RESEARCH ARTICLE

Effect of Root Extracts of Medicinal Herb *Glycyrrhiza glabra* on HSP90 Gene Expression and Apoptosis in the HT-29 Colon Cancer Cell Line

Seyed Manuchehr Nourazarian[1], Alireza Nourazarian[2]*, Maryam Majidinia[2], Elmira Roshaniasl[3]

Abstract

Colorectal cancer is one of the most common lethal cancer types worldwide. In recent years, widespread and large-scale studies have been done on medicinal plants for anti-cancer effects, including *Glycyrrhiza glabra*. The aim of this study was to evaluate the effects of an ethanol extract *Glycyrrhiza glabra* on the expression of HSP90, growth and apoptosis in the HT-29 colon cancer cell line. HT-29 cells were treated with different concentrations of extract (50,100,150, and 200 μg/ml). For evaluation of cell proliferation and apoptosis, we used MTT assay and flow cytometry technique, respectively. RT-PCR was also carried out to evaluate the expression levels of HSP90 genes. Results showed that *Glycyrrhiza glabra* inhibited proliferation of the HT-29 cell line at a concentration of 200 μg/ml and this was confirmed by the highest rate of cell death as measured by trypan blue and MTT assays. RT-PCR results showed down-regulation of HSP90 gene expression which implied an ability of *Glycyrrhiza glabra* to induce apoptosis in HT-29 cells and confirmed its anticancer property. Further studies are required to evaluate effects of the extract on other genes and also it is necessary to make an extensive *in vivo* biological evaluation and subsequently proceed with clinical evaluations.

Keywords: *Glycyrrhiza glabra* - HT-29 colon cancer cells - HSP-90 - apoptosis

Asian Pac J Cancer Prev, **16 (18)**, 8563-8566

Introduction

Colorectal cancer is one of the most common deadly cancer types in the worldwide (Gholamhoseinian Najar et al., 2013; Li et al., 2015). According to previous studies, the expression levels of heat shock protein 90 (HSP90) is increased in tumors and cancerous cells in comparison with normal tissues. This protein forms 4 to 6 % of total proteins in cancer cells and 1 to 2% of total proteins in normal cells.

In addition, it has been specified that the ATPase activity of HSP90 is higher and more complex in tumors, but are in a latent state in normal cells. This results the maintenance of homeostasis as well as growth and survival in counter settings (Cooper, 2011; Dobo et al., 2013; Mamede et al., 2014). Furthermore, this protein plays an significant function in most oncoproteins involved in the survival and growth of cancer cells, such as the serine/threonine kinase (Akt), expressed protein from v-raf-1, murine leukemia viral oncogene homolog 1 (RAF-1), human growth factor-1, apoptosis induction, and other activities such as angiogenesis, and cell cycle inhibition in cancer cells (Niknejad et al.,2014). Recent data have showed the critical role of Hsp90 in promotion of malignant transformation and therefore, it is necessary for the development of solid malignancies. Most importantly, the proliferative potential of malignant cells is relatively depends on Hsp90 activity in cancer cells, and has been reported to prevent tumor cells from digression of apoptotic death (Moser et al., 2009).

The expression levels of Hsp90 is higher in colon cancer cells as compared with normal cells (Drecoll et al., 2014). Recently, it is suggested that sophisticated conditions such as cancers should be treated by the combination of several methods including surgery, chemotherapy, hormonal therapy and biological therapy. The most important side effects of this methods are destroying of the some healthy cells in addition to cancer cells (Nourazarian et al.,2014). Furthermore, in recent years, medicinal plants have been used to conduct widespread and large-scale studies (Nobili et al., 2009). From a variety of medicinal plants, the anti cancer functions of Glycyrrhiza glabra have been reported in various studies (Sheela et al.,2006; Dong et al.,2007) .

The aim of this study was to evaluate the effects of G. glabra on the expression of HSP90 growth and apoptosis in the HT-29 colon cancer cell line.

*[1]Department of Laboratory Sciences, Faculty of Paramedical Sciences, [2]Department of Biochemistry and Clinical Laboratories, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, [3]Department of Biochemistry, Faculty of Medicine, Urmia University of Medical Sciences, Urmia, Iran *For correspondence: alinour65@gmail.com*

Materials and Methods

Extraction of G. glabra

The plant material was collected from the Agriculture and Natural Resources Research Center of East Azarbaijan, Iran. Dried licorice (Glycyrrhiza glabra) roots were freeze-dried and pulverized. The dried powder (500 g) was then soaked in 80% ethanol for 24 h. The extracts were collected and the same process was repeated three times. The total extract was collected, filtered, and evaporated under reduced pressure. The end product was freeze-dried and the powdered extract was kept in a deep freezer (-70°C).

Cell Culture and Treatment

HT29 cells (the human colorectal carcinoma cell line) was purchased from the Pasteur Institute Cell Bank of Iran (Tehran, Iran). The cells were grown in minimal essential tissue culture medium RPMI-1640 (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (FBS: Gibco-Life technologies) penicillin (100 units/mL), streptomycin (100 µg/mL : Sigma) at 37°C in a 5% CO_2 humidified atmosphere and the medium was exchanged. The cells were treated with different doses of G. glabra extract (50, 100, 150, and 200 µg/mL) for 24, 48, and 72 h. The viability of the treated cells was expressed as a percentage of that of the control cells.

MTT cell viability assay

The effect of G. glabra extraction in inhibiting the proliferation of the HT29 cell was determined by MTT assay. The cells were seeded in 96-well tissue culture plates at a density of 15,000 cells per well and incubated at 37°C in 5% CO_2 humidified incubator. After 60-70% confluency, the cells were treated with different concentrations of G. glabra extract (50-200 μg/ml). For MTT assay, 2 mg/ml of MTT solution was added to each well and incubated for 3 h at 37°C. The medium was removed and the blue formazan crystals were dissolved in 200 µl of DMSO and 25 µl Sorenson buffer. The absorbance was read in a microplate reader (Biotek, model Elx808) at 570 nm. Each experiment was repeated in a triplicate, and results are expressed as Mean±SEM.

RNA extraction and c-DNA synthesis

At 72 h after treatment, the medium was removed from the monolayer cancer cells and scrapped in 1 ml RNAX-PLUS (Cinagene, Iran). The Total RNA was extracted from the samples using Cinagene Kit following the manufacturer's instruction (RNX-Plus Solution, SinaClon, Iran). The genomic DNA contaminant was removed, and the resulting RNA was selected for DNase treatment using DNase, RNase-free (Fermentas, USA). After purification and quantification, RNA was determined by measuring the optical density at 260 and 280 nm using nanodrop (NanoDrop- ND-1000). The cDNA synthesis was performed with cDNA synthase kit (Qiagene).

Real-time PCR

Real-time PCR test was used to determine the effect of the G. glabra extract on the degree of gene expression of HSP90 from a real-time PCR with iCycler IQ5 Multicolor Real-time PCR Detection System (Bio-Rad, USA). GAPDH was used as a housekeeping gene. The sequence of used primers is indicated in Table 1. Each experiment was repeated thrice and results are expressed as Mean±SEM.

Flow cytometry

The apoptotic rate was detected by flow cytometry. HT-29 cells (1 × 10^6) cells /well were plated in 6-well plates for 24 h and then treated with G. glabra at concentrations of 50 µg/mL and 400 µg/mL for an additional 24 h in a 5% CO_2 humidified atmosphere at 37°C. At the end of the incubation period, the treated HT-29 cells and controls were harvested and incubated with Annexin V and PI for 15 min before being analyzed on flow cytometer with 488 nm excitation and 515 nm for Annexin V detection.

Statistics

Statistical analysis was performed with SPSS version 18.0 software, and ANOVA with the Tukey-Kramer multiple comparisons test was used to compare between groups. Data are represented as Mean±SEM. The differences were considered significant when $P<0.05$.

Results

Effects of Glycyrrhiza glabra extract on the growth of HT-29 colon cancer cell lines

In order to determine the effect of G. glabra extract on the growth of HT-29 colon cancer cell lines proliferation, MTT assay was used at 24, 48, and 72 h after incubation with different doses of G. glabra extract. The survival curve shows that the cytotoxic effects of G. glabra on treated cells are dose and time dependent. There was a significant decrease in the viability of cells incubated for 24, 48, and 72 h. The data suggests that the G. glabra extract significantly inhibited HT-29 cell proliferation in dose and time-dependent manners. Cell growth was inhibited considerably in 200 μg/ml and 72 h compared with control groups as shown in Figure 1, cell proliferation was decreased to 52, 62 and 68% ($P<0.05$) respectively

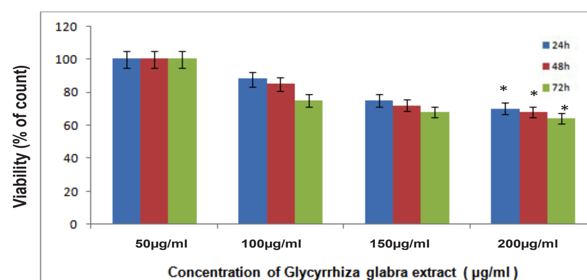

Figure 1. Effects of *Glycyrrhiza glabra* Extract on the Growth of HT-29 Colon Cancer Cell Lines

Table 1. Primers used for Real Time- PCR

Genes	Primer sequence (5' to 3')
HSP90	F 5'- TGTCATGAGCCTGAGGTGAAC-3'
	R 5'- GTGGATCCAGACACCAACAG-3'
GAPDH	F 5´AAGCTCATTTCCTGGTATGACAACG3´
	R 5´ TCTTCCTCTTGTGCTCTTGCTGG 3´

after 24, 48 and 72 h.

Cell proliferation (%) of Ht-29 human colon cancer cell lines evaluated after 24, 48 and 72 h of incubation with different dose of *Glycyrrhiza glabra* extract. Significantly different from Treated cells and controls (P<0.05)* Data are represented Mean ± SEM. The bars indicate mean ± standard deviation of three independent experiments performed in triplicate

Analysis of the effects of G. glabra on HSP90 expression

The down regulations of HT-29 cells were investigated by real time PCR. After 48 h of treatment, the mRNA levels of HSP90 gene were analyzed. The genes CT values were normalized against the mRNA level of GAPDH as a housekeeping gene and the relative expression for each group was measured. Figure 2 shows that, there is a significant decrease in the level of HSP90 expression by about 62%, when compared with the control group (P<0.05).

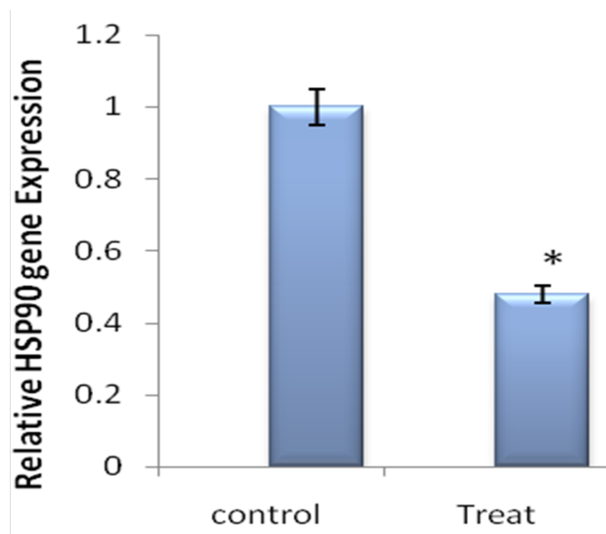

Figure 2. The Effects of *Glycyrrhiza glabra* Extract on the HSP90 gene Expression after48 h in HT-29 Colon Cancer Cells

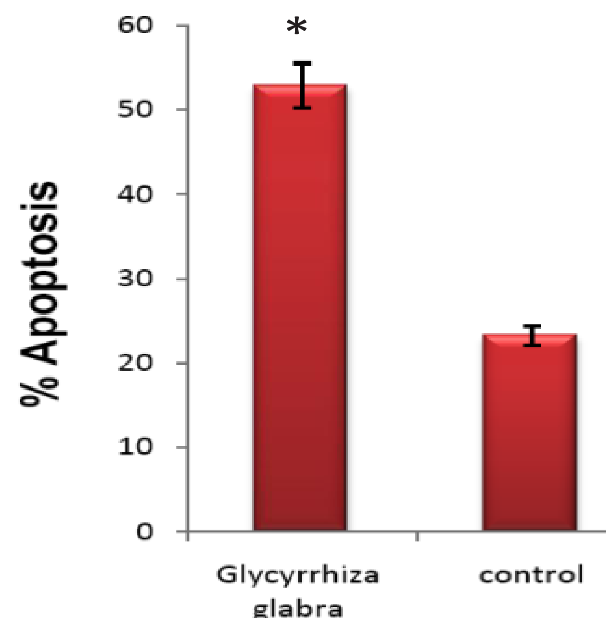

Figure 3. Effects of *Glycyrrhiza glabra* Extract in Apoptosis Rate

Significantly different from treated cells and controls was shown in the 200 μg/ml Glycyrrhiza glabra extract (P<0.05)*. Data are represented Mean ± SEM. The experiments were repeated as triplicate.

Flow cytometry

The results show that apoptosis occurred significantly in 52.85% of the cells treated with 400 μg/ml *Glycyrrhiza glabra* (p<0.05) extract compared to the control group (23.2%, Figure 3)

Apoptosis (%) of Ht-29 human colon cancer cell lines incubation with 200 μg/ml *Glycyrrhiza glabra* extract. Significantly different from Treated cells and controls (P<0.05)* Data are represented in percent.

Discussion

Because of their effective antitumor features, the researches about application and mechanism of anticancer medicinal herbs is becoming very prevalent (Vickers, 2006). Nowadays, the number of discovered medicinal plants available in nature, which have confirmed anti cancerous properties is increased (Rajandeep et al., 2001).

Several years ago, extracts of *Glycyrrhiza glabra* have been generally used in traditional Iranian medicine for the treatment of different diseases. Reactive oxygen species induce potent damages to different parts of cells and have various pathological effects such as, DNA damage, lipid and protein peroxidation, and cellular degeneration which are a pathophysiological dilemma of some disorders including ageing, cardiovascular disease, inflammatory diseases, cancer and a variety of other disorders (Crage et al., 1997). Due to unique action of G. glabra in scavenging free radicals and being a potent antioxidant, It was suggested that the extract of it could has protective effects against ischemic damages (Visavadiya et al., 2009; Nakagawa et al., 2014).

The anticancer effects of extracts of *Glycyrrhiza glabra* were reported by several studies. For example, Fu and et al., showed that extracts of G. glabra triggered the apoptosis in PC-3 prostate cancer cells. In addition, it suppressed the expression of cyclin B1 and cdc2 and as a result caused a potent arrest of cell in G2/M. other observed finding in the study are including inhibition of the phosphorylation of Rb, decreasing the expression of transcription factor E2F, reduction of cyclin D1, decrease in the expression of CDKs 4 and 6, and increased cyclin E expression (Fu et al., 2004). In another study by Chu et al. (2014) it is suggested that extracts of *Glycyrrhiza glabra* can mitigate the tumourigenic effects of Endocrine-disrupting chemicals in breast cancer cells by cell cycle arrest and inhibition of AhR expression (Chu et al., 2014). In addition, Nagaraj et al. (2012) revealed anti-angiogenesis, anti- migration and anti- proliferation effects for *Glycyrrhiza glabra*in MDA-MB-231 cells (Nagaraj et al., 2012). In agreement with these studies our results also showed that extracts of *Glycyrrhiza glabra* had anti-proliferative and pro- apoptotic effects in in HT-29 colon cancer cell line. Moreover, in according to the results of this study, it might be anticipated that *Glycyrrhiza glabra* is a cancer chemo protective agent, but more detailed

investigations are necessary for concerning its application as a supplementary anticancer agent.

Accumulating studies have shown that Hsp90 is required for the consistency of many signaling kinases. Therefore, from the target therapy points of view in cancer field, it allows the concurrent inhibition of several signaling pathways including MAPK, WNT, NF-kB and TGF-beta signaling (Haupt et al., 2012); as a result, the potential role for Hsp90 in tumourigenesis has also been suggested. Most tumors such as colon and breast cancer showed an increased levels of Hsp90 correlated with a poor prognosis (Yano et al., 1996; Drecoll et al., 2014). Hsp90 is mainly involved in the regulation of protein stability. Many Hsp90 inhibitors have been developed and many of them are presently undergoing clinical trials investigations (Porter et al., 2010). Hsp90 inhibition offers a multi-aspect treatment strategy, which is in contrast to current cancer therapies that target a single signaling pathway (Hall et al., 2014).

In this study, we investigated the effects of extract of *Glycyrrhiza glabra* on the levels of the Hsp90 in target cells. The treatment of cells with this medical herbal resulted in decrease in HSP90 levels in cancerous cells in comparison with untreated cell. Further studies are required to identify the molecule(s) by which their biological activity are mediated to design more effective molecules for its especial use as cancer chemo protective and/or therapeutic agents to target cell growth prevention. For performing accurate pharmacodynamic studies, it is essential to accomplish in vitro experiments with different solvents by using many different cancer cell lines. After isolation and characterization of known chemical structure(s) with significant activity in vitro, it is necessary to proceed with studies in vivo.

Acknowledgements

The authors wish to thank the all persons for participating in this study. All authors declare that they have no competing interests.

References

Chu XT, De la Cruz J, Hwang SG, Hong H (2014). Tumorigenic effects of endocrine-disrupting chemicals are alleviated by licorice (Glycyrrhiza glabra) root extract through suppression of AhR expression in mammalian cells. *Asian Pac J Cancer Prev*, **15**, 4809-13.

Cooper L (2011). The role of Hsp90 in the Wnt pathway of MCF7 breast cancer cells. Masters, Rhodes University.

Crage G, Newman D, Snader K (1997). Natural products in drug discovery and development. *J Natural Products*, **60**, 52-60.

Dobo C, Stavale JN, Lima FO(2013). HSP27 is Commonly Expressed in Cervical Intraepithelial Lesions of Brazilian Women. *Asian Pac J Cancer Prev*, **14**, 5007-10.

Dong S, Inoue A, Zhu Y, Tanji M, Kiyama R (2007). Activation of rapid signaling pathways and the subsequent transcriptional regulation for the proliferation of breast cancer MCF-7 cells by the treatment with an extract of Glycyrrhiza glabra root." *Food Chemical Toxicol*, **45**, 2470-8.

Drecoll E, Nitsche U, Bauer K, et al (2014). Expression analysis of heat shock protein 90 (HSP90) and Her2 in colon carcinoma. *Int J Colorectal Dis*, **29**, 663-71.

Foster FM , Streuli CH (2006). Inhibitor of apoptosis proteins in breast cancer. *Breast Can Res*, **8**, 9.

Fu Y, Hsieh TC, Guo J, et al (2004). Licochalcone-A, a novel flavonoid isolated from licorice root (Glycyrrhiza glabra), causes G2 and late-G1 arrests in androgen-independent PC-3 prostate cancer cells. *Biochem Biophys Res Commun*, **322**, 263-270.

Gholamhoseinian Najar A, Pashaei-Asl R, Omidi Y, Farajnia S, Nourazarian AR (2013). EGFR antisense oligonucleotides encapsulated with nanoparticles decrease egfr, mapk1 and stat5 expression in a human colon cancer cell line. *Asian Pac J Cancer Prev*, **14**, 495-8.

Hall AJ, Forsberg LK, Blagg SJ (2014). Alternative approaches to Hsp90 modulation for the treatment of cancer. *Future Med Chem*, **6**, 1587-1605.

Haupt A, Joberty G, Bantscheff M, Frohlich H (2012). Hsp90 inhibition differentially destabilises MAPkinase and TGF-beta signalling components in cancer cells revealed by kinase-targeted chemoproteomic. *BMC Cancer*, **12**, 1-12.

Li H, Zhu F, Boardman LA, et al (2015). Aspirin prevents colorectal cancer by Normalizing EGFR Expression. *EBio Med*, **2**, 447-55.

Mamede AC, Laranjo M, Carvalho MJ (2014). Effect of amniotic membrane proteins in human cancer cell lines: an exploratory study. *J Membr Biol*, **247**, 357-60.

Moser C, Lang S, Stoeltzing O (2009). Heat-shock Protein 90 (Hsp90) as a molecular target for therapy of gastrointestinal cancer. *Anticancer Res*, **29**, 2031-42.

Nagaraj SA, Lingaraj SM, Balaraju Y, Kumar A, Salimath BP (2012). MTA1 induced angiogenesis, migration and tumor growth is inhibited by Glycyrrhiza glabra. *IOSR J Pharmacy*, **2**, 34-43.

Nakagawa K, Kishida H, Arai N, Nishiyama T, Mae T(2004). Licorice flavonoids suppress abdominal fat accumulation and increase in blood glucose level in obese diabetic KK-A(y) mic. *Biol Pharm Bull*, **27**, 1775-8.

Niknejad H, Khayat-Khoei M, Peirovi H, Abolghasemi H (2014). Human amniotic epithelial cells induce apoptosis of cancer cells: a new anti-tumor therapeutic strategy. *Cytotherapy*, **16**, 33-40.

Nobili S, Lippi D, Witort E, Donnini M, Bausi L (2009). Natural compounds for cancer treatment and prevention." *Pharmacological Res*, **59**, 365-78.

Nourazarian AR, Kangari P, Salmaninejad A (2014). Roles of oxidative stress in the development and progression of breast cancer. *Asian Pac J Cancer Prev*, **15**, 4745-51.

Porter JR, Fritz CC, Depew KM (2010). Discovery and development of Hsp90 inhibitors: a promising pathway for cancer therapy. *Curr Opin Chem Biol*, **14**, 412-420.

Rajandeep K, Karan K, Harpreet K (2011). Plants as asource of anticancer agents. *J Nat Prod Plant Resour*, **1**, 119-24.

Sheela ML, Ramakrishna MK, Salimath BP (2006). Angiogenic and proliferative effects of the cytokine VEGF in Ehrlich ascites tumor cells is inhibited by Glycyrrhiza glabra. *Int Immunopharmacol*, **6**, 494-498.

Vickers A (2002). Botanical medicines for the treatment of cancer: rationale, overview of current data, and methodological considerations for phase I and II trials. *Cancer Invest*, **20**, 1069-79.

Visavadiya NP, Soni B, Dalwadi N (2009). Evaluation of antioxidant and anti-atherogenic properties of Glycyrrhiza glabra root using *in vitro* models. *Int J Food Sci Nutr*, **2**, 135-49.

Yano M, Naito Z, Tanaka S, Asano G (1996). Expression and roles of heat shock proteins in human breast cancer. *Jpn J Cancer Res*, **87**, 908-15.