University of Mazandaran

Journal of Genetic Resources
J Genet Resour2017;3 (1):47-53
http://sc.journals.umz.ac.ir
doi: 10.22080/jgr.2018.13284.1076

Iranian Biology Society

Evaluation of Cytotoxicity Activity and *NM23* Gene Expression in T47D Breast Cancer Cell Line Treated with *Glycyrrhiza glabra* Extract

Seyed Ataollah Sadat Shandiz[1], Ali Salehzadeh[2*], Mojgan Ahmadzadeh[2] and Kimia Khalatbari[2]
[1] Department of Biology, Central Tehran Branch, Islamic Azad University, Tehran, Iran
[2]Department of Biology, Rasht Branch, Islamic Azad University, Rasht, Iran

*Corresponding author: salehzadeh@iaurasht.ac.ir

Received: 01 November 2016 Accepted: 25 February 2017

Abstract

Glycyrrhiza is a genus of about 20 accepted species in the legume family (Fabaceae), with a distribution in Asia, Australia, Europe, and the Americas. Recently, about 30 species from the genus *Glycyrrhiza* have been found and used in traditional medicine for treating cancer. Different studies confirmed that down regulation of Non-metastatic protein (NM23), a metastasis suppressor gene is related to high metastatic potential. The aim of this study was to investigate the effect of *Glycyrrhiza glabra* extract on the expression of NM23 gene and against breast cancer (T47D) cell line. In this study, T47D cancer and MRC-5 normal cell lines were treated with different concentrations (0.1, 0.2, 0.5, 1, 2, 4, and 8 mg/ml) of *G. glabra* extract after 24, 48, and 72 h. 3-(4, 5-Dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) test was used to evaluate the effects of the extract toxicity against T47D cells. Quantitative real-time polymerase chain reaction (PCR) technique was used to evaluate NM23 gene expression in T47D cells. Gas chromatography/mass spectrometry (GC/MS) analysis revealed that 28 different compounds were found in the *G. glabra* water extract. Among the chemical constituents defined, the dominant constituents were Benzeneacetic acid, 4-hydroxy-, methyl ester (27.35%), Thiophene, Tetrahydro-2-methyl- (11.42%), Mome-Inositol (9.91%), and 5-Tridecanone (4.73%). The percent of cell toxicity revealed that the effect of toxicity is related to the time and dose. The mRNA levels of NM23 gene expression were significantly increased in the T47D cells treated with IC_{50} concentration of *G. glabra* ($P < 0.001$, 30.33 fold). This amount in sub-IC50 concentration of the extract was 5.06 ($p < 0.01$) fold, showing a positive effect of the extract in enhancing the NM23 expression as compared to the control groups after 72 h. The result showed that *G. glabra* has the potential to cure breast cancer by enhancing NM23 expression. Therefore, it is suggested that more researches are needed to find some effective combinations in the plant to design new and effective drugs to treat cancer.

Key words: Cell toxicity; *Glycyrrhiza glabra*; metastasis; *NM23*; T47D

Introduction

Breast cancer is a current malignant disease in women and the second reason of death (Siegel *et al.* 2015; Colagar *et al.*, 2015).The outbreak of this disease is more in developing countries except Japan, but in advanced countries it is reducing (DeVita and Chu, 2008). Breast cancer is a heterogeneous disease, because it is caused by genetic and environmental factors and this leads to advanced aggregation of genetic and epigenetic factors in malignant cells (Walsh and King, 2007; Jemma *et al.* 2009). Therefore, it is necessary to investigate a new and effective ways for the treatment of this cancer (Abolaji *et al*., 2014).

Metastasis is a common event in these patients and about 70% of patients who have developed breast cancer suffered from bone metastasis (Pytel and Sliwinski, 2009).

More than 20 genes, including *RKIP*, *JNKK/MKK4*, *KiSS1*, *E-cadherin*, *CD44*, *KAI1*, and *NM23* (NME 1) were identified as an inhibitor of metastasis process which decreases their expression during the metastasis (Jemma *et al*. 2009). Between these genes, the product of *NM23* makes a protein that functions as nucleoside diphosphate kinase or histidine kinase. It was specified that *NM23* gene expression decreases in many tumors including liver, breast, and prostate cancer (Prabhu, 2012).

In recent years, the use of herbal combinations to prevent and interfere in different stages of cancer has been embraced. Among the inhibitor combinations of cancer, natural polyphenolic antioxidants are the most effective combinations, because of the high efficiency and low systemic effect. *Glycyrrhiza glabra* is used in traditional medicine to treat inflammation, distension exanthema, and allergy. The root of *G. glabra* contains terpenes, glycyrrhizic acid, flavonoids, polyamine, coumarin, polysaccharides, volatile oils, sterols, phytoestrogen, and vitamins (Kinoshita *et al*. 2005). About 1 to 5% of the dry root of *G. glabra* consists of polyphenolic combinations with basic phenolic combination, liquiritin and liquiritigenin (Wang and Nixon, 2001; Nezamabadi, 2007).
In this research, the toxicity of different doses of *G. glabra* was evaluated against the breast cancer cell line (T47D) and normal cell line (MRC-5). Furthermore, the expression of *NM23* gene was analyzed in the T47D cell line after treatment with the root water extract of *G. glabra.*

Materials and Methods

Extraction the water extract of *G. glabra*

For preparing the water extract of *G. glabra*, each, 1 g powder and about 2 ml distilled water was poured in a beaker and after boiling, distilled water was added to the powder of the plant, and then it was boiled again for 15 min (Soltani *et al.* 2011). The fluid was filtered using Whatman filter paper and was transferred for elimination using solvent device, and elimination was done at 30ºC in Bain-Marie, and dry extraction was stored in fridge.

GC-MS analysis

The gas chromatography-mass spectrometric analysis of the *G. glabra* extract was done via an Agilent 7890 gas chromatograph (Agilent Technologies, USA) attached to Agilent 5977A mass spectrometer (Agilent Technologies, USA). The column used was HP DB-5 capillary column (30×0.25 mm×0.25 µm; Agilent Technologies). GC oven initial temperature was at 50°C for 2 min and it was later computed at 280°C at a rate of 5°C/min, and was finally held at 280°C for 2 min. A previous work has shown the operational conditions (Salehi *et al*., 2017).

Cell culture

Breast cancer T47D and normal MRC5 cell lines were bought from Institute of Pasture in Iran and cultured in medium RPMI1640 (Biosera) with 2 mM glutamine, 10 u/ml penicillin, 1.0 µ/ml streptomycin, 0.1 mM nonessential amino acids, and 1 mM pyruvate sodium using 10% fetal bovine serum (FBS). The cells were incubated in humid atmosphere with 5% CO_2 and 37ºC. Cell passage was done according to their rate of division. The medium was changed every 3 days for better growth of cells.

MTT assay

The 3-(4, 5-Dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to investigate the effects of the extract on cellular viability. Different concentrations (0.1, 0.2, 0.5, 1, 2, 4, and 8 mg/ml) were prepared from the extract of *G. glabra* and T47D and MRC-5 cell lines were treated at intervals of 24, 48, and 72 h. Thereafter, the content of wells in plate with 96-well plates was removed and was added to the MTT color with concentration of 0.5 mg/ml, and was kept at 37ºC and 5% CO_2. MTT color was isolated and 100 µL dimethyl sulfoxide (DMSO) was added for solubility of the formazan crystals medium. After incubation at room temperature for 30 min, the absorption of samples was measured at wavelength of 570 nm using ELISA reader (Oragcnon, ELISA reader teknika, Netherland).
The percentage of cell viability in the control groups (untreated cells) was evaluated according to equation 1.

$$\left[\text{Viability}(\%) = \frac{\text{Absorption of treated samples} \times 100}{\text{Absorption of controls}}\right]$$

(Equation 1)

The ratio of half maximal inhibitory concentration was calculated; positive and negative controls were noted in AU tests and they were repeated three times (Rafieian-Kopaei *et al*., 2014).

Study of NM23 gene expression by PCR

The ratio of *NM23* gene expression was investigated using real-time PCR with SYBER green method. First, the extraction of RNA was done from T47D cells according to the protocol of Cinna Pure RNA Purification Kit. After the determination of RNA concentration using nano drop (Implen GmbH, Germany), cDNA was made using the AidTm Lcit (first strand cDNA synthesis kit) of Fermentase Co. In this study, *β-actin* gene was used as the housekeeping gene (reference gene) to investigate the *NM23* gene expression in T47D cells. Thus, the primers sequence of *NM23* and *β-actin* genes were designed (Table 1).

PCR reaction was done using the Bioneer exicycler 96 according to the following duration: 95ºC for 10 min, denaturation stage at 95ºC for 20 s, multiplication stage at first was at 57ºC for 40 s, then 72ºC for 30 s; this was repeated for 40 cycles. The data were evaluated using the software of the device for evaluating the gene expression. The analysis of real-time PCR data was done based on the comparison of threshold cycle. In this study, the difference of threshold cycle was obtained from samples and control samples and the ratio of *NM23* to β-actin gene was calculated with ΔCT. Real-time PCR analysis was evaluated using REST 2009 software and expression fold change was determined by $2^{-\Delta\Delta ct}$.

Table 1: The sequences of primers were used in this study.

Primer	Sequence (5'→3')	Tm (ºC)	Product (bp)
NM23-F	5'-ATGGCCAACTGTGAGCGTACC-3'	58	190
NM23-R	5'-CATGTATTTCACCAGGCCGGC-3'	50	
β-actin-F	5'-TCCTCCTGAGCCAAGTA-3'	50	150
β-actin-R	5'-CCTGCTTGCTGATCCACATCT-3'	60	

Statistical analysis

Analysis of variance (ANOVA, Tukey's tests) was completed via GraphPad Prism 5.0 software version 6.0. P value less than p<0.05 of each sample was noted as the significant level and calculation of p-value was done using SPSS ver. 22 software. Also, the results of this study were based on at least three repetitions.

Results

Our data showed that the GC/MS analysis of all the chemicals identified in the *G. glabra* extract (Fig. 1). The mass spectra of the constituents with the NIST library were compared with GC/MS analysis of the *G. glabra* extract. About 27 different compounds were found in the *G. glabra* extract. Among the chemical constituents defined, the dominant constituents were Benzeneacetic acid, 4-hydroxy-, methyl ester (27.35%), Thiophene, Tetrahydro-2-methyl- (11.42%), Mome-Inositol (9.91%), and 5-Tridecanone (4.73%) (Fig. 2).

The treatment of T47D cells with different concentrations of *G. glabra* extract in different period showed that the survival of cells gradually reduced when the concentration increases (Fig. 3).

The concentration of extract in 8 mg/mL had the most toxicity. This concentration killed many of live cells and the minimum viability was seen after 72 h. This rate was observed when compared with the control group (p<0.001). To compare the influence of the extract on normal cells, normal MRC5 cells line was used. The influence of the concentrations (0.1, 0.2, 0.5, 1, 2, 4, and 8 mg/ml) of the extract was investigated. The results showed that the viability of the cells decreased as time increases.

The effects of different concentrations of the extract on normal MRC5 cell line treated with the extract were evaluated after 24, 48 and 72 h. In concentrations of 2, 4 and 8 mg/ml to the control group, there was a noticeable difference (p<0.001), whereas a low concentration of extracts showed no statistically significant differences when compared with the control groups (Fig. 4).

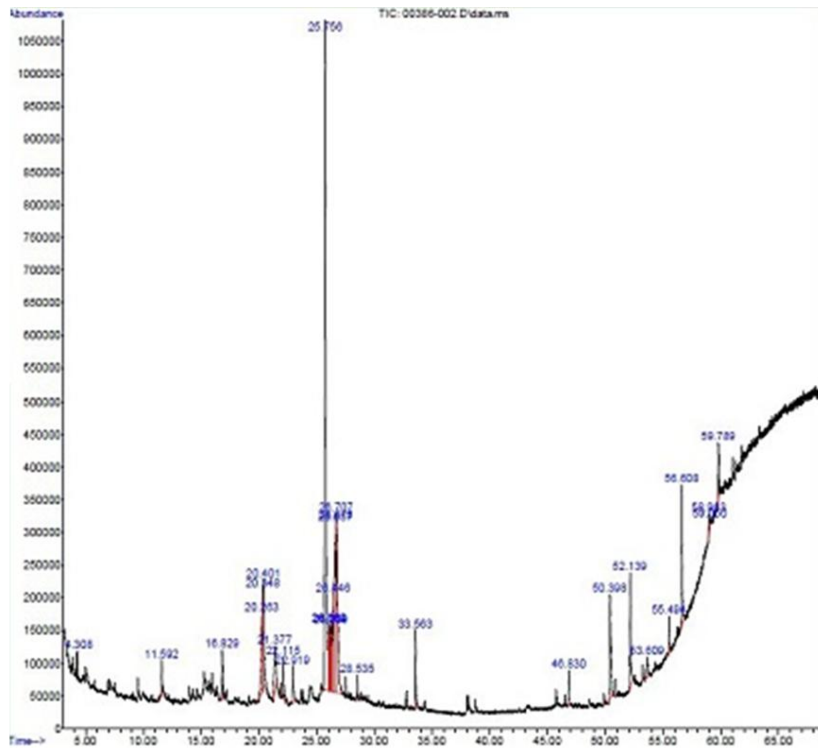

Fig. 1.The GC-MS Chromatogram of *Glycyrrhiza glabra* extract showing the peaks of the test compounds Vs retention time in minutes.

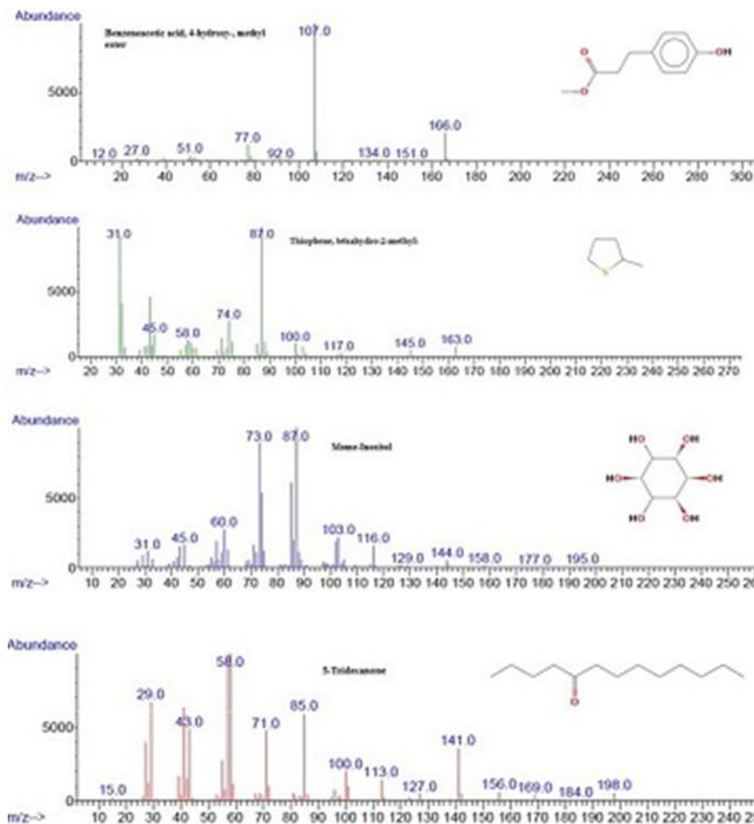

Fig. 2: Mass spectrum and structure of active phytocomponents identified by GC-MS in extract of *G. glabra*.

The comparison of diagrams 2 and 3 showed that the influence of different concentrations of *G. glabra* extract on the viability of the cancer cells was more than the normal cells; while in concentrations higher than 1 mg/ml, many of the cells were dead and a noticeable difference was observed between the two groups ($p<0.01$).

Real time PCR experiment was evaluated using primers that are related to *β-actin* and *NM23* genes. The cells were treated with the IC_{50} concentrations (0.75 mg/ml) and sub-IC_{50} (0.5 mg/ml) of the extract of *G. glabra*. The mRNA levels of *NM23* gene expression were significantly increased in the T47D cells treated with IC_{50} concentration of *G. glabra* ($P<0.001$, 30.33 fold). This amount in sub-IC_{50} concentration of the extract was 5.06 ($p<0.01$) fold, showing a positive effect of the extract in enhancing the *NM23* gene expression as compared to the control groups after 72 h. This showed a positive effect of the extract in increasing the *NM23* metastasis repressor gene (Fig. 5).

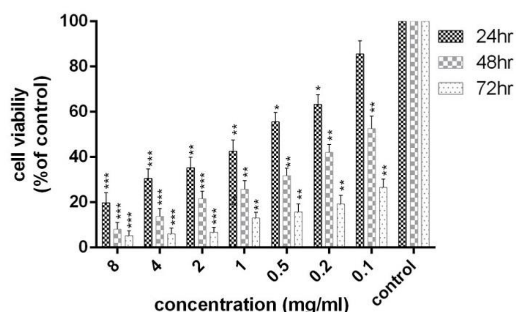

Fig. 3. Cytotoxicity effect of different concentrations of the extract on T47D cells after 24h,48h,72h (n=4, p<0.01**, p<0.01**, p<0.05*).

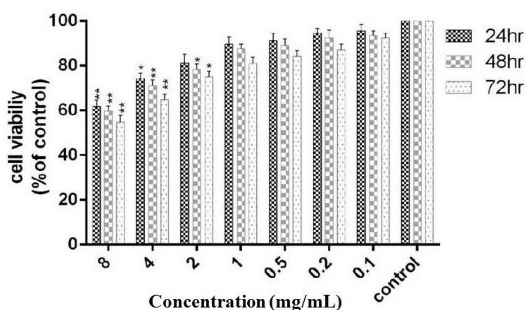

Fig. 4. Comparison the cytotoxicity effect of different concentrations of the extract on MRC5 cell line after 24h,48h,72h (n=4, p<0.01**, p<0.01**, p<0.05*).

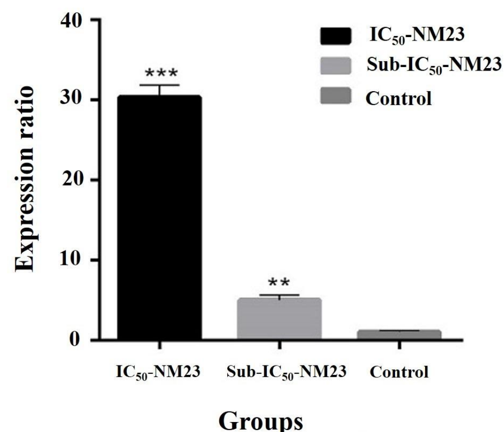

Fig. 5. *NM23* gene expression in treated and untreated cells with IC50 and sub – IC50 concentrations. The reference gene (control) is β-actin (p<0.01**, p<0.01**, p<0.05*).Note: sub-IC50 represent the concentration of extract that estimate under the IC50 concentration.

Discussion

The property of anti-cancer and other properties of herbal extracts were known and this knowledge led to the awareness that natural products like pharmaceutic herbs can be a positive way for treatment and control in a lot of diseases like cancer (Zaidi *et al*., 2009; Darakhshan *et al*., 2015; Mohadjerani *et al*., 2016). *G. glabra* is widely used in traditional medicine and the extract of this plant can be used as chemotherapy drug. Nowadays, many studies have shown that the extract of *G. glabra* inhibited different cancers like colorectal (Huang *et al.,* 2014), breast (Lorusso and Rüegg, 2012), prostate (Lee *et al*., 2013), glioblastoma (Li *et al*., 2014), liver (Zhang *et al*., 2012), stomach and uterus (Park *et al*., 2009), bladder (Yuan *et al*., 2014), and Leukemia cancers (Chueh *et al*., 2012). According to GC-mass results, the dominant constituents were Benzeneacetic acid, 4-hydroxy-, methyl ester (27.35%). Benzeneacetic acid, 4-hydroxy-, methyl ester is found in different plants with antibacterial, antifungal, and anticancer activities (Singh and Bhat, 2011). Thiophene, Tetrahydro-2-methyl, Mome-inositol, and 5-Tridecanone also have antimicrobial, antioxidant, and anticancer properties (Wei *et al*., 2011; Shokri, 2016; Neda and Rabeta, 2013; Chailungka *et al*., 2017). Flavonoids and triterpenes inhibit the progress of cancers and the use of these compounds with other substances resulted in

synergistic effect in chemotherapy (Sharma *et al*., 2011). Different researchers conducted on the anti-tumor activity of *G. glabra* extract against breast cancer. These studies have shown that the extract of *G. glabra* inhibits cyclin B_1 and cdc2 expression in cell cycle and finally results in reduction of G2/M cyclin. Fu et al. showed the apoptotic effect of *G. glabra* extract at 12.5 µM and 25µM concentration can arrest the cell cycle progression in G2/M phase in prostate PC-3 cancer cell line (Fu *et al*., 2004). This study showed that by increasing the concentration of the extract, the proliferation of breast cancer cell lines was reduced rather than normal cell lines; it was also observed that the maximum cytotoxicity of extract was seen at concentration of 8 mg/ml toward breast cancer cells. Also, the result of this study showed that the cell cytotoxicity effect depends on the time and concentration of the extract of *G. glabra*. Among all the genetic changes, inactivation of metastasis inhibitor genes is an important factor to form metastasis cancer. In recent decades, there was a lot of promotion about metastasis suppressor genes. These genes are effective in inhibition of metastasis *in vivo* condition without efficacy on the growth of primary tumors (Cheel *et al*., 2010). These investigations showed that *NM23* gene expression was reduced in metastasis cells; so the increase of its expression caused the reduction of the metastasis and this led to cell apoptosis. Yet, there is no information about *NM23* gene expression when the breast cancer cells were treated with the extract of *G. glabra*. In this study, *NM23* gene expression analysis was done in the concentration of IC_{50} of extract. Thus, it can be concluded that the extract of *G .glabra* might be modulate metastasis by up regulation of the *NM23* gene expression at mRNA expression level. Researchers have shown that the extract of *G. glabra* reduced the production of vascular endothelial growth factor (VEGF) cytokine and prevents angiogenesis (Sheela *et al*., 2006). New vessels are necessary for spreading the tumor and the growth of solid tumors, so reducing VEGF cytokine can be a way of treating cancer. Overall, the obtained data showed that *G. glabra* has the potential to cure breast cancer by enhancing *NM23* expression. Thus, it is suggested that more researches are needed to find some effective combinations in the plant to design new and effective drugs to treat cancer.

References

Abolaji AO, Eteng MU, Ebong PE, Dar A, Farombi EO, Choudhary MI. 2014. Artemisia annua as a possible contraceptive agent: a clue from mammalian rat model. *Nat Prod Res* 28: 2342-46.

Chailungka A, Junpirom T, Pompimon W, Nuntasaen N, Meepowpan P. 2017. Two flavonoids first isolated from the seed of *Syzygium nervosum* and preliminary study of their anticancer and anti- HIV-1 reverse transcriptase activities. *Maejo Int J Sci Technol* 11(01): 58-67.

Cheel J, Antwerpen PV, Tůmová L, Onofre G, Vokurková D, Zouaoui-Boudjeltia K, Vanhaeverbeek M, Nève J. 2010. Free radical-scavenging, antioxidant and immunostimulating effects of a licorice infusion (*Glycyrrhiza glabra* L.). *Food Chem* 122(3):508-517.

Chueh FS, Hsiao YT, Chang SJ, Wu PP, Lin JJ. 2012. Glycyrrhizic acid induces apoptosis in WEHI-3 mouse leukemia cells through the caspase-and mitochondriadependent pathways. *Oncol Rep* 28(6): 206976.

Colagar AH, Moradi Firouzjah H, Halalkhor S. 2015. Vitamin D receptor poly (A)-microsatellite polymorphism and serum levels of 25-hydroxyvitamin D: association with susceptibility to Breast cancer. *J Breast Cancer* 18(2): 119-125.

Darakhshan S, Bidmeshki Poura A, Colagar AH, Sisakhtnezhad S. 2015. Thymoquinone and its therapeutic potentials. *Pharmacol Res* 95-96: 138-158.

DeVita VT, Chu E. 2008. A history of cancer chemotherapy. *Cancer Res* 68(21): 8643-53.

Fu Y, Hsieh TC, Guo J. 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: 263-70.

Huang W, Tang S, Qiao X, Ma W, Ji S, Wang K, Ye M, Yu S. 2014. Isoangustone A induces apoptosis in SW480 human colorectal adenocarcinoma cells by disrupting mitochondrial functions. *Fitoterapia* 94: 36-47.

Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ. 2009.

Cancer statistics. *CA Cancer J Clin* 55: 10-30.

Kinoshita T, Tamura Y, Mizutani K. 2005. The isolation and structure elucidation of minor isofl avonoids from licorice of *Glycyrrhiza glabra* origin. *Chem Pharm Bull* 53:847-49.

Lee SK, Park KK, Park JH, Lim SS, Chung WY. 2013. The inhibitory effect of roasted licorice extract on human metastatic breast cancer cell-induced bone destruction. *Phytother Res* 27(12): 1776-83.

Li S, Zhu JH, Cao LP, Sun Q, Liu HD, Li WD, Li JS. 2014. Growth inhibitory *in vitro* effects of glycyrrhizic acid in U251 glioblastoma cell line. *Neurol* 35(7): 1115-20.

Lorusso G, Rüegg C. 2012. New insights into the mechanisms of organ-specific breast cancer metastasis. *Semin Cancer Biol* 22(3): 226-33.

Mohadjerani M, Naqinezhad A; Aghasizadeh Sharbaf M. 2016. *Platycladus orientalis* extracts with antioxidant activity from north of Iran. *J Genet Resour* 2(2): 60-66

Neda GD, Rabeta MS. 2013. Chemical composition and anti-proliferative properties of flowers of *Clitoria Ternatea*. *Int Food Res J* 20(3): 1229-34.

Nezamabadi H, Rahimiyan Mashhadi H, Zand A, Alizadeh H. 2007. Investigation of some ecophysiological aspects of Licorice (*Glycyrrhiza glabra*) rhizome. *Appl Entomol Phytopathol* 74(2):45-62.

Park I, Park KK, Park JHY, Chung WY. 2009. Isoliquiritigenin induces G2 and M phase arrest by inducing DNA damage and by inhibiting the metaphase/anaphase transition. *Cancer Lett* 277(2): 174-81.

Prabhu V. 2012. Targeting Tumor Metastasis by Regulating *NM23* Gene Expression. *Asian Pacific J. Cancer Prev* 13: 3539-48.

Pytel D, Sliwinski T. 2009. Tyrosine Kinase Blockers: New Hope for Successful Cancer Therapy. *Anticancer Agents Med Chem* 9 (1): 66-76.

Rafieian-Kopaei M, Setorki M, Doudi M, Baradaran A, Nasri H. 2014. Atherosclerosis: process, indicators, risk factors and new hopes. *Int J Prev Med* 5(8): 927-46.

Salehi S, Mirzaie A, Shandiz SAS, Noorbazargan H, Rahimi A, Yarmohammadi S, Ashrafi F. 2017. Chemical composition, antioxidant, antibacterial and cytotoxic effects of *Artemisia marschalliana* Sprengel extract. *Nat Prod Res* 31: 469-72.

Sharma H, Parihar L, Parihar P. 2011. Review on cancer and anticancerous properties of some medicinal plants. *J Med Plants Res* 5(10): 1818-35.

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(3): 494-8.

Shokri H. 2016. A review on the inhibitory potential of *Nigella sativa* against pathogenic and toxigenic fungi. *Avicenna J Phytomed* 6(1): 21-33.

Siegel RL, Miller KD, Jemma A. 2015. Cancer statistics. *CA Cancer J Clin* 65(1): 5-29.

Singh SA, Bhat VS. 2011. Antimicrobial potential of 3-hydroxy-2-methylene-3-phenylpropionic acid derivatives. *Acta Pharm* 61: 447-455.

Soltani N, Karami R, Ranjbar M. 2011.The interaction of salicylic acid and cold stress on antioxidant enzyme activities in licorice (*Glycyrrhiza glabra*). *J Herbal Drugs* 2(1): 7-13.

Walsh T, King MC. 2007.Ten genes for inherited breast cancer. *Cancer Cell* 11:103-5.

Wang ZY, Nixon DW. 2001. Licorice and cancer. *Nutr Cancer* 39:1-11.

Wei LS, Wee W, Siong JY, Syamsumir DF. 2011. Characterization of antimicrobial, antioxidant, anticancer properties and chemical composition of *Sauropus androgynus* stem extract. *Acta Med Litu* 18(1): 12-16.

Yuan X, Li T, Xiao E, Zhao H, Li Y, Fu S, Wang Z. 2014. Licochalcone B inhibits growth of bladder cancer cells by arresting cell cycle progression and inducing apoptosis. *Food Chem Toxicol* 65: 242-251.

Zaidi SH, Huddart RA, Harrington KJ. 2009. Novel targeted radiosensitisers in cancer treatment. *Curr Drug Discov Technol* 6(2): 103-34.

Zhang JF, Li H, Li CQ, Tang HM, Yan RG. 2012. Effect of diammonium glycyrrhizinate on proliferation in liver cancer cell SMMC-7721 and p53 expression. *Chongqing Med* 41(27): 2852-2856.