore, plants are used today as medicinal and therapeutic compounds to inhibit some of the inflammatory factors involved in the path of colorectal cancer. For example, two interleukins 6 and 4 are included in the group of inflammatory cytokines, and it is known that interleukin 6 is produced by macrophages stimulated by cancer cells, and this interleukin is able to activate STAT3 in cancer cells. IL-6 as a growth factor for intestinal cancer cells is known to inhibit negative signals IL-6 interferes with the growth of cancer cells. On the other hand, IL-4 as one of the systemic suppressive factors has been identified. IL-4 is an inflammatory toxin that alters the fate of innate and adaptive spermatocytes, mainly of the phenotype Th2 cells. It supports T cells that in this way can exert its influence on the patient's immune and adaptive system and also faces challenges in the treatment approach. For this reason, plants such as licorice that contain biologically active compounds such as glycyrrhizin are currently considered as a therapeutic approach. They are specifically positioned; Because these biologically active compounds have important properties such as anti-inflammatory properties (anti-interleukin 4 and 6 activity) and antioxidants that can successfully treat diseases (such as cancer) associated with these conditions. Given the importance of this topic, in this research we will try to use the CaCO-2 cell line with the anti-inflammatory effect of glycyrrhizin extract to reduce the expression of anti-inflammatory genes IL-4 and IL-6.

2. Materials and Method

The new and innovative aspect is in the merit

According to the investigations conducted on the Internet, there is no study yet that investigates the changes in genes associated with the inflammatory cytokines IL-6 and IL-4. Cell line CaCO-2 treated with glycyrrhizin not found

Objectives

To study the change between genes associated with inflammatory cytokines IL6 and IL4 in CaCO-2 treated with special purpose glycyrrhizin (partial):

- Collection and extraction of plants
- Culture of CaCO-2 cell line
- Study of toxicity of licorice plant extract on CaCO-2 level by MTT method
- IL-4 inflammatory gene expression in the presence of licorice extract (glycyrrhizin) by real-time PCR method
- IL-6 inflammatory gene expression in the presence of licorice extract (glycyrrhizin) by real-time PCR method

S Objectives

- Licorice extract (glycyrrhizin) does CaCO-2 have toxicity on the cell line?
- Licorice extract (glycyrrhizin) reduces gene expression Does IL-4 convert to CaCO-2 in the cell line?
- Licorice extract (glycyrrhizin) reduces gene expression Does IL-6 convert to CaCO-2 in a cell line?

Hypotheses of merit

- Licorice extract (glycyrrhizin) CaCO-2 is cytotoxic.
- Licorice extract (glycyrrhizin) causes a decrease in the IL-4 gene in a CaCO-2 cell line.
- Licorice extract (glycyrrhizin) causes a decrease in the IL-6 gene in a cell line CaCO-2.

3. Discussion

Chinese medicinal plants have been widely used in Chinese medicine. S.D.G.S.R.Z.K (GA) or glycyrrhizin is the main active ingredient in the Chinese plant licorice root. Previous studies have shown that GA has anticancer activity against various types of cancer. For example, in one study, GA increased cell proliferation by inducing G1/S phase arrest and apoptosis. It reduces GC (Wang et al., 2020). Previous studies have shown that GA and its derivatives have various medicinal effects such as detoxification, anti-inflammatory, bronchodilator, antitumor, anti-wound and anti-inflammatory. The antitumor effect of GA has been reported in various types of tumors, such as lung, liver, breast and cervical tumors, as well as in hematological malignancies such as leukemia. (Wu et al., 2018; Lin et al., 2018). In one study, gamma-aminobutyric acid showed low toxicity and the LD50 value in rats was estimated after a single oral dose of 2000 mg/kg. (Nazari et al., 2017). In general, leave Turkey gamma-aminobutyric acid is widely used in the treatment of Russian hepatitis and liver cancer. (Yang et al., 2017; Li et al., 2014a). Previous studies have shown that. GA can regulate many important signaling proteins, including: proteins associated with the aspartate-dependent protease (caspase) and Bcl-2 families, nuclear factor-kappa B (NF-κB), high-mobility group box-1 (HMGB1), extracellular-regulated protein kinases (ERK), phosphodiesterase 3-kinase (PI3K)/AKT, and glycogen-3-kinase (GNK) (Wang and Du, 2016; Afnan et al., 2016). However, a limited number of reports have examined the toxic site through which GA affects a variety of cancer cell lines.

Cell proliferation inhibition can occur by cell cycle arrest. Cyclins and cyclin-dependent enzymes (CDKs) are vital proteins that regulate cell cycle progression. The study results showed that T. Mar GA reduces the level of G1 phase-associated proteins (Wang et al., 2020). Activated D-type cyclins (cyclins D1, cyclins D2 and cyclins D3) bind to CDK4 and CDK6 to form cyclin D-CDK4/CDK6 complexes, leading to phosphorylation of the neoblast receptor protein and subsequent degradation

of the transcription factor C becomes E2F. After release, Ronno factor Y.S E2F promotes downstream target genes and facilitates S phase entry. Mazda's regulation of D-type cyclins, especially cyclin D1, can directly lead to proliferation and progression of the cell cycle from G1 to S phase (Musgrove et al., 2011). E-type cyclins (cyclin E1 and cyclin E2) are first synthesized in the middle G1 phase. Their expression reaches its maximum levels at the G1/S transition, and then degrades at the end of the S phase. They serve as key regulators of the transition from G1 to S phase. A similar action has also been observed for D-type cyclins. E-type cyclins bind to and activate CDK2, as a result of which cells enter the S phase (Teixeira and Reid, 2017). Changes in the level of cyclin proteins induced by treatment of GA cells are associated with the arrest of the G1/S phase of the cell cycle (Wang et al., 2020(.

In this study, the toxicity level of GA on the cell line was determined. CaCO-2 assay was performed at different concentrations by MTT assay. Different concentrations of GA were used to treat cells and induce apoptosis on CaCO-2 cell lines. Their effect was evaluated. The results showed that GA significantly ($P < 0.05$) reduced the viability of treated cancer cells compared to untreated cells, a concentration-dependent reduction. The required concentration of GA reduces 50% of cell viability (IC 50) for evaluating the IL-4 and IL-6 genes. In this assay, the IC 50 value was recorded, which is equivalent to 59.90 µg/µl. As mentioned earlier, unfortunately, there are limited studies on the toxic effects of GA on cancer cells, especially CaCO-2. This has been done and this topic makes discussing the results obtained in this study with the results of previous studies difficult. In a similar study, Al-Sandoni et al. (Al-Sandoni et al., 2019) showed that the cytotoxicity test of glycyrrhizin derivatives ME-GA (18β-glycyretinic acid -30-methyl ester) and AKBA (3-acetyl-11-keto-β-butylic acid) showed stronger cytotoxicity against the hepatocellular carcinoma cell line HepG-2. The IC50 to Tert B was 1/06 ± 5/25 and 0.89 ± 73/19. Both were turkey porcine rather than human fibroblast cell line. WI-38 showed higher selectivity for hepatocellular carcinoma. The presence of methyl ester at C-30 increased the cytotoxicity of ME-GA, which may be attributed to its higher activity and selectivity. Both ME-GA and AKBA played a role in inhibiting cancer cell migration in the wound healing process and preventing the formation of clones. (Senduny et al., 2019). Using flow cytometry to analyze and analyze the cell cycle and identify the site of action of potential apoptotic toxins, ME-GA arrested the cell cycle at G2/M, inhibited liver cancer and induced apoptosis through the extrinsic pathway and its ability to increase activation was p53 (Senduny et al., 2019). Although the cell lines and doses studied in this study are different from Senduny et al.'s research, both confirm the toxicity of GA on cancer cells. Also, in the study of Chueh et al. (Chueh et al., 2012), it was reported that GA causes morphological changes, G0/G1 phase arrest, apoptosis and DNA damage in WEHI-3 cancer cells, which is consistent with this study. The flow cytometry results also showed that GA increased the level of ROS, decreased the mitochondrial membrane potential ($\Delta\Psi m$), and stimulated the activity of caspase-3 in WEHI-3 cells. GA regulates the expression of intrinsic and extrinsic apoptosis-related proteins. In addition, the endoplasmic reticulum (ER) stress responses in WEHI-3 cells treated with GA were observed (Chue et al., 2012). Nita J. Wang and colleagues (Wang et al., 2020) demonstrated that GA had a time- and dose-dependent inhibitory effect on GC cell proliferation with the same trend as the results obtained in the following study. Flow cytometry analysis showed that GA induces G1/S phase arrest and apoptosis. GA treatment increased the levels of G1 phase-related proteins, including cyclin D1, D2, D3, E1, and E2. B.N. The levels of Bcl-2, survivin, and p65 decreased after treatment. In addition, GA reduces phosphorus from copper and copper PI3K/ACT.

Also, the toxic effect of GA with other compounds has been reported before. For example, Lallemand et al. (2011) prepared GA compound coupled with vanillyldynastone at C-30 and had significant effects on cancer cells. A549, SKMEL, T98G, HS683, U373, PC3, and MCF7 were reported with IC50 ≥ 3. In particular, this combination had significant effects on cancer cells. A5349 and PC3 with IC50: 2.0 M/cromol. Li et al. (2016) prepared GA derivatives coupled with isofluronic acid and trans-4-hydroxylacid derivatives at C-30. This compound was found to have cytotoxic effects against breast cancer cell lines (MDA-MB-231) with IC50 between 5 and 5.9 M chromol, while GA was only active against MCF-7 with IC50: 8.5 M chromol. The results of one study showed that both the

early and late apoptosis rate of gastric MGC-803 cancer cells seeking treatment were decreased by GA addition (Ershad et al., 2022). Current studies have shown that GA has different effects on inducing apoptosis and necrosis under different conditions. In tumors, GA induces apoptosis, while it attenuates apoptosis during liver and brain injury (Pang et al., 2016; Zhao et al., 2016). The caspase family of proteins is known to play an important role in the process of apoptosis. Caspase-dependent apoptosis is a sequential reaction "cascade model" that begins with the activation of initiator enzymes such as caspase-2, 8-, 9-, and 10- and with the increase in their expression level forever the auto-activation of copper activates the intrinsic and extrinsic apoptosis. After activation, the initiators are that they stimulate a series of downstream caspase proteins and finally activate caspase-3, 6, 7, and 14, which are factors in the apoptotic process (Kiraz et al., 2016). However, a study by Ershad et al. (Ershad et al., 2022) showed that GA treatment did not change the levels of cleaved caspase proteins in MBC-803 cells, suggesting a caspase-independent apoptosis of gastric cancer cells. In addition, the levels of other key apoptosis-related proteins changed, including Bcl-2, Bax, cleaved PARP, Survivin, and p65. Bcl-2 Bax is an anti-apoptotic protein that prevents the release of apoptotic factors into the cytoplasm, while Bax penetrates the outer mitochondrial membrane to release increased apoptotic factors and accelerate the apoptosis process (Singh et al., 2019). Similar results were observed in breast cancer in the study of Lin et al. (Lin et al., 2018). The surface ratio of BN Bcl-2 and Bax is used to reflect the apoptotic cascade (Edlich, 2018). PARP as a DNA repair enzyme and substrate of the caspase family of enzymes. During the induction of apoptosis, the N protein The full-length PARP is cleaved into a catalytic domain (89 kDa) and a binding domain (24 kDa), which is considered the hallmark of apoptosis. (Jobin et al., 2016). N protein Survivin exhibits anti-apoptotic effects by inhibiting caspase family proteins. (Perry et al., 2017). Previous studies have shown that GA treatment by blocking the AKT/mTOR/STAT3 signaling pathway downregulates serotonin (He et al., 2015). p65 is one of the active subunits of NF-kB protein responsible for regulating cell proliferation and apoptosis (Xia et al., 2018). GA has been reported to inhibit cell growth and possibly induce apoptosis by downregulating p65. (Li et al., 2014b).

RS Genal PI3K/AKT plays a role in regulating many biological processes, including inhibition of apoptosis and cell proliferation. PI3K can be activated by T receptors and G protein-coupled receptors activated by Ras. This activation leads to lysine phosphorylation and subsequent activation of AKT at residues Ser473 and Thr308 (Ersahin et al., 2015). Phosphorylated AKT targets more than 100 proteins and leads to tumorigenesis. It can protect cardiomyocytes from H2O2-induced damage by reducing apoptosis. (Li et al., 2018). Phosphorylated AKT regulates p21cip1 and p27kip1, leading to induction of G1/S phase arrest. It occurs in thyroid cancer cells (Zhang et al., 2019). In addition, reduction of phosphorylated AKT inhibited proliferation and caused G1/S phase arrest and apoptosis in glioma cells, resulting in significant decreases in Bcl-2 and total N d1.39 levels (Li et al., 2017). In the study of Ershad et al., PI3K and AKT were decreased, which may be responsible for cell cycle arrest and apoptosis in gastric cancer cells. M1 macrophages secrete pro-inflammatory cytokines such as interleukin-12 (IL-12), TNF-α, and IFN-γ and express high levels of iNOS. In contrast, M2 macrophages produce anti-inflammatory cytokines such as interleukin-10 (IL-10), IL-14, and IL-4 (IL14, IL4). (Lin et al., 2019). Macrophages that encounter cancer cells are activated to convert them to the tumor phenotype. M1 polarization by iNOS induction and simultaneous production of large amounts of NO has not been determined to cause cancer cell death (Weigert and Brun, 2008). Furthermore, NO increases the polarization of M1 macrophages in the TME leading to the production of NO through positive regulation of iNOS. However, TME is a KBN toxin that inhibits iNOS and M2 macrophage-derived anti-inflammatory mediators. The activity of macrophages is neutralized by M1, causing repolarization of M1 macrophages to M2 macrophages. Therefore, the fall of the M1 macrophage induction method may be an effective way to increase cancer cell death (Mintz et al., 2021). Recent studies have shown that glycyrrhetinic acid (GA), the hydrophobic part of GL, causes macrophages to polarize to the P phenotype becoming M1. In addition, it has been shown that GA M2 macrophages will change to M1 macrophages (Mao et al., 2015(.

E-interleukin belongs to the group of cytokines, which are perhaps the most important messenger molecules produced by leukocytes to modulate their activity. Or paracrine Several groups of ILs have

been identified (Oyang and O'Garra, 2019). Among them we can mention IL-1, IL-2, IL-3, IL-5, IL-6, IL-10, IL-12, IL-13 and many others. Although more beautiful ILs under the influence of R GL and GA are IL-12 has the greatest effect IL-12 C is a heterogeneous cytokine produced mainly by macrophages and monocytes [8]. Its main function is to modulate cytokines and T cell subsets (Dai et al., 2021). A study showed that infertility D Endogenous IL-12 affects the development of immunodeficiency in HIV-infected patients (Dai et al., 2021). Studies have shown that IL-12 rescues many activities of HIV-infected cells (Al-Shahimi et al., 1992). Several studies have shown that IL-12 can affect T and Y cells The natural NK cells have an effect that leads to cell proliferation, cytolytic activities and stimulation. IFN-γ becomes (Dai et al., 2021 , Trincheri, 2014). Other studies have shown that the polarization of the T helper response to the dominant Th1 form through IL-12 is promoted by IFN-γ and leads to the inhibition of its production IL-4 becomes (Trincheri, 2013). GA is able to block the subsets of IL-1β, IL-3, IL-5, IL-6, IL-10, IL-12, IL-13, evotaxin and TNF-α and is able to promote lymphocyte proliferation and act as a signal transduction facilitator during lymphocyte development. It induces the action of IL-2 (Bordbar et al., 2012; Raphael and Cottan, 2003). Fu et al. showed that GA induces apoptosis in PC-3 prostate cancer cells. In addition, BNC completely suppressed B1 and cdc2, and as a result, caused cell arrest in G2/M. The effects observed in this study can be attributed to inhibition of phosphorylation in solution. Rb reduced the BNC transcription factor E2F, reduced total cyclin D1, reduced CDKs 4 and 6 and increased total BNCs (Fu et al., 2004).

In another study by Zhou et al. (Zhou et al., 2014) it was suggested that licorice root extracts can reduce the carcinogenic effects of endocrine disrupting chemicals in breast cancer cells by arresting the cell cycle and inhibiting the expression of AhR. In continuation of this study, the expression of IL-4 and IL-6 genes before and after the effect of GA, with the use of real-time PCR for the purpose of measuring the turnover. Based on the available results, the expression of IL-4 gene in the samples treated with GA extract compared with the untreated cells, there was a decrease of 2.097 times, which means that the treatment was effective in reducing the IL-4 gene. The PCR efficiency of IL-4 gene was equal to 09/86. In addition, the ratio of IL-6 gene to cell in untreated animals reduced the fall by 2.094 times and the PCR efficiency of DNA of this gene was equal to 85.01. There is no precise information available regarding the mechanism of downregulation of the expression of these genes, but since there are regulatory elements in the promoter region of these genes, regulation at the transcriptional level by these elements could be one of the factors. Studies suggest that IL-6 in the Caco-2 cell line by activating multiple signals including copper release, JAK-STAT, PI3K-Akt, and MAPK regulate cell processes such as proliferation, migration, apoptosis, and inflammation. (Kim et al., 2016) . In addition, IL-4 as one of the systemic suppressive factors has been identified. This inflammatory cytokine alters the fate of innate and adaptive immune cells and regulates the growth and proliferative capacity of cancer cells. (Matsui et al., 2006). IL-4 is mainly dependent on the phenotype of Th2 cells. T cells and IL-4 share similar receptor patterns across cancer cells. (Merlekar, 2022) . The control of growth of IL-4 and IL-6 by glucocorticoids, which depends on the inhibition of NF-kB, is linked to their anti-inflammatory effects (Mills et al., 1999). In one study, data clearly showed that GA, as well as Dex, tol IL-6 and GL production, inhibited IL-6 induced by TNF-α and IL-1β (Taki et al., 2008). Recently, it was reported that gallic acid directly binds to high-mobility group box-1 (HMGB1) protein and inhibits its chemotactic activity. HMGB1 is a nuclear protein that acts as a binding factor for architectural chromatin (Bianchi and Agresti, 2023).

Zhang et al. also showed that GA has a selective effect on signal transduction to produce IL-2 and IL-2R secretion and facilitate TCR-mediated T cell proliferation (Zhang et al., 2013). They also showed that GA exhibits two separate activities on the surface of immature cells, facilitating IL-2 production on the one hand and inhibiting the growth response on the other hand (Zhang et al., 2013). Wang and Du demonstrated that GA is able to inhibit the increased levels of TNF-α, IL-1β, and IL-6 as well as the high levels of IL-10 in mice with cancer, which is consistent with the present study (Wang and Du, 2016). This was stated by Safipour Afshar et al. (Safipour Afshar et al., 2017). With the increase of licorice extract concentration, the cell death rate, especially in two types of cancer, increases significantly. The fall also increased with increasing concentration of ODC1 gene extract

in breast cancer lines, there was a significant decrease, and comparing three and two cancer lines, the MDA-MB-231 line was more affected by licorice extract in this study. Also, the anti-inflammatory and anti-cancer properties of GA obtained from licorice are shown in this study. These results are also consistent with the study of Matsui et al. (Matsui et al., 2004) who reported that GA-bound plant extracts are effective in reducing chemokine production, and GA-modified plant extracts among them, hetero-30-OH-GL and homo-30-OH-GL seem to be more beneficial due to their inhibitory ability with less cytotoxicity. Finally, Nourazarian et al.'s research showed that GA-induced proliferation of HT-29 cell lines is inhibited by chloroctal at a concentration of 200 µg/ml, with increased cell death rate measured by trypan blue and MTT assays. Neta J. and RT-PCR showed downregulation of HSP90 gene, indicating the ability of JA to induce apoptosis in HT-29 cells and confirmed its anticancer properties. Most tumors, such as colon cancer and breast cancer, showed increased levels of Hsp90, which is associated with poor prognosis. Hsp90 is mainly involved in the regulation of protein stability. Several Hsp90 inhibitors have been developed and several are currently under clinical research (Nourazarian et al., 2016).

4. Conclusion

In this study, the toxicity level of glycyrrhizin extract on the cell line was studied. We analyzed CaCO-2 at different concentrations by MTT assay. Treatment of cells with different concentrations of this extract (obtained from licorice plant) caused the induction of apoptosis on the cancer cell line and toxicity on cancer cells. In addition, the relative expression of cytokine genes IL-4 and IL-6 (as a growth factor for colon cancer cells) in samples treated with glycyrrhizin extract compared to untreated cells was established. The sequence decreased by 2.097 and 2.094 times. Therefore, it can be concluded that this extract has anticancer properties against CaCO-2 cells and can be considered as a drug or anticancer agent with more complete clinical studies.

Suggestions

1) From licorice root on CaCO-2 cell lines that have similar properties to glycyrrhizin and have been examined before.
2) Study the effect of glycyrrhizin on other cancer cell lines in the stomach, prostate, breast and cervix.
3) Study the effect of glycyrrhizin on the expression of genes involved in programmed cell death
4) Further studies are necessary to identify the molecule(s) through which the biological activity occurs, in order to find more effective molecules for use. They can be designed as preventive and/or therapeutic agents for cancer or to prevent cell growth.

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