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Effects of Anethum graveolens, Urtica dioica, Milk thistle Aqueous Extract and Deferoxamine on total iron binding capacity, iron and ferritin levels

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Abstract

***Background & Aims*:** Iron overload is one of the medical problems in some diseases. Recent reports have indicated a need for iron chelator. In this study, the possible iron chelating properties of aqueous extracts of A.graveolens, Urtica dioica and milk thistle on total iron binding capacity, iron and ferritin levels in iron overloaded rats were investigated.

***Materials & Methods*:** Fresh A.graveolens and Urtica dioica leaves and milk thistle seeds were obtained from the local market. Fortyeight male rats were randomly divided into six groups: negative control (normal), positive control (iron overload) and groups treated with deferoxamine (DFO), aqueous extracts of A.graveolens, Urtica dioica and milk thistle. Iron dextran was injected intraperitoneally (i.p.) at 50mg/kg body weight/day to establish the iron overload for twelve weeks. Normal group rats received normal saline, while the rats of the treated groups received (orally) aqueous extracts of A.graveolens, Urtica dioica and Milk thistle and DFO daily for eight weeks. Changes in biochemical factors were measured at the end of the experiment.

***Results*:** After twelve weeks of iron dextran treatment, we found a significant increase in iron and ferritin levels and a decrease in total iron binding capacity (TIBC) level compared to normal group (229.0±5.85, 181.22±5.53 and 200.54 ±1.51 vs. 131.90±6.85, 50.25 ±4.01 and 291.71, respectively). After eight weeks of treatment with extracts and DFO, there was significant reduction in serum iron level of extracts of A.graveolens, Urtica dioica and milk thistle and DFO treated groups compared to iron overloaded group (185.81±3.5, 180.88±2.73, 200.6±2.44, 176.48 ±2.29 vs. 229.0±85.5). Also, there was a significant increase in serum TIBC level in the extracts of A.graveolens, Urtica dioica and milk thistle and DFO treated groups compared to iron-overloaded rats (218.62±2.44, 226.74±2.71, 211.06±1.86, 231.57 ±2.05 vs. 200.54±1.51, respectively). Furthermore, there was a significant reduction in serum ferritin level in the extracts of A.graveolens, Urtica dioica and milk thistle and DFO treated groups compared to iron-overloaded rats (130.49±4.24, 121.96±4.31, 140.63±3.82, 112.87 ±4.60 vs.181.22±5.53). So after eight weeks treatment with aqueous extracts of

A.graveolens, Urtica dioica and milk thistle, we found a significant reduction in iron and ferritin and an increase in TIBC level. These effects indicated the following hierarchy: Urticadioica >Anethum graveolens > milk thistle.

***Conclusion*:** Anethum graveolens, Urtica dioica and milk thistle extracts may be a potential herbal plant to reduce liver damage caused by iron overload. These results indicated that Anethum graveolens, Urtica dioica and milk thistle extracts can preserve liver function.

***Keywords*:** Aqueous extract, Anethum graveolens, desferrioxamine, Urtica dioica and milk thistle

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Introduction

Iron is a basic requirement for the survival and growth of cells. Iron is a required trace element in both prokaryote and eukaryote organisms, because it plays a role in some cellular processes such as oxygen transport, electron transport, and energy metabolism(1). Serum iron concentration normally is between 3-5 µg/ml in which its dominant form is binding to transferrin (as trivalent Fe) and albumin to a lesser extent(2). Free iron content should be kept in the least required amount to prevent the dangerous effects caused by oxidative stress(3). Iron overload may cause by regular blood transfusions in patients with different types of anemia or high intestinal iron absorption due to genetic defects(4). Humans have no physiological pathway for excess iron removal in iron overload conditions(5). Besides, excess iron deposits in various tissues lead to dysfunction in heart, liver, spleen, endocrine tissues(4). Chelators are called molecules that could form complex with metals and excrete through urine and stool(4). Iron chelation with chelator agents is a suitable method in eliminatingexcess iron and its complications in diseases that develop iron overload(5). Abnormal accumulation of iron develops clinical complications such as diabetes, heart failure, cirrhosis and finally death(6). Iron participates in the reactions producing reactive oxygen species (ROS) such as fenton reaction; this reaction is done slowly in normal conditions (7). ROS leads to biomolecular damage such as proteins, lipids and DNA(8). The purpose of using chelator agents is to maintain normal levels of iron in the tissues and cells to prevent iron overload complications(9). Nowadays, industrial drugs are used to treat disorders like deferoxamine and 1,2-dimethyl-3-hydroxypyrid-4-one (deferiprone, L1)and deferasirox. Despite the beneficial effects, these drugs have harmful effects on body. Since deferoxamine is not absorbed dietary and requires repeated injections to obtain better effect, it can lead to suffering for patients.(10). Long-term use of deferiprone leads to reduced number of agranulocytes, especially neutrophils(4). Moreover, deferasirox causes gastrointestinal disorders such as nausea, vomiting, and abdominal pain(11). So, it seems a chelator with fewer complications and absorbing dietary is required (10). Since the extracts of medicinal plants have less harmful effects than synthetic drugs, thus, researchers are trying to find more effective and safer herbal treatments(12). From ancient times, the plants were used for their therapeutic properties. Plants of chelating activity is attributed to their constituents including phenolic compounds that are plant secondary metabolites(4).Secondary metabolites include phenolics, flavonoids and terpenoids. These compounds function as antioxidant and immune-modulators(13).

From a chemistry perspective, phenolic compounds are molecules containing hydroxyl groups attached to the benzene ring. Plants are source of 8000 polyphenol for humans that are classified into two flavonoid and non-flavonoid compounds(14). Flavonoids act as protective constitutes, because they have properties such as electrons transfer of free radicals, metal chelation, activation of antioxidant enzymes, inhibition of oxidases and reduction of alpha-tochopherol radicals(15). The antioxidant capacity and chelating activity of polyphenols are related to a number of hydroxyl groups and their place in the benzene ring(16).

Urticadioica Linn of family Urticaceae is a wildling, annual and durable plant that is also introduced as stinging nettle(17). From thousands of years ago, due to urtica dioica of treatment capabilities, its extract is used

for amelioration of disorders like rheumatism, eczema, gout and anemia. Many researches indicated that its medicinal features are related to secondary metabolites(18). These metabolites are abundant in leaves among 3-caffeoylquinic acid ،caffeoyl malic acidkaempferol, isorhamnetin and quercetin(19). The milk thistle or silybummarianum is an annual or biennial plant which belongs to asteraceae family(20). Some of the important phenolic compounds include silybin A (12.2%), silybin B (17.67%), isosilybin A (21.9%), isosilybin B (12.8%), silychristin (7.9%) and silydianin (7.5%). these compounds are rich in seeds(21).Anethumgraveolens L. is another plant that the existence of phenolics and flavonoids in it was investigated. This plant known as dill, is annual and native in Europe, Mediterranean region, and Asia having famous compounds such as flavonols, alkaloids, antocianin, tannin and saponin(22). Administration of Anethum graveolens, Urtica dioica, and Silybum marianum extracts have beneficial effects on liver enzymes activity (23).This study aimed to examine the effect of Anethum graveolens ,Urtica dioica and milk thistle, on iron-overloaded rats in comparison to deferoxamine(DFO).

Materials and Methods

All solvents and reagents used for this study were measurable. AlCl3, Potassium acetate, sodium carbonate, galic acid, ethanol, quercetin, normal saline were purchased from Asia Pajohesh Co., Iran.

Iron dexetran was purchased from Viforco.,DFO was obtained from Exir Pharmaceutical Co, made in Iran. Iron and TIBC kits were purchased from Darman Kave and Darmanfarazkave Co. (Tehran, Iran),respectively. Ferittin kit was purchased from Immunology Consultants Laboratory Inc.

Instruments:

Vortex mixer labnet, single beam UV/visible spectrophotometer (UNICO, 2100, USA)**,** centrifuge (Clement 2000**,** Australia), waterbath (Fanazmagostar Co WM22), Dionizer (HastaranTeb Co). Tazu (A&D) (AND Japan), Shaker (J-kottermann-KG,3165, Hanigsen, type 4010 Germany), Elisa reader (AWERENESS Technologynic, Stat Fax -2100), HPLC; (Unicam Crystal-200, UK)

Plant materials:

Aerial parts of A.graveolens, Urtica dioica and seeds of milk thistle were purchased freshly from local market on July 2016 to May 2017. The plant was taxonomically identified by botanical experts at the department of agriculture. Leaves were dried in the shade at room temperature.

Preparation of plant leaves:

Fresh leaves of A.graveolens, Urtica dioica and seeds of milk thistle were washed with water and rinsed with distilled to remove possible pesticide and preservative residue.

Aqueous extraction of Plants:

The leaves of A.graveolens, Urtica dioica and seeds of milk thistle were dried at room temperature for 10 days, finally powdered and used for extraction. The powder (20 g) of A.graveolens, Urtica dioica and seeds of milk thistle was respectively mixed with 550, 800 and 440 ml distilled water using a shaker for 24 hours, then this process was repeated again with the precipitated pellet. The sediments were filtered and collected. All samples of the extract were stored at −4◦C until used for the further analysis.

Animal:

Forty-eight male albino Wistar rats (weighing 185 to 195 g) were originally supplied by the Central Animal House Babol, Iran. The animals were housed in a climate-controlled room (23°C ± 2°C) with a 12-hour light and dark cycle. The animals were fed on pellet diet and water was freely available. All procedures involving animals were carried out according to the guidelines for care and use of experimental animals, and this study was approved by the Ethics Committee for Research of Babol University of Medical Sciences (MUBABOL.HRI.REC. 1395.38 -3031).

Experimental design:

Rats were randomly divided into six groups, each containing eight samples. Group 1(negative control group) received normal saline (0.5 ml/kg) by i.p. route during the project. To induce iron overload in 2-6 groups, rats were given iron dextran 50mg/kg(4) by i.p rout for 12 weeks (each week once time). One week after

iron overload induction, 3-6 groups were treated respectively with A.graveolens, Urtica dioica and milk thistle extracts (orally) and DFO (by subcutaneous) at dose 50mg/kg for eight weeks (once each week). Serum samples collected before and after treatment. At the end of the experiment, the rats were fasted overnight, anaesthetized by diethyl ether and blood was collected from the eyes (venous pool) by standard venous puncture with glass capillary tubes in dry, clean and screw capped tubes and left to clot.

Determination of Biochemical parameters:

Blood samples were collected and serum was separated for biochemical parameters analysis. The levels of serum iron and the serum total iron binding capacity were determined using quantitative diagnostic kits. The serum ferritin was measured using enzyme immune assay Eliza kit. The assay and the outcomes of assaying biochemical markers employed here have been typically validated against other methods and had good inter- and intra-assay coefficients of variation (23).

Determination of total phenolic compounds:

Total phenol content of the extracts was assessed by folinciocalteu method and results were expressed as acid galicmgr/gr extract. This assay is the most common method for the measurement of phenolic compounds. Based on this method, folinciocalteu reagent is reduced by phenolic compounds in alkaline environment that leads to blue complex formation. This complex has absorption maximum wavelength of 760 nm. Extract (0.5ml) was mixed with folinciocalteu reagent 0/2 N (2.5 ml). After 5 minutes, sodium carbonate 75 g/l (2ml) was added. Then solution was incubated in room temperature for 2 hours. The absorption reaction was measured in wavelength 760 nm(24).

Determination of flavonoid compounds:

Flavonoid content of the extracts was assessed by ALCL3 method and the results were expressed as quercetin mgr/gr extract, in which the extract (0.5 ml) was mixed with methanol (1.5ml), aluminum chloride10% (0.1ml), potassium acetate 1M(0.1) and distilled water (2.8ml), then this solution was kept in room temperature for 30 minutes. Solution absorption was measured in wavelength 415 nm(24).

The analysis of silymarin samples was carried out using a Unicam crystal-200 liquid chromatograph (England), equipped with a Nucleosil C18 (150 × 4.6 mm I.D, 5 μm) column. A mixture of methanol-water (50:50, v/v) served as the mobile phase. The elution has been made in an isocratic mode at a flow rateof 1mL/min and the detection made at 288 nm. One analysis requires 20 min.

Chemical analysis of A.graveolens, Urtica dioica and milk thistle extracts composition was performed by HPLC method with the cooperation of Faculty of Department of Biological Sciences and Department of Cell and Molecular Biology of Kharazmi University, Tehran, Iran.

Another part of the our results entitled the effect of the aqueous extract of anethum graveolens, urticadioica, milk thistle on the liver function in iron over loaded rats is being developed and will be reported in another paper.

Statistical analysis:

All values were expressed as the mean ± standard deviation of the mean. Statistical analysis was performed using SPSS 18. Probability of p.value was below 0.001. The sample size was based on the outcome of previous studies.

Results

Figure 1 shows the serum iron, ferritin and TIBC Levels of normal and experimental animals. There was a significant ($P<0.001$) decrease in serum iron and ferritin compared to the positive control group. TIBC levels significantly increased in experimental groups compared to the positive control.Intraperitoneal injections of iron-dextran (50 mg/kg body wt.) was evenly distributed over a 12-week period that resulted in iron overload condition (serum iron 229.0±5.85μg/dl). The normal rats were injected with an equal volume of normal saline at the same time showed normal level of iron (serum iron 131.90 ± 6.85μg/dl) in rats. There was a significant increase in serum ferritin level in iron overloaded group rats (181.22 ± 5.53 ng/dl) compared to normal control group rats (50.25 ± 4.01 ng/dl). There was a significant decrease in serum TIBC level in iron overloaded group rats (200.54 ± 1.51 μg/dl) compared to normal control group rats (291.71 ± 5.34 μg/dl).

Administration of aqueous extracts of A.graveolens, Urticadioica and milk thistle at dose 50 mg/kg tended to bring the value closer to normal. These effects showed the following hierarchy: Urticadioica >A.graveolens >milk thistle.

There was a significant difference between A. graveolens, Urtica dioica and milk thistle extracts of the iron chelating capacity.

The correlation of mean value of iron, TIBC and ferritin levels in iron overload rats in different treated rats serum are illustrated in Figure 2.

The concentrations of the main compounds were signified with HPLC method in aqueous extracts of Urticadioica (Rutin, Chlorogenicacid, and Elagic acid), milk thistle (Silimarine and Silidianin) andA.graveolens (Apigenin, Luteolin and Kampferol). Total phenolic and flavonoid compounds of A.graveolens, Urtica dioica and milk thistle aqueous extract are shown in table 1.

Table 1. Total phenolic and flavonoid compounds aqueous extract of A.graveolens, Urtica dioica and milk thistle

aqueous extract	Total phenolic(µg/ml)	flavonoid(µg/ml)
A.graveolens	42.83±4.00	20.73±3.50
Urticadioica	86.64±3.50	38.53±1.50
milk thistle	23.04±3.49	12.65±2.51

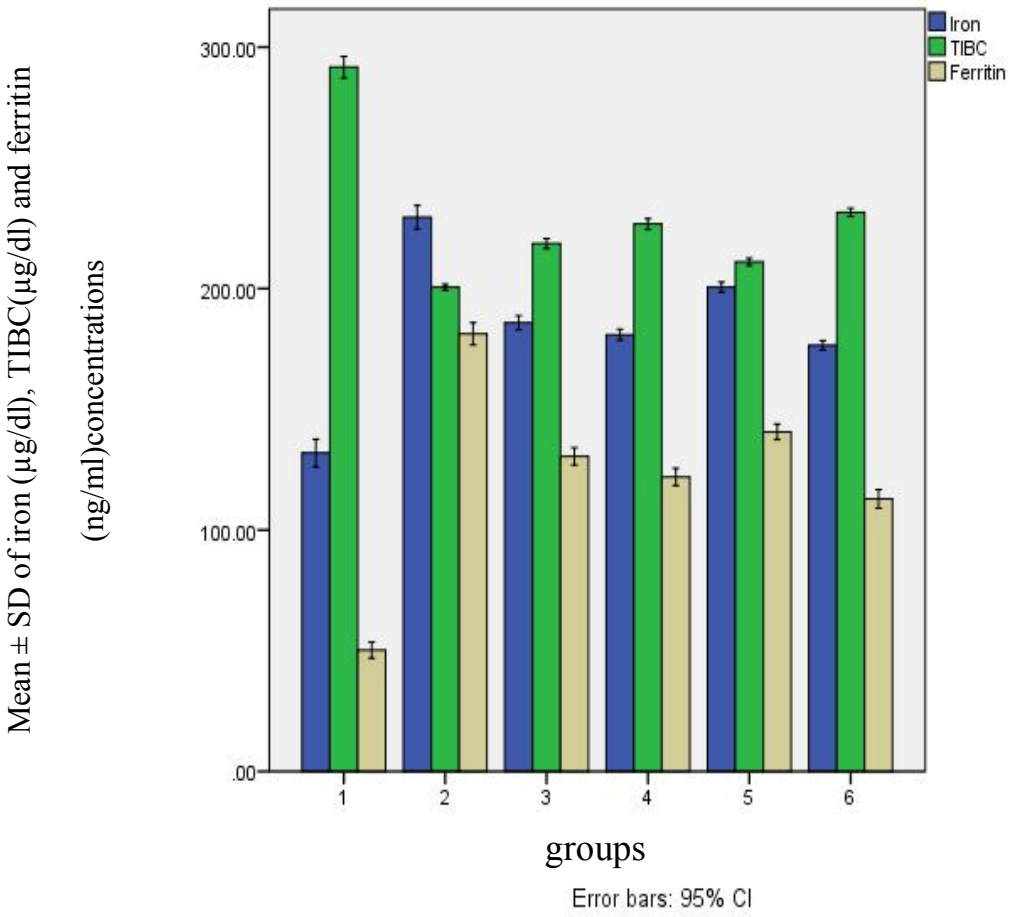

Figure 1. Iron, TIBC and ferritin levels in iron overload rats in different treated groups.

Rats were randomly divided into six groups: (1)negative control group, (2)positive control group (iron overload group), (3) Iron-dextran plus A.graveolens extract, (4)Iron-dextran plus Urticadioica extract, (5)Iron-dextran plus milk

thistle extract group, (6) Iron-dextran plus DFO group(50mg/ kg/1day). Column 1, Iron (µg/dl); column 2, TIBC(µg/dl);column 3,ferritin(ngdl/). Values are expressed as mean ± SD of eight rats. P <0.001 compared with control.

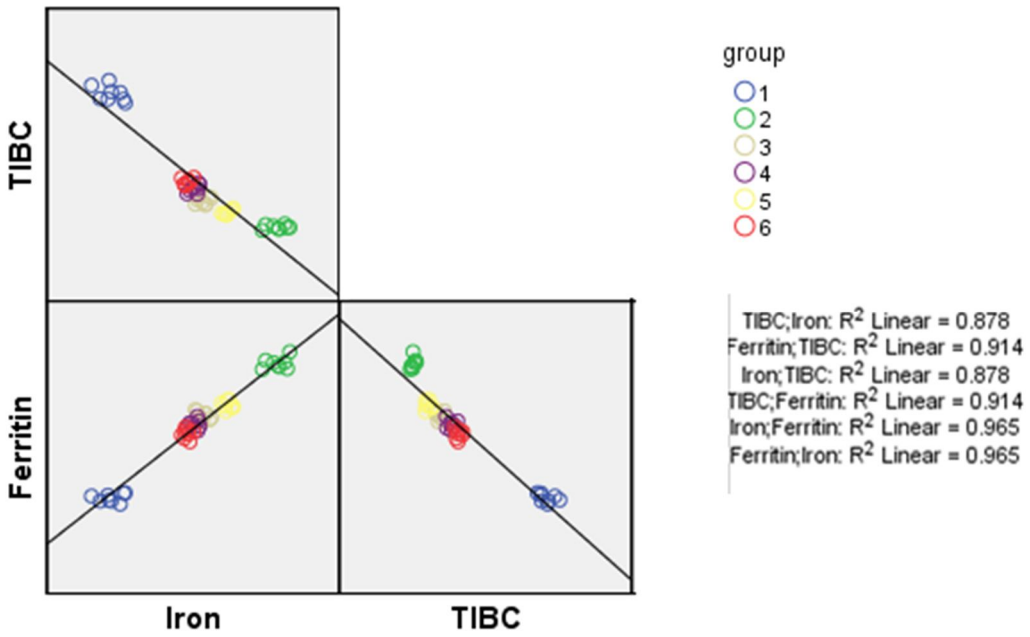

Figure 2. The correlation of mean value of Iron, TIBC and ferritin serum levels in iron overload rats in different treated groups. (1) negative control group, (2)positive control group (iron overload group), (3) Iron-dextran plus A.graveolens extract, (4)Iron-dextran plus Urticadioica extract, (5)Iron-dextran plus milk thistle extract group, (6) Iron-dextran plus DFO group(50mg/ kg/1day).

Discussion

Iron overload mediated reactions have been found to be involved in the disturbed liver function. Nowadays, drugs are used to treat iron overload including DFO, deferiprone and deferasirox. Despite the beneficial effects, these chemical drugs have side effects on the consumer body. For instance, DFO dose not have oral absorption ability, likewise, for sufficient effect requires repeated injections which may cause patients to suffer (10). Long-term use of deferiprone leads to reducing the number of agranulocytes especially neutrophils(4). In addition, deferasirox causes gastrointestinal disorders such as nausea, vomiting, and abdominal pain(11). So, it seems a chelator is required with less complications and also could be absorbed dietary(10). As the extracts of medicinal plants develop less harmful effects than synthetic drugs, researchers are trying to find more effective and safer treatments (12). During recent years, there has been a growing interest in identifying the potential iron chelators against iron over load, especially those compounds obtaining from natural substances. In this study, the aqueous extracts of A. graveolens, Urtica dioica leafs and milk thistle seed extracts were assessed for their iron chelating ability and compared with DFO. A. graveolens, Urtica dioica and milk thistle extracts administration in iron overloaded rats reduced the serum iron levels.

The elevated serum iron level was found in iron-overloaded rats compared to normal rats. Administration of A.graveolens, Urticadioica and milk thistle extracts and DFO reduced the serum iron level to the 18.86%, 21.01%, 12.4% and 22.93%. The obtained data suggested that A.graveolens, Urticadioica and milk thistle extracts have chelating power nearer to synthetic iron chelating agent DFO. The iron chelating activity of anethum graveolens, urtica dioica and milk thistle extracts could be due to scavenging or chelating properties of compounds present in these plants such as flavonoids and phenols. Ebrahimzadeh et al had studied iron chelating activity of Danshen and milk thistle

extract in iron overloaded mice and observed significant reduction in plasma iron levels in iron-overloaded mice treated with the extracts.Also,we indicated iron chelating effect of milk thistle extract on iron- overload treated rats. Silymarin is the most important compounds in the milk thistle extract, it can be attributed the protective effect of this plant extract to the composition of the silymarin(25). Another hypothesis is suggested that these plant extracts decrease absorption of iron and have iron chelator capabilities because of their scavengering properties.

The decrease in serum iron content induced by A. graveolens, Urtica dioica and milk thistle extracts treatment supports theirs iron-chelating potency which was established previously.

The present study showed that a variety of iron scavengers are presented in A.graveolens, urtica dioica and milk thistle including flavonoids, carotenoids.

Our results showed that iron chelating activity of Urtica dioica extract with the dose of 50 mg/kg in iron over loaded rats was more effective than A. graveolens and milk thistle extracts. This effect may be related to the presence of more iron scavengers in Urticadioica.Bahramikiaet al analyzed inhibitory effects of the plant extracts against ROS formation and LPO to varying degree. According to their study Teucrium Rotundus has Polyphenol compounds more than another plant and showed more protective effect, therefore the protective effects of the extracts have been attributed to the content of their polyphenols(26). More studies should be conducted to elucidate the mechanism of action details of the A. graveolens, Urticadioica and milk thistle extracts in iron over load treatment.

According to the results, because A. graveolens, Urtica dioica and milk thistle extracts are herbal products, and their side effects were limited, so they might be used as an easily accessible source of natural food supplement in subjects with iron-overload condition, such as beta-thalassemia. In future, these products could be considered as products that will enhance the life quality of b-thalassemia patients. Nevertheless, it is suggested to perform further research on the isolation and identification of the chelating components in A. graveolens, Urticadioica and milk thistle.

Conclusion

According to the results of this study, it can be concluded that A.graveolens, Urtica dioica and milk thistle extracts have protective effect against iron overload-induced liver toxicity as evidenced by biochemical studies. Due to the benefits provided and the absence of toxicity, A. graveolens, Urticadioica and milk thistle extracts could be useful therapeutic substitutions to reduce the liver toxicity induced by iron overload status.

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Author Contribution:

DQ, Designed the experiments, MH performed the experiments, T analyzed the results and DQ wrote the manuscript.

Disclosure of Potential Conflicts Of Interest:

The authors declare that they have no conflict of interests.

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