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Iron chelation and liver disease healing activity of edible mushroom (*Cantharellus cibarius*), *in vitro* and *in vivo* assays

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ABSTRACT

Cantharellus cibarius, an edible mushroom, has been reported to display a wide variety of biological properties, including iron-chelation activity. In the current study we evaluated chelating capacity of ethyl acetate and methanolic extracts of *Cantharellus cibarius*, on iron-overloaded mice. Extracts and defroxamine were injected for a frequency of 5 times a week for 4 weeks. Total iron and Fe^{3+} content of plasma was determined by atomic absorption spectroscopy and kit respectively. Liver sections were stained by haematoxylin and eosin and Perls' stain. Iron- overloaded animals treated with the extract, showed a dramatic decrease in plasma iron content when compared with the control group. The highest activity was observed in the methanolic extract. A high-performance liquid chromatography was performed to simultaneously separate 5 phenolic acids and 2 flavonoids in extracts. *p*-Coumaric acid and ferulic acid were discovered to be major phenolic acids in ethyl acetate extract and methanol extract, respectively. Both methanolic and ethyl acetate derived extracts of mushroom *Cantharellus cibarius* exhibit satisfactory potency to chelate excessive iron in mice.

Keywords: Iron overload, *Cantharellus cibarius*, Hepato protection, Iron chelation, Thalassemia, liver disease.

Highlights

- Methanolic extract of Cantharellus cibarius shows significant iron chelation activity both in vitro and in vivo.
- Ethyl acetate extract of Cantharellus cibarius displays significant iron chelation activity both in vitro and in vivo.
- Methanolic extract of Cantharellus cibarius is more efficient than its ethyl acetate extract in chelation of excessive iron from plasma and liver.

1. Introduction

Patients with major thalassemia require red blood cell transfusion to reduce the anemia and to expand the bone marrow, guaranteeing the normal growth during childhood and extended survival. However, after a year of transfusion iron accumulates in some tissues such as liver and leads to a number of problems, including hepatocellular necrosis, inflammation, and fibrosis and hepatocellular lipid peroxidation of polyunsaturated fatty acids in cell membranes [1-5]. A fraction of iron that does not bind transferrin (iron carrier protein) may endorse the generation of free hydroxyl radicals, spreading oxygen-related damage [6]. In such conditions an iron-chelating agent can be an alternative to eliminate excessive iron and neutralize free radical damages [1, 3, 6-7]. Among natural compounds, flavonoids and phenols have been reported for iron chelation capacity [8-11].There are some iron chelators currently used to manage and treat thalassemia and other transfusion-dependent anemia among which are Deferoxamine (DFO), Deferiprone (L1), Deferasirox (ICL-670) [3, 12]. Although these drugs are very effective, they exhibit many side effects [2-4], necessitating to be replaced by safer alternatives. In ancient medicine, mushrooms have an extensive use in treatment of human diseases including liver diseases [13-14]. In recent years, consumption of wild edible mushrooms has been increased. In fact, wild-growing mushrooms have been a popular delicacy in many countries as they add flavor and texture to the food. The chanterelle (*Cantharellus cibarius*) is widely viewed as among the most desirable of edible mushrooms. Chanterelle's activity has been reported from North America, Europe, North Africa, the Himalayas, Thailand and Iran [14-16]. Previous studies on Canterelle, was focused on its minerals, amino acids, vitamin D, ash, protein, fat, fiber, ascorbic acid and trace elements contents. The biological activities of two fatty acid derivatives, their mutagenic and insecticidal properties have been reported. Phenolic compounds (3-, 4-, and 5-O-caffeoylquinic acids, caffeic

acid, *p*-coumaric acid, and rutin) and organic acids are known to contribute to the organoleptic characteristics of fruits and vegetables [15]. The *C. cibarius* fruit body has been shown to protect against various diseases through its antioxidant activity. Several fatty acids, phenolic compounds, organic acid, carotenoids and steroids from this mushroom have been shown to exhibit antioxidant and anti-inflammatory properties [17]. *C. cibarius* has been found to show various biological properties such as antimicrobial activity, scavenging of lipid peroxidation, antioxidant, analgesic, anti-aging and anticancer activities [15-16].

Nowadays, there are increasing interests for use of natural products instead of chemical ones. Studying local plant foods is of particular interest as they often contain high amounts of bioactive compounds [18]. Because of phenol and flavonoids contents and strong iron chelating activities of *C. cibarius* [16], this mushroom was nominated for assay of *in vivo* chelating test. The current study was carried out to study the ability of ethyl acetate and methanolic extracts of *C. cibarius* to chelate excessive iron in iron-overloaded mice.

2. Materials and methods

2.1. Chemicals and standards

Phenolic acid standards, including gallic, chlorogenic, Caffeic, *p*-coumaric, and ferulic acids, and flavonoid standards, including rutin and quercetin were purchased from Sigma-Aldrich (St. Louis, MO). Ferrozine, Methanol and Acetic acidwere obtained from Merck Co. (Darmstadt, Germany). Distilled deionized water was prepared by Ultrapure™ water purification system.

2.2. Mushroom materials

Cantharellus cibarius edible wild mushrooms were obtained throughout the fall of 2012 from indigenous people who collect edible mushrooms from forest of Sari, Iran. The sample was authenticated by Dr Bahman Eslami and the voucher specimens were deposited in the Sari School of Pharmacy herbarium (No. 1382). The macroscopic descriptions e.g. size, shape, color, texture and odour were recorded. Color of the carpophore, shape of cap and stipe, color of flesh and latex, smell and its habitat were also documented. The mushroom samples were transported to the laboratory and kept at < 4 °C within 24 h prior to sample preparation. Samples were dried in the oven at 45-46 °C for 48 h.

2.3. Mushroom Extracts

200 g derided powder of *C. cibarius* was fractionated by successive solvent extraction at room temperature by percolation with ethyl acetate (400 mL×3) and methanol (400 mL×3), consecutively for 48 h. Extracts were concentrated under reduced pressure at 40°C using a rotary evaporator until a crude solid extract was obtained which was then freeze-dried for complete solvent removal(MPS-55 Freeze-drier, Operon, South Korea) [16].

2.4. Metal Chelating Activity Assay

To assay metal chelating activity, 0.5 mL of 2 mM $FeCl_2$ solution was added to 1 mL of each fraction (800 µg mL^{-1}). The reaction was initiated by adding 5 mM ferrozine (0.2 mL), and the absorbance of the solution was measured by spectrophotometer at 562 nm. EDTA was used as a standard. The ratio of inhibition of ferrozine–Fe^{2+} complex formation was calculated using the following equation:

$$I\ (\%) = \left[\frac{A_{blank} - A_{sample}}{A_{blank}}\right] \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test sample), and A_{sample} is the absorbance of fraction [16].

2.5. HPLC analysis

The phenolic compounds present in the *C. cibarius* extracts were analyzed by HPLC method described by Lin et al. (2010) by some modifications [19]. The HPLC system consisted of a model K-1001 solvent delivery system equipped with a Rheodyne injection valve (20 µL sample loop inserted) and a UV-vis spectrophotometric detector model K-2600 set at 290 (for gallic acid) and 320 nm (for chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, rutin and quercetin) (all from Knauer Assoc., Germany). The analysis was performed using an ODS-C_{18} column (250 mm×4.6 mm I.D., 5 µm particle size, Shim-pack VP-ODS). All solvents were filtered and degassed before entering the column. A gradient solvent system consisting of solvent A (MeOH) and solvent B (H_2O with 9% glacial acetic acid) (conditions: 5% A from 0 to 3 min and kept at 10% A from 3 to 15 min, kept at 30% A from 15 to 27 min and kept at 80% A from 27 to 45 min) were used for separation. The flow rate was set at 0.8 mL min^{-1} and all the measurements were done at ambient temperature.

2.6. Animals and experimental design

Thirty five male mice samples NMRI (20- 25 g) were purchased from Pastor Institute (Amol, Iran) and housed in polypropylene cages at an ambient temperature, 23±1 °C and 45-55% relative humidity, with a 12 h light: 12 h dark cycle (lights on at 7 a.m.). The animals had free access to standard pellet and water. Experiments were conducted between 8:00 and 14:00 h.

Experiments were conducted following the approval and in accordance with the guidelines set by the Animal Experimental Ethical Committee, The Mazandaran University of Medical Sciences (Approval No:911, 2012). The mice were divided into 5 groups as: (A) control (negative control), (B) iron- overloaded (positive control), (C) DFO (D) ethyl acetate, (E) methanolic groups. To induce iron overloading, the mice were given 100 mg kg^{-1} day^{-1} iron by *i.p.* injections of iron dextran with frequency of 5 days a week for 4 subsequent weeks and then left for 1 month to equilibrate the excessive iron. DFO used as a positive control group (1 mg kg^{-1} day^{-1}, *i.p.* injections, with frequency of 5 days a week for 4 subsequent weeks and then left for 1 month) To evaluate the iron-chelation capacity of *C. cibarius*, the extracts were applied as *i.p.* injection with a frequency of 5 times a week at concentrations of 400 mg kg^{-1} day^{-1} for ethyl acetate and methanolic extracts for 4 weeks. Normal saline instead was given to the control iron-overloaded group, with the same frequency and duration [20-21].At the end of the experiment, the mice were euthanized by diethyl ether. Blood samples obtained directly from the heart chamber of the anaesthetized mice. Their plasma was separated. Livers were removed and preserved in 10% buffered formalin specified for histopathological study.

2.7. Determination of iron in plasma by atomic absorption spectroscopy

The iron content was determined by atomic absorption spectroscopy at 248.3 nm using an air/acetylene flame (Perkin–Elmer AAS 100 Wellesley, MA). Iron standard solutions for calibration were prepared from single-element stock solutions (Merck, Darmstadt, Germany) in 0.2% (w/v) nitric acid. The mouse plasma samples were analysed directly after 1+4 dilution with ultrapure water for iron contents [20, 22]. Iron was expressed as mg L^{-1}.

2.8. Determination of plasma ferric ion (Fe^{3+})

Plasma Fe^{3+} content was determined by kit (ZiestChem, Iran). Ferric content was expressed as μmol L^{-1}.

2.9. Histology

Formalin-fixed liver specimens for histology were embedded in paraffin wax. Tissue sections were cut at 5μm and stained by H&E and Perls' stain.

2.10. Statistical analysis

Statistical analysis of the results was performed using GraphPad Prism 5 for Windows. The results are expressed as Means± SD. One way analysis of variance (ANOVA) was performed. Newman-Keuls multiple comparison test was used to determine the differences in means. All p values less than 0.05 were regarded as significant.

3. Results and Discussion

3.1. HPLC separation of flavonoids and phenolic acids

The established HPLC method using a C_{18} column (250×4.6 mm, 5 μm) and a binary gradient solvent system (MeOH/H_2O with 9% glacial acetic acid, flow rate = 0.8 ml min^{-1}) could simultaneously separate 5 phenolic acids and 2 flavonoids within 40 min (Figure 1). Ethyl acetate extract contains all phenolic and flavonoids compounds tested including: gallic acid (0.05 mg g^{-1} of extract), chlorogenic acid (0.66 mg g^{-1} of extract), caffeic acid (1.23 mg g^{-1} of extract), *p*-coumaric acid (2.12 mg g^{-1} of extract), ferulic acid (0.55 mg g^{-1} of extract), rutin (1.28 mg g^{-1} of extract) and quercetin (1.35 mg g^{-1} of extract). Methanol extract contained mainly ferulic acid

(30.00 mg g^{-1} of extract) and lower amounts of gallic acid (0.08 mg g^{-1} of extract), chlorogenic acid (0.46 mg g^{-1} of extract), caffeic acid (0.48 mg g^{-1} of extract), *p*-coumaric acid (1.08 mg g^{-1} of extract), rutin (0.17 mg g^{-1} of extract) and quercetin (0.23 mg g^{-1} of extract).

3.2. Ferrous ion (Fe^{2+}) Chelating Activity Assay

The *in vitro* chelating activity was measured by monitoring the colour reduction of the red Fe^{2+}/ferrozine complex. The percent iron chelating activities in 800 µg mL^{-1} were60.33±1.453% in ethyl acetate extract and 76.00±2.00% in methanolic extract (Figure 2).

EDTA was used as a standard and their IC_{50} chelating activity was 4.324±1.146 µg mL^{-1}.

3.3. Content of total iron in the plasma of iron overloaded and control mice

The maximum amount of iron in iron overloaded group obtained by atomic absorbance analysis was (2750±353.6 mg L^{-1}) which shows a significant difference with control group at statistical probability level of $p<0.001$. In control group, iron amount was calculated to be 901.3±41.66 mg L^{-1}. Tha iron overloaded mice treated with the extract of *C. cibarius* showed decrease in their plasma iron level, among which those treated with 400 mg kg^{-1} of methanolic extract displayed the maximum reduction (986.7 ± 147.0 mg L^{-1}). There is no difference between control and 400 mg kg^{-1} of methanolic extract treated groups ($p> 0.05$). Total iron plasma level was found to decrease in the mice treated with ethyl acetate extract, showing a significant difference ($p<0.001$) with that of iron-overloaded mice.

Compared to DFO-treated mice, the mice that received either ethylacetate extract or methanolic extract showed lower levels of iron in their plasma ($p<0.01$ and $p<0.001$ respectively), (Figure 3).

3.4. The content of Fe^{3+} in the plasma iron- overloaded mice

The maximum amount of iron (133.2±23.3 μmol L^{-1}) has been shown in iron- overloaded group by Fe^{3+} analysis by iron kit which reveals significant difference with control group (38.9±6.7 μmol L^{-1}) at statistical probability level of $p<0.001$. Iron-overloaded mice treated by the extract displayed obvious decrease in Fe^{3+} content when compared with the control group. Again, methanolic extract showed the highest activity. Fe^{3+} content of group receiving 400 mg kg^{-1} of methanolic extract was 53.95±7.46 μmol L^{-1}. There is no difference between control and 400 mg kg^{-1} of methanolic extract treated groups ($p> 0.05$) (Figure 4).

As the figure 3 indicates, methanolic extract decreases the plasma iron to a level comparable to that DFO does, both decreasing the iron content approximately to the level that occurs in normal saline- receiving group.

3.5. Study of morphological changes

3.5.1. Morphological changes of the liver observed by H&E staining

Light microscopy analysis of H&E stained liver sections showed that hepatocellular plates in the control group were spoke-like in shape. There was a main vein in the center around which hepatic cells were dispersed radially (Figure5A). In the iron- overloaded group, the hepatocytes were necrotic, and the hepatic lobule structure appeared disintegrated; meanwhile, proliferating fibrous tissues, pseudo lobules, bile duct proliferation and extensive portal tract inflammation were seen, and haemorrhagic necrosis was detected around the fibrotic tissues and dark brown particles (iron deposits) in the liver tissues. Furthermore, obvious fatty degeneration of hepatocytes and scattered lymphocyte in filtration in the liver interstitium were observed (Figure

5B).The result of H&E staining indicated that periportal inflammation, focal necrosis and genesis of brown pigments occur in the mice receiving DFO, but in very less degree than that observed in iron-overloaded mice (Figure 5C). Methanolic and ethyl acetate (400 mg kg^{-1} day^{-1}) extract treatment markedly reduced the extent of necrotic hepatocytes, fibrous tissues and pseudolobules, with little accompanying inflammatory cell infiltration (Figure5D and E).

3.5.2. Morphological changes of the liver observed by Prussian blue staining

Pathological changes in liver tissue were monitored by Prussian blue staining method. Persian blue stained iron deposits are visible in the cytoplasm of hepatocytes in the iron- overloaded group (Figure 6B).The iron content decreases in liver tissue upon treatment with DFO, the methanolic extract and the ethyl acetate extract. The two later are even more potent than DFO (Figure 6 C, D and E). Meanwhile, only minor change was observed in the amount of iron deposited in the control group (Figure 6A).

There are several human disease conditions resulting from opportunistic deposition of toxic metal ions leading to pathological states, including death. Of special interest is Fe^{3+} intoxication because it may be amenable to treat by in vivo chelation. For example, thalassemia, aplastic anemia and sickle cell anemia are all conditions treated by regular blood transfusions, which frequently lead to Fe^{3+} overload, ferritin deposition in body compartments or tissues; and degeneration especially of the myocardium, liver, endocrine organs and brain [23]. Excess iron can be highly toxic, leading, through generation of reactive oxygen species, to iron overloading disorders. Excess iron deposited chronically in hepatic parenchymal cells is associated with hepatic injury, fibrosis, and ultimately cirrhosis [24]. The preferred treatment in such conditions is using iron chelators [23]. Iron chelating therapy has long been the standard care for patients

suffering from Thalassemias and other anemias, caused *e.g.* by renal dysfunction, and for patients receiving transfusion iron supplementation. Iron chelators are used to treat a number of diseases, including cardiac diseases, malaria, and iron poisoning. Iron chelators are also able to stimulate EPO production. They can inhibit tumour cell growth too [25].

In the current work we studied the iron cheleating activity of edible mushroom *C. cibarius* as a natural compound. Among all the body organs, liver is more susceptible to iron overloading damages; therefore we used liver tissue in a pathological study to show the iron overloading damages and to display the potential inhibitory effects of mushroom extract. Our results revealed that mushroom extract, specially its methanolic extract, could decrease iron level in blood plasma. In addition, methanolic extract was also able to diminish the iron accumulation in liver tissue, resulting in decreased tissue injury. Ethyl acetate extract of mushroom was also found to exhibit useful properties against iron accumulation in mouse liver tissue. This type of extract was able to reduce tissue inflammation. Compared to DFO, mushroom extracts chelate more iron in liver tissues, as evidenced by Prussian blue staining (figure 6C, D and E). Generally, both methanolic and ethyl acetate extracts of *C. cibarius* were found to be efficient compounds to prevent, or at least reduce, the deleterious effects of excessive iron in mice. The pharmacological effects may be attributed to the presence of polyphenols in the extracts.

4. Conclusion

Both methanolic and ethyl acetate derived extracts of mushroom *C. cibarius* exhibit satisfactory potency to chelate excessive iron in mice, potentially offering new natural alternatives to treat diseases correlated with iron overload. Further investigation of individual compounds for their iron chelating activity is needed.

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Conflict of Interest

The authors declare no conflict of interest.

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Figure 1: HPLC profiles of *C. cibarius* extracts analyzed at 320 nm. (A) Ethyl acetate extract, (B) methanol extract and (C) *Standards*. 1, Gallic acid; 2,Chlorogenic acid; 3, Caffeic acid; 4, *p*-Coumaric acid; 5, Ferulic acid; 6, Rutin and 7, Quercetin.

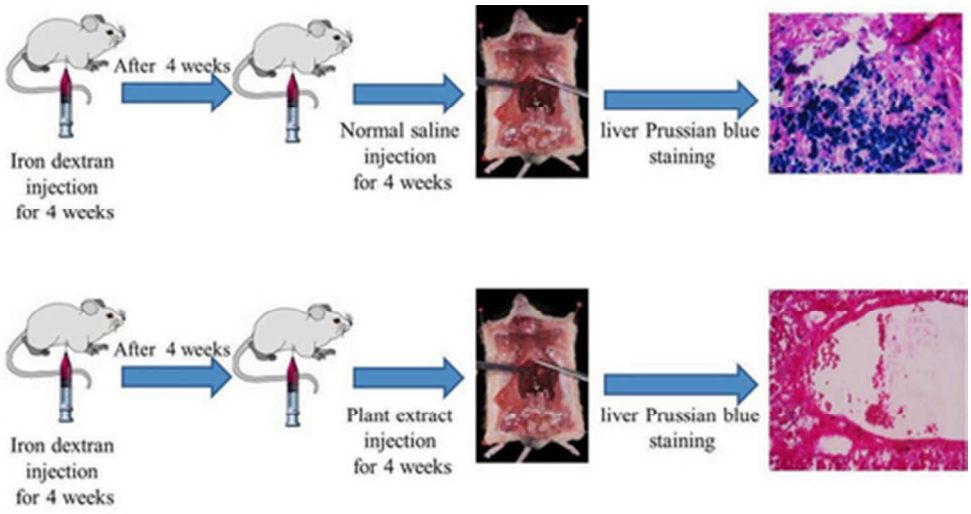
Figure 2: Percent of ferrous ion (Fe^{2+}) Chelating Activity in ethyl acetate and methanolic extract of *C.cibarius*(concentration of extract and EDTA were 800 and 30 µg/ ml respectively) (means ± SD, $p<0.05$)

Figure 3: Total iron plasma iron concentration that was determined by atomic absorption ((A) Control group, (B) Iron overloaded group, (C) DFO group, (D) Ethyl acetate extract group, (E) Methanolic extract group) (means ± SD, $p<0.05$)

Figure 4: Fe^{3+} of plasma iron concentration that was determined by iron kit ((A) Control group, (B) Iron overloaded group, (C) DFO group, (D) Ethyl acetate extract group, (E) Methanolic extract group) (means ± SD, $p<0.05$)

Figure 5: Effects of *C. cibarius* on iron deposition in mouse liver with H&E. Representative microscopic photographs of livers stained with H&E (magnification 400×). ((A) Control group, (B) Iron overloaded group, (C) DFO group, (D) Ethyl acetate extract group, (E) Methanolicextract group)

Figure 6: Effects of *C. cibarius* on iron deposition in mouse liver. Representative microscopic photographs of livers stained with Prussian blue (magnification 400×). ((A) Control group, (B) Iron overloaded group, (C) DFO group, (D) Ethyl acetate extract group, (E) Methanolic extract group)



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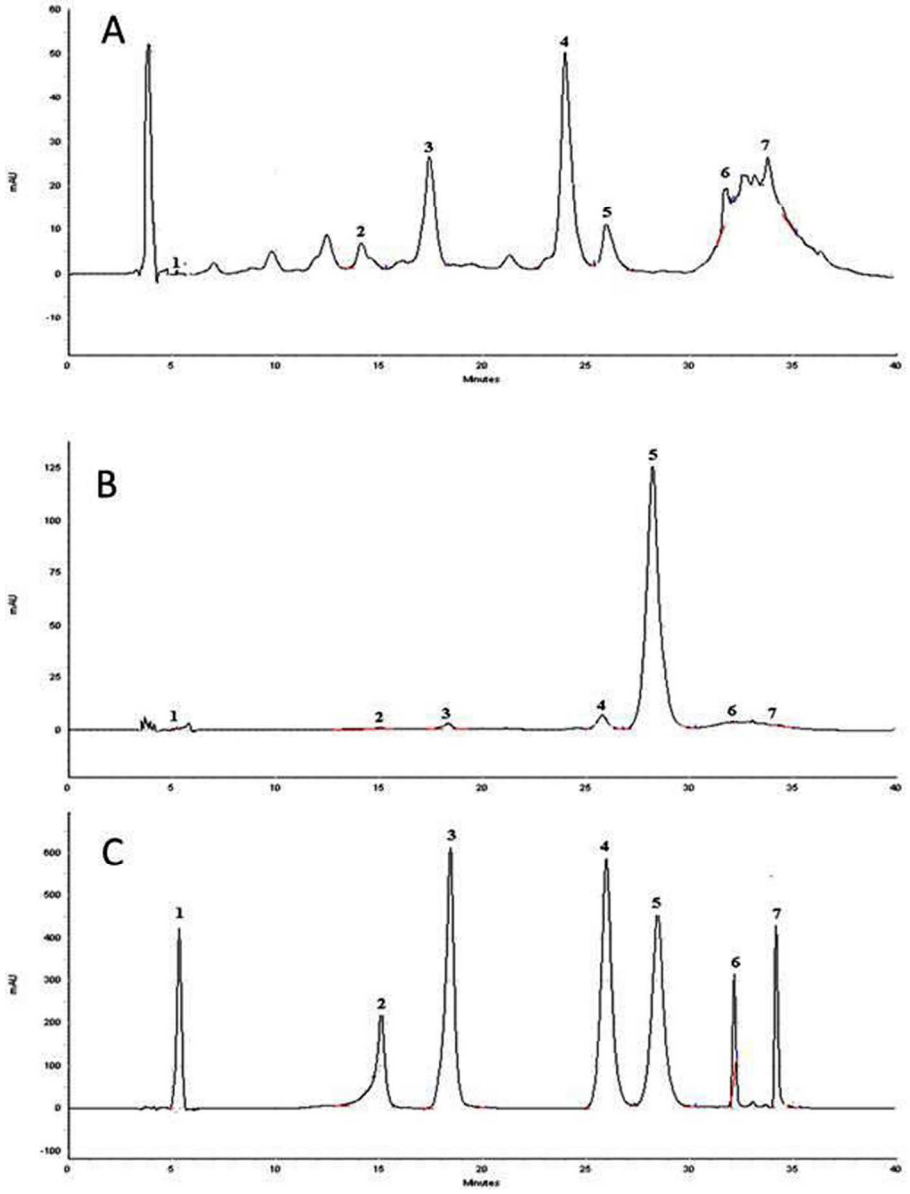

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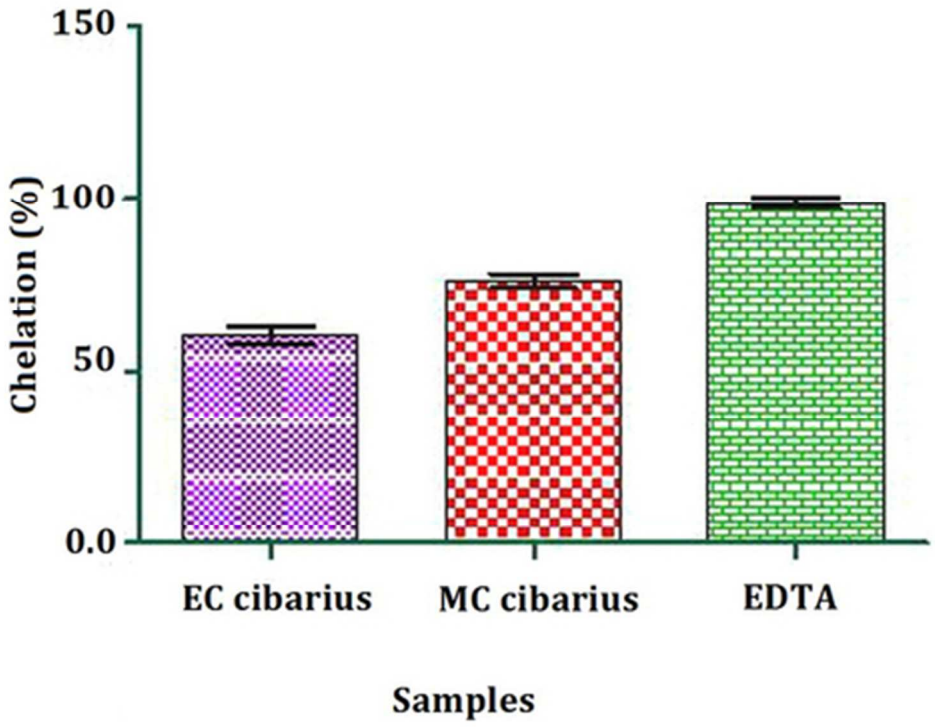

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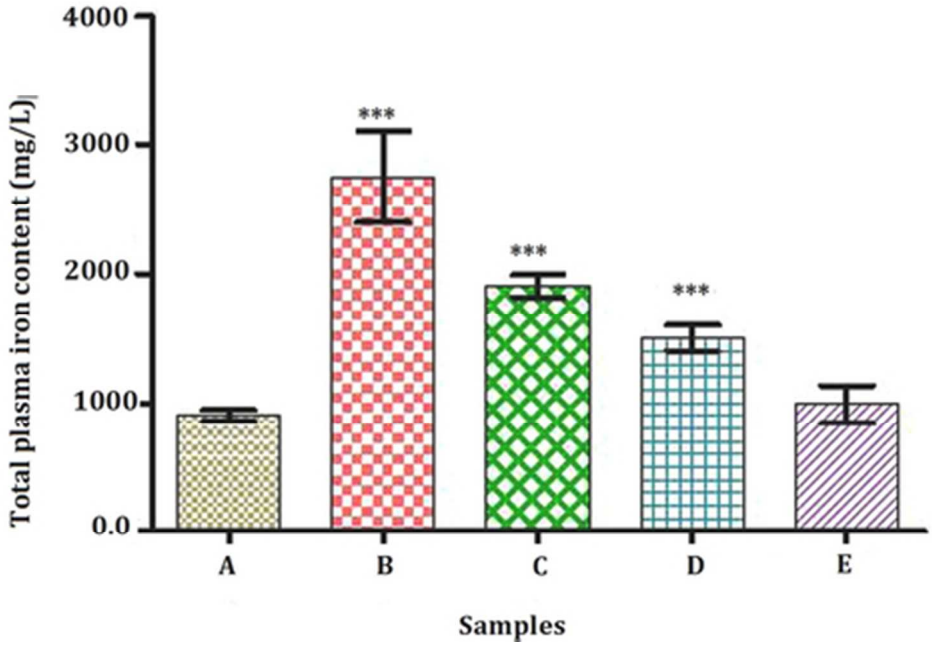

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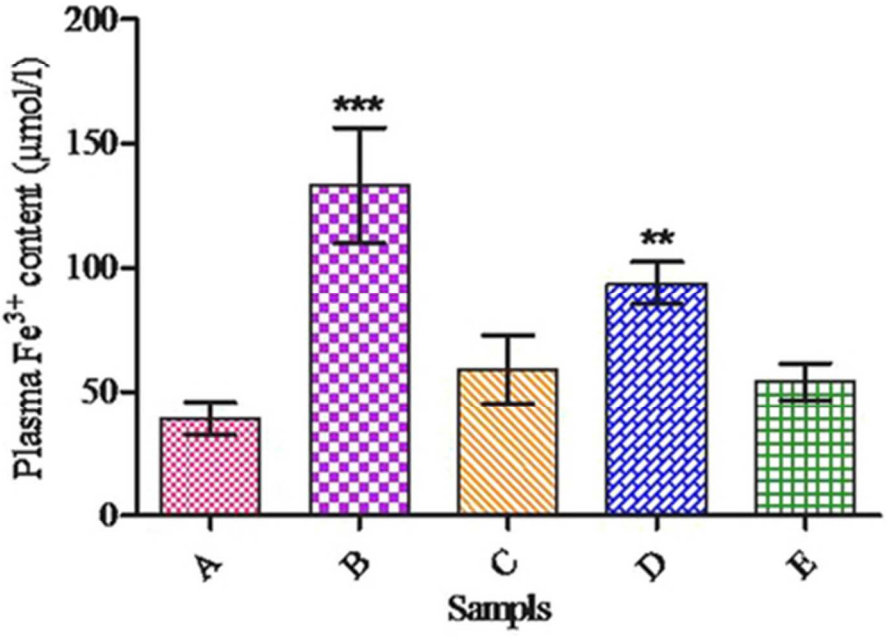

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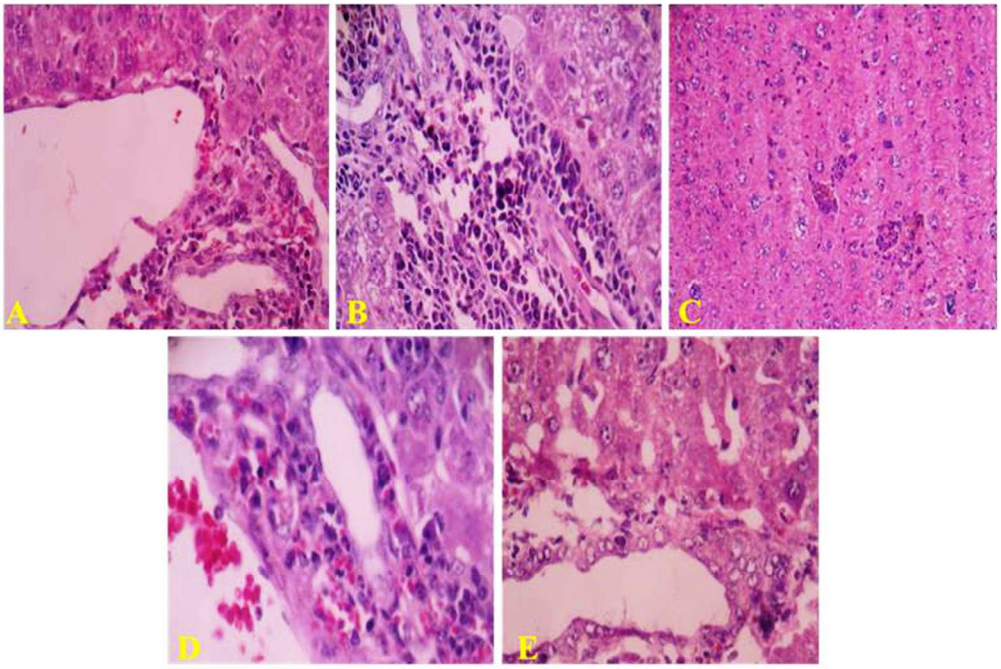

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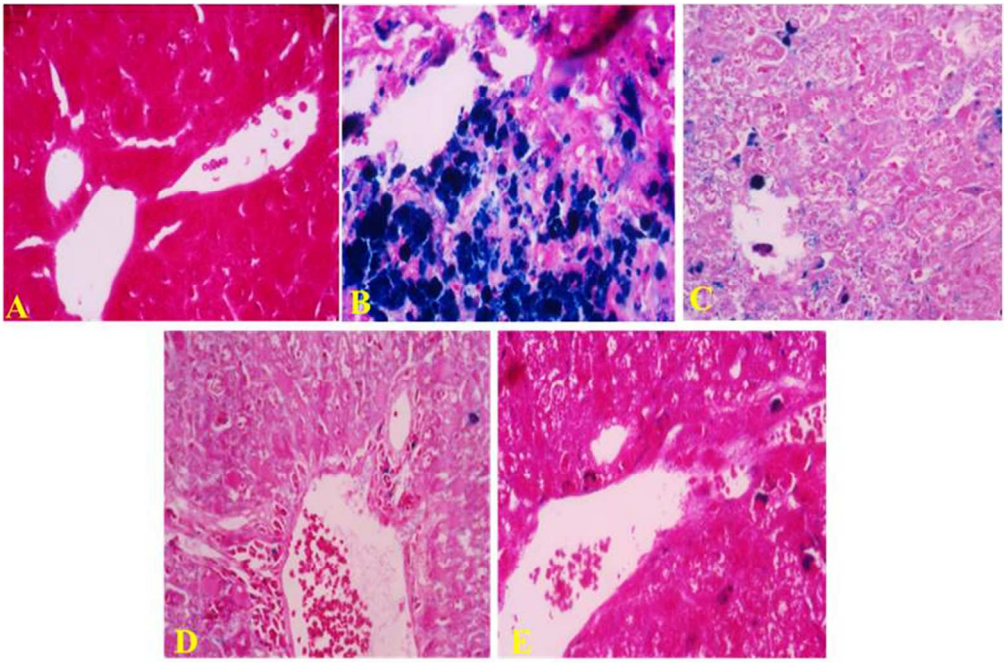

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