INTRODUCTION

Iron is an essential microelement, which requires several biochemical and physiological processes (e.g., hemoglobin synthesis and mitochondrial enzymatic activity) in the living organisms (Khalili et al., 2015b). However, its excessive amount in the human body causes several problems, including endocrine disrupting, nervous system, lung and vascular diseases, Alzheimer’s disease, Parkinson’s disease, atherosclerosis and aging. Patients with genetic disorders like hemochromatosis thalassemia major and sickle cell anemia may have excessive iron in their bodies (Pari, Prasath, 2008; Farrar et al., 2008; Gilbert, Colton, 1999). Excessive iron leads to Fenton reaction, a reaction in which reactive oxygen species (ROS) cause oxidative stress (Farrar et al., 2008). Iron-chelating compounds can help patients to get rid of the excessive iron. Deferoxamine and deferiprone are two FDA- approved compounds that are currently used as iron chelator agents. These compounds, while being effective, show various side effects that may limit their application for some patients (Grady et al., 2013; Lai et al., 2010; Porter, 2009). Iron- caused liver injuries resulted in the significant increase in the levels of alanine aminotransferase (ALAT) and aspartate aminotransferase (ASAT) enzymes (Sarkar, Hazra, Mandal, 2012). Studies show that cellular enzymes enter the bloodstream when hepatic cells are injured by excessive iron, resulting in increased levels of serum these enzymes, (Reddy, Lokesh, 1996). Studies show that some iron chelation compounds like desferrioxamine (DFO) and deferiprone are caused to lowering serum content of ferritin, serum and ALAT (Chen, Scholl, Stein, 2006).

Transferrin and ferritin are plasma proteins that carry iron ions. Each transferrin and ferritin can carry up to six ions. surpasses Iron ions sediment in hepatocytes when the iron concentration in the serum is over the

Naringin is a promising natural compound for therapy of iron-overload disorders

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Naringin has been shown to exhibit satisfying iron chelation capacity. Considering the side effects of routinely-used iron chelator (desferrioxamine, DFO), we decided to evaluate the iron chelation potency of naringin to discover whether or not it can be a promising natural substitute for treatment of excessive iron-related diseases. Therefore, we provided 35 mice were classified into and they were divided into 5 five groups of 7 and subjected to iron dextran administration to induce the iron-overload condition. Iron-overloaded mice were then treated with normal saline (as control), naringin or DFO (n=7). Group A treated by normal saline, the others treated with iron dextran. After that group A and B treated with normal saline, group C received desferal, group D and E received high and low dose of naringin respectively. Morphology changes, and iron deposition in liver tissues were studied using H&E and Perl’s staining after. The results revealed that naringin is more potent than DFO in removing excessive iron ions deposited in liver tissues, indicating indication that naringin is a promising natural compound for therapy of iron overload disorders.

Keyword: Iron chelation activity. Naringin. Deferoxamine. Iron overloaded mice.

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capacity of iron-carrying molecules. In the patients that receive high amounts of iron, the ions deposit in liver parenchymal cells, leading to emergence of fibrosis and cirrhosis, increasing the risk of hepatocellular carcinoma (Andrews, 1999; El-Shanshory et al., 2018).

Flavonoids are are produced by almost all plant species. These compounds interfere with several cellular processes which shows (Jagetia, Reddy, 2011). Naringin is a flavonoid compound found in citrus (Grazul, Budzisz, 2009). Studies show that naringin likes other flavonoids have iron chelation activity, free radicals scavenging, antioxidant activity, and protection against lipid peroxidation, to inhibit radiation (Jagetia, Reddy, 2005; van Acker et al., 1998; Grazul, Budzisz, 2009).

The currently-used iron-chelating compound (DFO), while being effective, shows several side effects. Naringin has been shown to exhibit satisfying iron chelation capacity in in vivo studies. In the current study, we decided to compare the capacities of these two compounds to see whether or not the latter can be a promising compound with natural origin for therapy of iron-overloaded disorders.

**MATERIAL AND METHODS**

**Animals and experimental design**

Thirty-five male mice NMRI (20-25 g) were purchased from Pastor Institute (Amol, Northern Iran). The mice were kept under controlled conditions: temperature of 24±2 °C, humidity of 45-55%, and the daily light cycle of 12 h light 12 h dark. All the experiments were carried out in the context of ethical guidelines approved by the Ethical committee of Golestan University of Medical Sciences (approval number: ir.goums.rec.1395.278).

The animals were divided into five groups: the control group (group A, n=7), the iron overloaded group (group B, n=7), the DFO group (group C n=7), the group that was treated by naringin 30 mg/kg/day (group D, n=7), and the group that was treated with naringin 60 mg/kg/day (group E, n=7). The group A was treated with normal saline, group C treated by DFO (25 mg/kg/day), and group D and E treated with naringin 30 and 60 mg/kg/day respectively. Treatment was done four days a week for four subsequent weeks (Khalili et al., 2015b). After concluding the experiments, the mice were anesthetized by injection of ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.). Blood samples were taken from their hearts were the. Liver tissues were excised and maintained in formalin buffer (10%).

**Quantification of ferric iron ions (Fe3+) in serum samples**

Serum ferric iron ions were quantified using Pars Azmoon iron quantification kit (Tehran, Iran).

Shortly, Summarily, 100 µl serum samples or standard (calibrator TruCal U, Pars Azmoon, Tehran, Iran) was added to the 1000 µl solution one. After 10 minutes the absorbance was measured at 600 nm. Then, Afterward, 250 µl of the second solution 2 was added to the mixture. After 10 minutes incubation the and absorbance was measured absorbance was read at 600 nm the same wavelength after 10 min at. The ferric iron ions content was calculated according to the following formula:

$$[	ext{Fe}^{3+}] = \left( \frac{\Delta A_{\text{Sample}}}{\Delta A_{\text{Cal}}} \times 187 \right) \times 0.1791$$

**Activity assay of serum marker enzymes**

ALAT and ASAT enzymatic activities were tested using Pars Azmoon enzymatic activity assay kit (Tehran, Iran). Briefly, 100 µl of the serum was added to the 1000 µl mix of the solution 1 and 2. After one minute incubation the absorbance should be read at one was measured at 340 nm after 1, 2, and 3 min utes, respectively incubation at 340 nm (Solutions 1 and 2 were different for ALAT and ASAT test). The average of three replicates was multiplied by 1985.

**Histology**

The paraffin blocks of tissues prepared and then they were cut into 4-micron sections. Samples stained with H&E and Perls’ stains. Pictures were taken by using a
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digital camera (Model: DP73, Olympus, Japan) attached to a light microscope (Model: BX 53, Olympus, Japan) at a 40× magnification.

**Statistical analysis**

Variance analysis (one-way ANOVA) of the data was carried out using Graph Pad Prism 5. (Means were compared using Newman–Keuls multiple comparison tests. Means were reported ± SD.

**RESULTS**

**Serum content of ferric iron (Fe3+)**

As expected, Fe³⁺ seromic level increased significantly \( (p<0.001) \) following *i.p.* iron dextran injection (Figure 1). Iron content increased up to 209.8±4.70 µmol/L, approximately 4-fold increase compared to seromic level of iron in control mice (51.50±4.24 µmol/L). Both DFO and naringin were found to significantly reduce plasma iron content. Plasma iron content in iron-overloaded mice following treatment with DFO, naringin 30, and 60 mg/kg/day were calculated to be 127.0±2.94, 139.4±12.30, and 138.6±3.83 µmol/L respectively.

**The effect of iron dextran on ALAT and ASAT liver enzymes**

Both ASAT and ALAT enzymes were found to increase significantly after treating with the iron dextran \( (p<0.001) \). Concentration of ASAT in iron-overloaded mice was calculated to be as high as 50.31±2.29 IU/L. Both DFO and naringin (30 mg/kg/day) were found to significantly decrease seromic level of ASAT (42.15 ± 1.72 IU/L) \( (p<0.05) \). Naringin at a higher level (60 mg/kg/day) was unable to decrease ASAT content (Figure 2).

**Iron-overloaded mice had the highest level of ALAT, 82.94±2.19 IU/L (group B, Figure 3). Following treatment with DFO and 30 and 60 mg/kg/day of naringin, ALAT level diminished to (61.1±1.92, 50.01±0.15, 52.65±1.03 IU/L, respectively), it is while the content of ALAT in negative group (group A) was 48.74±3.08 IU/L (Figure 3).**
Prussian blue staining

Prussian blue staining indicated that Iron ions deposits in liver tissues of iron-dextran receiving mice as blue spots in the cytoplasm of the hepatocyte. As Figure 5-B shows, large amounts of iron accumulate in the liver. Both DFO and naringin can reduce iron deposition in iron-overloaded mice (Figure 5-C, D, and E respectively).
DISCUSSION

The current study showed that excessive iron could damage liver tissue and lead to an increase in ALAT and ASAT levels in serum. We found that naringin is able to decrease serum iron level in an effective manner, even more potent than the gold standard, desferal. These data indicate that naringin can be a new alternative for therapy of iron-related disease, although more studies are still needed to introduce the compound as a remedy.

Although mammalians need iron for many biological systems, evolved to store, and transform this microelement, however, excessive iron can be toxic for human body; it can produce free radicals, which in turn lead to lipid peroxidation and subsequent diseases (Jagetia, Reddy, 2011; El-Shanshory et al., 2018; Khalili, Ebrahimzadeh, Kosaryan, 2015a). In iron-overloaded patients, iron is found in high levels in both serum liver tissues (Ebrahimzadeh et al., 2016; Khalili et al., 2015b; Khalili, Ebrahimzadeh, Kosaryan, 2015a; El-Shanshory et al., 2018). Iron chelators harness excessive iron and neutralize its side effects. Deferasirox and deferiprone are currently used for iron chelation therapy. Studies have shown that long-term use of these compounds may give rise to some undesirable effects (Grady et al., 2013; Kontoghiorghes, 2007; Badria et al., 2015). There is an increasing interest in the use of natural iron chelators for therapy of iron-related diseases. Flavonoids are among the natural compounds that are able to chelate Fe³⁺ (Mira et al., 2002; Mladěnka et al., 2011; Badria et al. 2015). One of these flavonoids is naringin, whose potency in reducing lipid peroxidation and harnessing superoxide and hydroxide radicals has been reported earlier (Cavias-Saiz et al., 2010; Jagetia, Reddy, 2005). In the current study, we report that naringin can effectively chelate excessive serum iron in iron-overloaded mice.

**FIGURE 5** - Perls’ Prussian blue staining of liver tissues (40x magnification). A: control, B: liver tissues of iron-overloaded mice, C: liver tissues of iron-overloaded mice treated with DFO, D: liver tissues of iron-overloaded mice treated with 30 mg/kg/day of naringin, and E: liver tissues of iron-overloaded mice treated with 60 mg/kg/day of naringin.
Increased level of serum enzymes is associated with some diseases (Chaudhuri et al. 2016). We found that the serum level of ALAT and ASAT enzymes increased in iron-overloaded mice. We also found that excessive iron and liver damage lead to the release of intracellular enzymes into the blood (Figures 2, 3). In comparison to DFO, the gold standard chelator, naringin was found to be more effective in reducing the enzymes in iron-overloaded mice. Reduction of enzymatic activity following treatment with natural products has been reported earlier. For instance, Pari and Prasath (2008) reported that caffeic acid can reduce both Ni content and enzymatic activity (Pari, Prasath, 2008). Iron chelation activity has been detected in the extracts of Spondias pinnata and Colocasia esculenta, as well. These extracts have been shown to decrease serum enzyme level (Chaudhuri et al., 2016; Chinonyelum et al., 2015). We found that naringin is able to protect hepatocytes from the side effect of iron and reduce iron deposition, even more efficiently than DFO. There are a number of natural products shown to reduce iron deposition in liver more effectively than DFO (Ebrahimzadeh et al., 2016; Khalili, Ebrahimzadeh, Kosaryan, 2015a; Khalili et al., 2015b; Eslami, Ebrahimzadeh, Biparva, 2018). The results of our study are consistent with the findings of previous studies (Chaudhuri et al., 2016; Basu et al., 2018; Eslami, Ebrahimzadeh, Biparva, 2018). Naringin iron chelation capacity may be due to its molecular structure; it has two functional groups (Verdan et al., 2011) which may contribute to iron chelation (Figure 6).

In the conclusion, the results of our study indicate that Naringin can significantly chelate excessive iron deposited in mice liver tissues. Also we concluded that Naringin can be a new alternative for therapy of iron-related disease, although more studies are still needed to introduce the compound as a remedy.

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**REFERENCES**

Andrews NC. Disorders of Iron Metabolism. N Engl J Med. 1999;341(26):1986-1995.

Badria FA, Ibrahim AS, Badria AF, Elmarakby AA. Curcumin attenuates iron accumulation and oxidative stress in the liver and spleen of chronic iron-overloaded rats. PLoS One. 2015;10(7):e0134156.

Basu T, Panja S, Shendge AK, Das A, Mandal N. A natural antioxidant, tannic acid mitigates iron-overload induced hepatotoxicity in Swiss albino mice through ROS regulation. Environ Toxicol. 2018;33(5):603-618.

Cavia-Saiz M, Busto MD, Pilar-Izquierdo MC, Ortega N, Perez-Mateos M, Muñiz P. Antioxidant properties, radical scavenging activity and biomolecule protection capacity of flavonoid naringenin and its glycoside naringin: a comparative study. J Sci Food Agric. 2010;90(7):1238-1244.

Chaudhuri D, Ghate NB, Panja S, Basu T, Shendge AK, Mandal N. Glycoside rich fraction from Spondias pinnata bark ameliorate iron overload induced oxidative stress and hepatic damage in Swiss albino mice. BMC Complement Altern Med. 2016;16(1):262.

Chen X, Scholl TO, Stein TP. Association of elevated serum ferritin levels and the risk of gestational diabetes mellitus
Naringin is a promising natural compound for therapy of iron-overload disorders in pregnant women: The Camden study. Diabetes Care. 2006;29(5):1077-1082.

Chinonyelum AN, Uwadiegwu AP, Nwachukwu OC, Emmanuel O. Evaluation of hepatoprotective activity of Colocasia esculenta (L. Schott) leaves on thioacetamide-induced hepatotoxicity in rats. Pak J Pharm Sci. 2015;28(6 Suppl):2237-2241.

Ebrahimzadeh MA, Khalili M, Azadbakht M, Azadbakht M. Salvia Virgata Jacq., and Silibum Marianum L. Gaertn Display Significant Iron-Chelating Activity. Int J Pharm Sci Res. 2016;7(9):3756.

El-Shanshory M, Hablas NM, Aboonq MS, Fakhreldin AR, Attia M, Arafa W, Mariah RA, Baghdadi H, Ayat M, Zolaly M, Nabo MMH, Almaramhy HH, El-Sawy SA, Zidan M, Elshazl M, Alharbi R, Moustafa S, Abu-el Naga M, El-Sayed SM et al. Nigella sativa improves anemia, enhances immunity and relieves iron overload-induced oxidative stress as a novel promising treatment in children having beta-thalassemia major. J Herb MedJournal of Herbal Medicine. 2018;100245.

Eslami S, Ebrahimzadeh MA, Biparva P. Green synthesis of safe zero valent iron nanoparticles by Myrtus communis leaf extract as an effective agent for reducing excessive iron in iron-overloaded mice, a thalassemia model. RSC Advances. 2018;8(46):26144-26155.

Farrar JE, Nater M, Caywood E, McDevitt MA, Kowalski J, Takemoto CM, Talbot CC, Meltzer P, Esposito D, Beggs AH et al. Abnormalities of the large ribosomal subunit protein, Rpl35a, in Diamond-Blackfan anemia. Blood. 2008;112(5):1582-1592.

Gilbert DL, Colton CA (1999) Reactive oxygen species in biological systems: an interdisciplinary approach. Springer. doi:10.1016/S0144-8617(01)00197-7

Grady RW, Galanello R, Randolph RE, Kleinert DA, Dessi C, Giardina PJ. Toward optimizing the use of deferasirox: potential benefits of combined use with deferoxamine. Haematologica. 2013;98(1):129-135.

Grazul M, Budzisz E. Biological activity of metal ions complexes of chromones, coumarins and flavones. Coord Chem Rev. 2009;253(21–22):2588-2598.

Jagetia GC, Reddy TK. Modulation of radiation-induced alteration in the antioxidant status of mice by naringin. Life Sci. 2005;77(7):780-794.

Jagetia GC, Reddy TK. Alleviation of iron induced oxidative stress by the grape fruit flavanone naringin in vitro. Chem Biol Interact. 2011;190(2):121-128.

Khalili M, Ebrahimzadeh MA, Kosaryan M. In Vivo Iron-Chelating Activity and Phenolic Profiles of the Angel’s Wings Mushroom, Pleurotus porrigenus (Higher Basidiomycetes). Int J Med Mushrooms. 2015a;17(9):847-856.

Khalili M, Ebrahimzadeh MA, Kosaryan M, Abbasi A, Azadbakht M. Iron chelation and liver disease healing activity of edible mushroom (Cantharellus cibarius), in vitro and in vivo assays. RSC Advances. 2015b;5(7):4804-4810.

Kontoghiorghes GJ. Deferasirox: uncertain future following renal failure fatalities, agranulocytosis and other toxicities. Expert Opin Drug Saf. 2007;6(3):235-239.

Lai ME, Grady RW, Vacquer S, Pepe A, Carta MP, Bina P et al, Sau F, Cianciulli P, Maggio A, Galanello R, Faciri P. Increased survival and reversion of iron-induced cardiac disease in patients with thalassemia major receiving intensive combined chelation therapy as compared to desferoxamine alone. Blood Cells Mol Dis. 2010;45(2):136-139.

Mira L, Tereza Fernandez M, Santos M, Rocha R, Helena Florêncio M, Jennings KR. Interactions of flavonoids with iron and copper ions: a mechanism for their antioxidant activity. Free Radic Res. 2002;36(11):1199-1208.

Mladěnka P, Macáková K, Filipský T, Zatloukalová L, Jahodár L, Bovicelli P et al. In vitro analysis of iron chelating activity of flavonoids. J Inorg Biochem. 2011;105(5):693-701.

Pari L, Prasath A. Efficacy of caffeic acid in preventing nickel induced oxidative damage in liver of rats. Chem Biol Interact. 2008;173(2):77-83.

Porter JB. Optimizing iron chelation strategies in β-thalassaemia major. Blood Rev. 2009;23 Suppl 1:S3-S7.

Reddy ACP, Lokesh B. Effect of curcumin and eugenol on iron-induced hepatic toxicity in rats. Toxicology. 1996;107(1):39-45.

Sarkar R, Hazra B, Mandal N. Reducing power and iron chelating property of Terminalia chebula (Retz.) alleviates iron induced liver toxicity in mice. BMC Complement Altern Med. 2012;12(1):1-10.

van Acker SA, van Balen GP, van den Berg DJ, Bast A, van der Vijgh WJ. Influence of iron chelation on the antioxidant activity of flavonoids. Biochem Pharmacol. 1998;56(8):935-943.

Verdan AM, Wang HC, García CR, Henry WP, Brumaghim JL. Iron binding of 3-hydroxychromone, 5-hydroxychromone, and sulfonated morin: implications for the antioxidant activity of flavonols with competing metal binding sites. J Inorg Biochem. 2011;105(10):1314-1322.

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