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Article

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. is being used 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 threated 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 slaine, 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 thant 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.

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- certifiedapproved 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 injuriesy 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 serumdecrease 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

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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 citruses (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; the other groups were given iron dextran (100mg/kg/day) as *i.p.* injections for four weeks, and four days each week. After that all animals were left to their own devices for one month, then the group A and B cv 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. were 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 heartswere 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).

ShortelySummarily, 100 µl serum samples or standard (calibrator TruCal U, Pars Azmoon, Tehran, Iran) was added to the 1000 µl solution one1., After 10 min,utes the absorbance was measured at 600 nm. ThenAfterward, 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 nmthe same wavelength after 10 min at.. The ferric iron ions content was calculated according to the following formula:

$$Iron(\mu moll^{-1}) = (\frac{\Delta A\ Sample}{\Delta A\ Cal} \times 187) \times 0.1791$$

Activity assay of serum marker enzymes

ALAT and ASAT enzymatic activities were tested using Pars Azmoon enzymatic activity assay kit (Tehran, Iran). In briefBriefly, 100 µl of the serum was added to the 1000 µl mix of the solution 1 and 2. After one minute incubation the aAbsorbance should be read at onewas measured at 340 nm after 1,, 2, and 3 min utes, respectivelyincubation 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

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, Fe^{3+} 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.

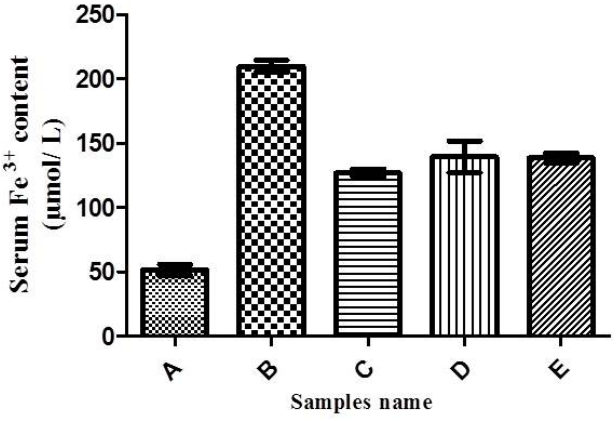

FIGURE 1 - The effect of iron chelators on serum Fe^{3+} content. A: the control group, the mice receiving normal saline B: Iron-overloaded mice with no iron chelator, C: Iron-overloaded mice receiving DFO, D: iron overloaded mice receiving 30 mg/kg/day of naringin, and E: iron overloaded mice receiving 60 mg/kg/day naringin.

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 (fFigure 2).

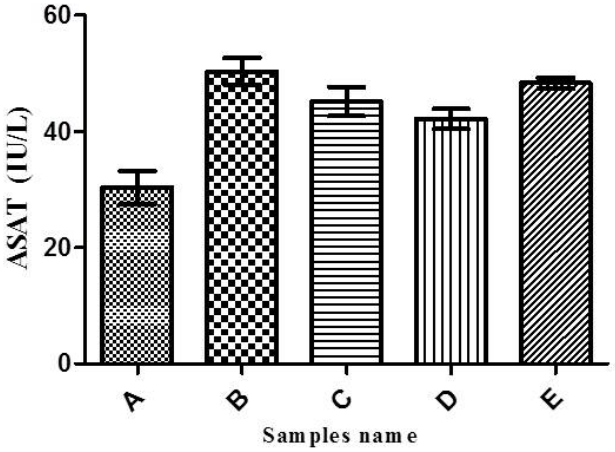

FIGURE 2 - ASAT content in serum samples following treatment with iron dextran and iron chelators. A: control group, B: iron-overloaded mice, C: iron- overloaded mice receiving DFO, D: iron-overloaded mice receiving 30 mg/kg/day naringin and E: iron- overloaded mice receiving 60 mg/kg/day naringin.

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).

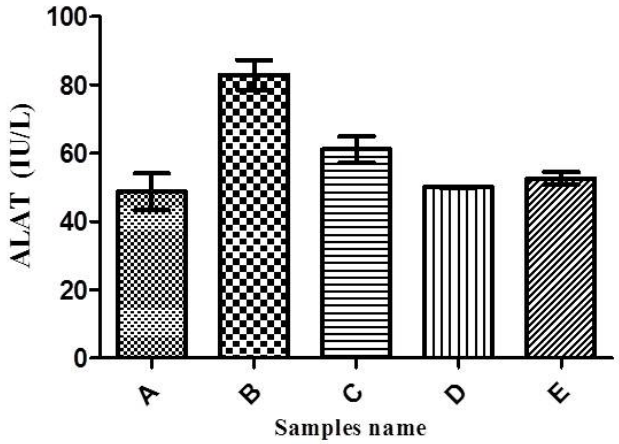

FIGURE 3 - ALAT content in serum samples following treatment with iron dextran and iron chelators A: control group, B: iron-overloaded mice, C: iron-overloaded mice receiving DFO, D: iron-overloaded mice receiving 30 mg/kg/day naringin, and E: iron-overloaded mice receiving 60 mg/kg/day naringin.

Histology

H&E staining

H&E staining revealed that iron deposits in liver tissues and causes morphological abnormalities (Figure 4); hepatic cells undergo morphological distortion, lobules disintegrate, bile ducts and pseudo- lobules emerge, and portal tract become inflamed (Figure 4-A and 4-B). Focal necrosis and periportal inflammation are seen in liver tissues of DFO-treated mice (Figure 4-C). A lower degree of necrosis and inflammation occurs in liver tissues of those mice treated with narengin (Figure 4-D and 4- E).

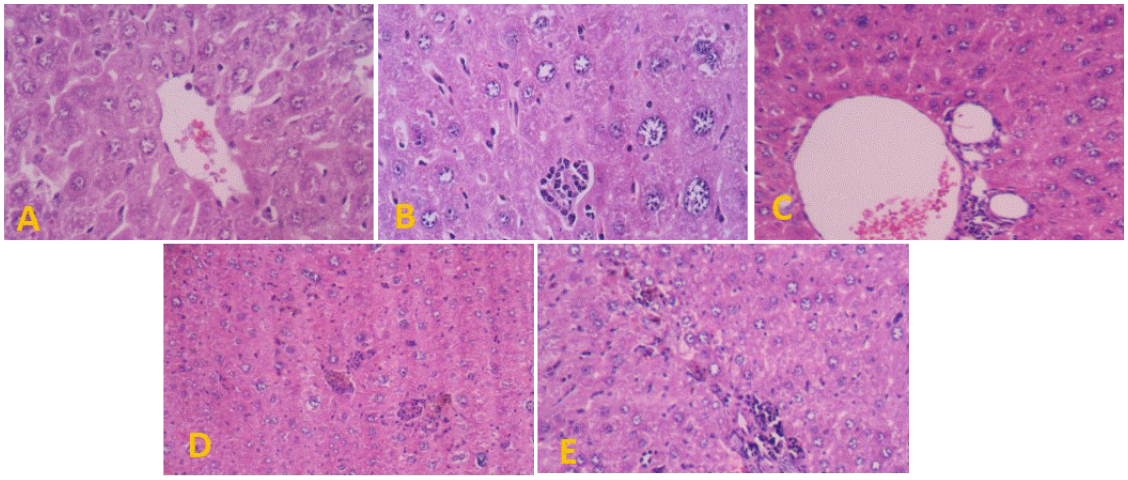

FIGURE 4 - H&E staining of liver tissues of normal and iron-overloaded mice (40× magnification). A: normal liver tissue, B: liver tissue From Iron-overloaded mice, C: liver tissue from an iron- overloaded mice receiving DFO as *i.p.* injection, D: liver tissue from iron-overloaded mice receiving 30 mg/kg/day naringin, and E: tissue from an iron- overloaded mice receiving 60 mg/kg/day naringin.

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).

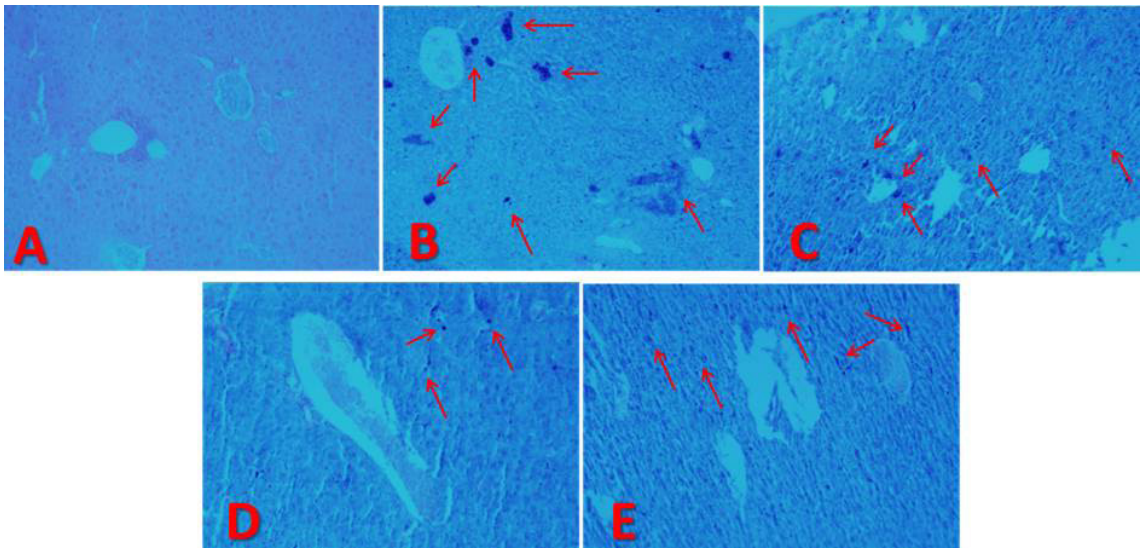

FIGURE 5 - Perls' Prussian blue staining of liver tissues (40x magnification). A: control, B: liver tissues of iron-overloaded mice, C: liver tissues of iron-overloaded mice treated with DFO, D: liver tissues of iron-overloaded mice treated with 30 mg/kg/day of naringin, and E: liver tissues of iron-overloaded mice treated with 60 mg/kg/day of naringin.

DISCUSSION

The current study showed that excessive iron could damage liver tissue and lead to an an increase in of 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; Ebrahimzadeh *et al*., 2016).

Dietary iron as well as some diseases (e.g. hereditary hemochromatosis, chronic liver diseases, and diseases associated with hemolytic anemia such as β-thalassemia) can lead to abnormal iron overloading in human body (Badria *et al*., 2015). Myelodysplastic syndromes, heart and cardiovascular diseases, gastrointestinal tract dis- orders, aging, diabetes, autoimmune nephrotic syndromes, cataract genesis, degenerative retinal damage, Alzheimer and Parkinson, bronchopulmonary dysplasia, and cancer are among the diseases that are associated with excessive iron in the body (Farrar *et al*., 2008; Khalili *et al.,* 2015b; Jagetia, Reddy, 2011; 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 Fe^{3+} (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 (Cavia-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.

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.

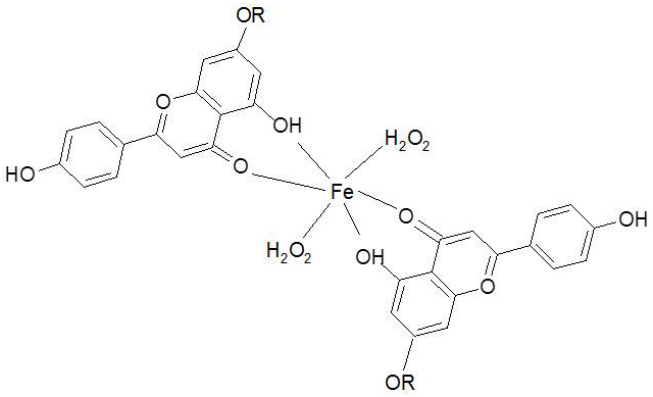

FIGURE 6 - Molecular structure of naringin and potential mechanism of iron chelation. Hydroxyl groups may be responsible for iron chelation activity. Two naringin molecules may be involved in trapping one iron ion.

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