

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

K. Mehdinejadiani (MSc)¹, H. Shirzad (PhD)², Sh. Fakhari (MSc)³, A. Jalili (PhD)^{*4}

1. Cellular-Molecular Research Center, Faculty of Medicine, Shahrekord University of Medical Sciences, Shahrekord, I.R.Iran

2. Department of Immunology, Faculty of Medicine, Shahrekord University of Medical Sciences, Shahrekord, I.R.Iran

3. Immunology Research Center, Faculty of Medicine, Kurdistan University of Medical Sciences, Sanandaj, I.R.Iran

4. Liver & Digestive Research Center, Kurdistan University of Medical Sciences, Sanandaj, I.R.Iran

Received: Oct 30th 2014, Revised: Feb 4th 2015, Accepted: May 6th 2015

ABSTRACT

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

METHODS: In this experimental study, adenocarcinoma gastric cell lines were prepared from cell bank and were cultured. After passage, the cells were transferred into a 96-well plate. In each well, approximately 2,000 cells in RPMI-1640 culture medium with FBS (10%) were placed. The cells were repeatedly exposed to different concentrations of Glycyrrhetic acid (0, 1, 10 and 100.1 μ M) and Glycyrrhizic acid (10, 1, 100 and 0.1 μ M) for 24 and 48 hours. Finally, the obtained results of the experimental and control groups were compared with each other.

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

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

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

Please cite this article as follows:

Mehdinejadiani K, Shirzad H, Fakhari Sh, Jalili A. An Evaluation the Effect of Glycyrrhetic and Glycyrrhizic Acids Derived from Licorice Extract on Gastric Cancer Cell Lines. J Babol Univ Med Sci. 2015;17(9):52-8.

Introduction

Gastric cancer is the second most prevalent carcinogenic disease. Epidemiological studies have

demonstrated that gastric cancer is the second leading cause of death (due to cancer) in the world

*Corresponding Author: A. Jalili (PhD)

Address: Kurdistan University of Medical Sciences, Pasdaran Boulevard, Sanandaj, I.R.Iran

Tel: +98 87 33613129

Email: Ali130@gmail.com

following lung cancer (1). In 2014, the number of patients with gastric cancer in the USA was reported to be 1665540, out of which 585720 cases died (2). Gastric cancer is often asymptomatic in the early stages and the lack of clinical symptoms in these patients leads to delayed diagnosis. Almost 80-90% of these patients refer to hospitals in the advanced stages of the disease with adjacent metastasis. According to epidemiological studies, chance of survival in the patients with advanced stages of gastric cancer with metastasis is very low despite undergoing chemotherapy and aggressive treatments such as surgery for five years (3). Current studies have focused much attention on the use of natural products for the treatment of different diseases and have reached significant results in this regard. Many studies showed that the use of natural products could be useful in numerous areas such as strengthening the immune system (4, 5), treating incurable diseases such as Alzheimer's (6, 7), atherosclerosis (8,9), diabetes (10, 11), gastrointestinal diseases (12, 13) and cancer (14, 15). In fact, it has created high hopes for prevention and treatment of several diseases. Licorice is one of effective plants in the treatment of numerous diseases. Flavonoids derived from licorice root are also applied for treatment of many diseases including microbial infections causing hepatitis C, stomach ulcer, skin and respiratory infections; it is also used as a carcinogenic treatment (16). Licorice is from the Fabaceae family (also known as *Glycyrrhiza glabra*). In European and Asian traditional medicine, it was used for treatment of gastritis, peptic ulcers and respiratory infections (16). This plant is grown in many countries and is known as an anti-inflammatory, antitumor and antioxidant substance. Anti-tumor activities of the licorice compounds include cell cycle arrest, apoptosis inducing and antioxidant effects. According to the studies conducted on the lab rats, some licorice compounds inhibit PI3-K, MKK4, MKK7, JNK1, mTOR, and Cdk2 activities through reducing carcinogenesis in several cells (4). Additionally, licorice is used for the treatment of viral hepatitis and cytomegalovirus (CMV) infection. Moreover, Glycyrrhizic acid derived

from licorice prevents the growth of viruses and viral components (5, 6). Glycyrrhetinic acid and Glycyrrhizic acid are two compounds isolated from licorice. Glycyrrhetinic acid is a pentacyclic triterpenoid derivative of the Glycyrrhizic acid (formula: $C_{30}H_{46}O_4$) which is obtained from licorice. Glycyrrhizic acid is a natural substance comprising of Glycyrrhetinic acid and two molecules of Glucuronic acid (7). Glycyrrhizic acid and Glycyrrhetinic acid can effect on the androgen steroids' activity through acting on glucocorticoid receptors and preventing the conversion of cortisol to cortisone in liver and kidney. In addition, compounds in licorice extract can cause lipoxygenase and cyclooxygenase disruption as well as arachidonic acid reduction. Glycyrrhetinic acid is used as an analgesic and anti-inflammatory. It is also used for treatment of allergy, gastric ulcer, hepatitis, liver toxicity and tumors. Glycyrrhetinic acid is an active pharmaceutical ingredient. It helps with reducing lipids through elevating the level of corticosterone (8, 9). In rats, Glycyrrhetinic acid plays a key role in prevention of development and progression of tumors by means of DMBA (7, 12-Dimethyl Benz (a) Anthracene) (trans-7, 12-dimethyl benzene) and TPA (12-o-Tetradecanoylphorbol-13-Acetate) (10), while Glycyrrhizic acid prevents the formation of tumors only by means of DMBA and TPA has no role (11). Given the efficacy of licorice compounds in treatment of various diseases with few side effects, this study aimed to determine the effects of Glycyrrhetinic acid and Glycyrrhizic acid derived licorice extract on gastric carcinogenic cell line.

Methods

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

incubated at 37 °C containing 5% CO₂. The culture medium was changed every 24 hours. Three or four days following the primary culture, cell density was evaluated under an inverted microscope. When the cells were grown to 80%, the passage was performed.

Cell viability: Approximately 2,000 cells in 50 µL RPMI-1640 culture medium containing 10% Fetal Bovine Serum (FBS) were added to each well in a 96-well plate and were kept in the CO₂ incubator overnight. Subsequently, the cells were repeatedly exposed to different concentrations of Glycyrrhetic acid (0.1, 1, 10 and 100 µM) and Glycyrrhizic acid (0.1, 1, 10 and 100 µM), which were dissolved in dimethyl sulfoxide. A control group was considered for each concentration of Glycyrrhetic and Glycyrrhizic acids. The control group was only located in a RPMI-1640 culture medium with FBS (10%). The cells were incubated with different amounts of the Glycyrrhetic acid and Glycyrrhizic acid for 24 and 48 hours. After the incubation time, 10 ml of XTT solution (salt, 2, 3-bis, (2-methoxy-4-nitrobenzonitrile-5 sulfosuccinates) 2 tetrazolium-5 carboxyaniline) was added to each well in a 96-well plate in order to evaluate cell viability. After a 4-hour period, supernatant reading was evaluated by dint of ELISA (Stat Fax, Palm City, FL) and mean optical density (MOD) at 540 nm in each well was performed (12). The optical density of cells determined by ELISA was converted into percentage of cell viability using the following formula:

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

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

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

Result

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

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

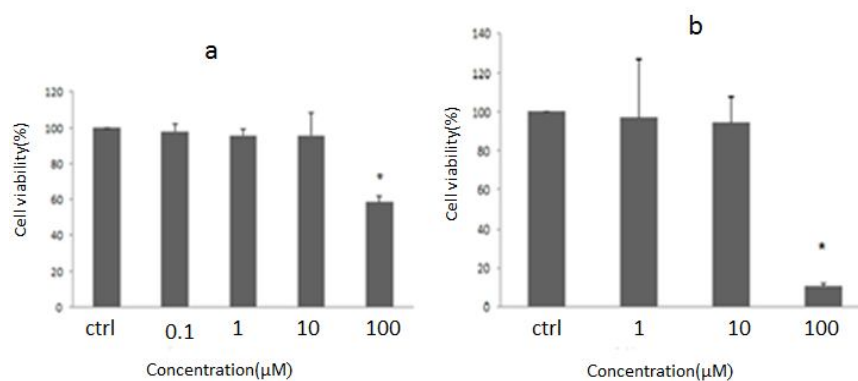


Figure 1. The percentage of cell survival in AGS cells after 24 (a) and 48 (b) hours of exposure to different concentrations of Glycyrrhetic acid

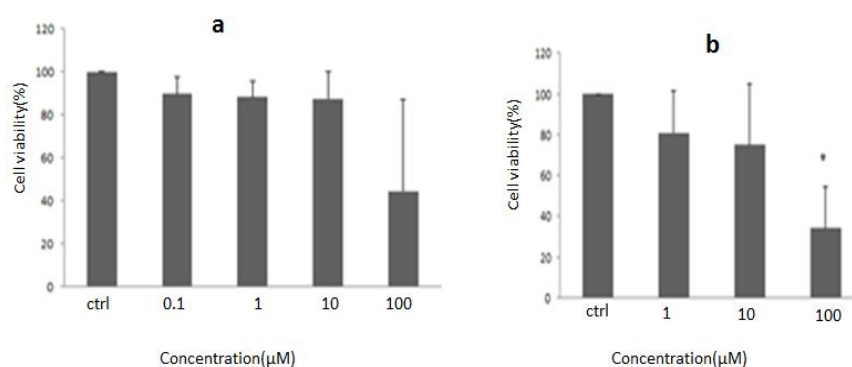


Figure 2. The percentage of cell survival in AGS cells after 24 (a) and 48 (b) hours of exposure to different concentrations of Glycyrrhizic acid

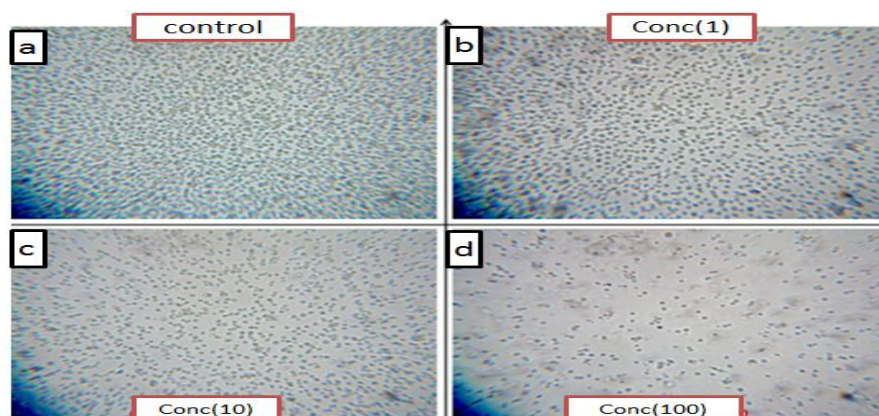


Figure 3. The number of AGS cells decrease by increasing the dose of Glycyrrhetic acid and Glycyrrhizic acid(a-d). (The greatest amount of cell death in 100 μM concentration(d))

Discussion

According to the obtained results, two compounds derived from licorice (Glycyrrhetic acid and Glycyrrhizic acid) increased the rate of carcinogenic cells death following 24 and 48 hours of incubation. A study performed to determine the effect of Glycyrrhetic acid on NCI-H460 NSCLC

cells showed that inducing apoptosis and cell cycle arrest occurred in G1 phase. This might be due to an increase in the PARP (Poly ADP-ribose polymerase) and caspase-3 expression and a decrease in Bcl-xl, BCL2, cyclin D1 and cyclin E expression. Moreover, Glycyrrhetic acid leads to

decreased amount of protein kinase C-beta (PKC-beta) phosphorylates and extracellular signal-regulated kinase (ERK) phosphorylates and increased phosphorylation of protein kinase Cδ (PKCδ) and c-Jun N-terminal kinases (JNKs), both of which can affect cell death (13). The aforementioned processes are the possible mechanisms involved in gastric cancer treatment. In an investigation of the anti-carcinogenic effects of licorice extract components on gastric cancer cells and immortalized gastric epithelial cell lines, it was found that Licochalcone A, which is isolated from root of *Glycyrrhiza glabra* (licorice), is the most toxic component of the licorice that has the greatest impact on cell cycle arrest and inducing apoptosis. Some researchers have indicated that licorice extract Licochalcone A prevents the growth of carcinogenic cells in a dose-dependent manner. Moreover, they reported that the Licochalcone A component of licorice extract induced lowered expression of cyclin A, cyclin B and murine double minute 2 homolog (MDM2) oncogene. Additionally, it leads to increased expression of retinoblastoma protein (Rb) in the carcinogenic cells. Furthermore, Licochalcone A component of licorice extract causes apoptosis through affecting on the expression of Bcl-2, Bax and caspase 3. These findings suggest that licorice extract is effective in treatment of gastric cancer (14). Another study pointed out that the licorice extract induces elevated expression of Bax and decreased expression of Bcl2, MKN-28 (significantly), AGS (moderately), and MKN-45 cells (slightly). Therefore, the increase in the ratio of Bax/Bcl-2 leads to release of cytochrome C from mitochondria to the cytosol where cytochrome C can bind to apoptotic protease, activating factor1 that is also known as APAF-1. This causes inducing apoptosis in cells and caspase-3 and poly (ADP-ribose) polymerase (PARP) activation (15). The obtained results of our study showed two compounds derived from licorice (Glycyrrhetic acid and Glycyrrhizic acid) had a toxic effect on carcinogenic cells. According to our study, Glycyrrhetic acid is more effect than Glycyrrhizic acid in treatment of gastric cancer. Therefore, Glycyrrhetic acid as an

effective combination treatment can be employed for treatment of gastric cancer. Although the combination of Glycyrrhizic acid prevents the growth of carcinogenic cells, its influence is more gradual than Glycyrrhetic acid. These differences might lead to various neurological pathways to prevent the growth of carcinogenic cells and could induce different effects on the AGS cell line. Impacts of oxidative stress on numerous diseases such as cancer (16,17) and also the effects of antioxidants on various diseases, especially the diseases associated with free radicals and oxidative stress such as cancer (19, 20), diabetes (21-23), infection (24,25) and atherosclerosis (26) (27) have been determined. In other words, the antioxidant effects of Glycyrrhetic acid and Glycyrrhizic acid might result in carcinogenic cells' death. There are numerous plants with antioxidant properties (25, 28-31), the anti-carcinogenic effects of which must be examined.

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

Acknowledgments

We wish to thank the research and student chancellor and staff of Shahrekord University of Medical Sciences, Sanandaj University of Medical Sciences and Dr. Mahmoud Rafieeian for their cooperation with this study.

References

1. Weis VG, Goldenring JR. Current understanding of SPEM and its standing in the preneoplastic process. *Gastric Cancer*. 2009;12(4):189-97.
2. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. *CA Cancer J Clin*. 2014;64(1):9-29.
3. Oliveira FJ, Ferrão H, Furtado E, Batista H, Conceição L. Early gastric cancer: report of 58 cases. *Gastric Cancer*. 1998;1(1):51-6.

4. Bode AM, Dong Z. Chemopreventive effects of licorice and its components. *Curr Pharmacol Rep*. 2015;1(1):60-71.
5. Lehtihet M, Nygren A. Licorice--an old drug and currently a candy with metabolic effects]. *Läkartidningen*. 2000;97(36):3892.
6. Arora R, Chawla R, Marwah R, Arora P, Sharma R, Kaushik V, et al. Potential of complementary and alternative medicine in preventive management of novel H1N1 flu (Swine flu) pandemic: thwarting potential disasters in the Bud. *Evidence-Based Complementary and Alternative Medicine*. 2010;2011.
7. Gottschalck TE, McEwen NM. *International cosmetic ingredient dictionary and handbook*. 10th ed. Washington DC: CTFA; 2004.
8. Whorwood CB, Sheppard MC, Stewart PM. Licorice inhibits 11 beta-hydroxysteroid dehydrogenase messenger ribonucleic acid levels and potentiates glucocorticoid hormone action. *Endocrinology*. 1993;132(6):2287-92.
9. Armanini D, De Palo CB, Mattarello MJ, Spinella P, Zaccaria M, Ermolao A, et al. Effect of licorice on the reduction of body fat mass in healthy subjects. *J Endocrinol Invest*. 2003;26(7):646-50.
10. Wang ZY, Agarwal R, Zhou ZC, Bickers DR, Mukhtar H. Inhibition of mutagenicity in salmonella typhimurium and skin tumor initiating and tumor promoting activities in SENCAR mice by glycyrrhetic acid: comparison of 18 α - and 18 β -stereoisomers. *Carcinogenesis*. 1991;12(2):187-92.
11. Agarwal R, Wang ZY, Mukhtar H. Inhibition of mouse skin tumor initiating activity of DBMA by chronic oral feeding of glycyrrhizin in drinking water. *Nutr Cancer*. 1991;15(3-4):187-93.
12. Pirzadeh S, Fakhari S, Jalili A, Mirzai S, Ghaderi B, Haghshenas V. Glycyrrhetic acid induces apoptosis in leukemic HL60 cells through upregulating of CD95/CD178. *Int J Mol Cell Med*. 2014;3(4):272-8.
13. Song J, Ko HS, Sohn EJ, Kim B, Kim JH, Kim HJ, et al. Inhibition of protein kinase C α/β II and activation of c-Jun NH2-terminal kinase mediate glycyrrhetic acid induced apoptosis in non-small cell lung cancer NCI-H460 cells. *Bioorg Med Chem Lett*. 2014;24(4):1188-91.
14. Xiao XY, Hao M, Yang XY, Ba Q, Li M, Ni SJ, et al. Licochalcone A inhibits growth of gastric cancer cells by arresting cell cycle progression and inducing apoptosis. *Cancer Lett*. 2011;302(1):69-75.
15. Del Poeta G, Bruno A, Del Principe MI, Venditti A, Maurillo L, Buccisano F, et al. Deregulation of the mitochondrial apoptotic machinery and development of molecular targeted drugs in acute myeloid leukemia. *Curr Cancer Drug Targets*. 2008;8(3):207-22.
16. Darani HY, Shirzad H, Mansoori F, Zabardast N, Mahmoodzadeh M. Effects of *Toxoplasma gondii* and *Toxocara canis* antigens on WEHI-164 fibrosarcoma growth in a mouse model. *The Korean journal of parasitology*. 2009;47(2):175-7.
17. Bagheri N, Rahimian Gh, Salimzadeh L, Azadegan F, Rafieian-Kopaei M, Taghikhani A, et al. Association of the virulence factors of *Helicobacter pylori* and gastric mucosal interleukin-17/23 mRNA expression in dyspeptic patients. *EXCLI J*. 2013;12:5-14.
18. Rahimian G, Sanei MH, Shirzad H, Azadegan-Dehkordi F, Taghikhani A, Salimzadeh L, et al. Virulence factors of *Helicobacter pylori* vacA increase markedly gastric mucosal TGF- β 1 mRNA expression in gastritis patients. *Microb Pathog*. 2014;67-68:1-7.
19. Shirzad H, Kiani M, Shirzad M. Impacts of tomato extract on the mice fibrosarcoma cells. *J HerbMed Pharmacol*. 2013;2(1):13-6.
20. Shirzad H, Taji F, Rafieian-Kopaei M. Correlation between antioxidant activity of garlic extracts and WEHI-164 fibrosarcoma tumor growth in BALB/c mice. *J Med Food*. 2011;14(9):969-74.
21. Asgary S, Rafieian-Kopaei M, Shamsi F, Najafi S, Sahebkar A. Biochemical and histopathological study of the anti-hyperglycemic and anti-hyperlipidemic effects of cornelian cherry (*Cornus mas* L.) in alloxan-induced diabetic rats. *J Complement Integr Med*. 2014;11(2):63-9.
22. Nasri H, Rafieian-Kopaei M. Protective effects of herbal antioxidants on diabetic kidney disease. *J Res Med Sci*. 2014;19(1):82-3.
23. Rafieian-Kopaei M, Nasri H. The ameliorative effect of *Zingiber officinale* in diabetic

nephropathy. Iran Red Crescent Med J. 2014;16(5):e11324.

24.Asadi-Samani M, Bahmani M, Rafieian-Kopaei M. The chemical composition, botanical characteristic and biological activities of *Borago officinalis*: a review. Asian Pac J Trop Med. 2014;7 S1: S22-8.

25.Bahmani M, Rafieian-Kopaei M, Hassanzadazar H, Saki K, Karamati SA, Delfan B. A review on most important herbal and synthetic antihelmintic drugs. Asian Pac J Trop Med. 2014;7 S1: S29-33.

26.Madihi Y, Merrikhi A, Baradaran A, Rafieian-Kopaei M, Shahinfard N, Ansari R, et al. Impact of Sumac on postprandial high-fat oxidative stress. Pak J Med Sci. 2013;29(Suppl 1):340-5.

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

28.Asgary S, Kelishadi R, Rafieian-Kopaei M, Najafi S, Najafi M, Sahebkar A. Investigation of

the lipid-modifying and antiinflammatory effects of *Cornus mas* L. supplementation on dyslipidemic children and adolescents. Pediatr Cardiol. 2013;34(7):1729-35.

29.Delfan B, Bahmani M, Hassanzadazar H, Saki K, Rafieian-Kopaei M. Identification of medicinal plants affecting on headaches and migraines in Lorestan Province, West of Iran. Asian Pac J Trop Med. 2014;7 S1:S376-9.

30.Khosravi-Boroujeni H, Sarrafzadegan N, Mohammadifard N, Sajjadi F, Maghroun M, Asgari S, et al. White rice consumption and CVD risk factors among Iranian population. J Health Popul Nutr. 2013;31(2):252-61.

31.Bahmani M, Saki K, Rafieian-Kopaei M, Karamati SA, Eftekhari Z, Jelodari M. The most common herbal medicines affecting *Sarcomastigophora* branches: a review study. Asian Pac J Trop Med. 2014;7 S1:S14-21.