SALVIA VIRGATA JACQ. AND SILIBUM MARIANUM L. GAERTN DISPLAY SIGNIFICANT IRON-CHELATING ACTIVITY

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ABSTRACT: In some disease like thalassemia major long-term transfusion alone inexorably produces the clinical problem of iron overload. Danshen and Milk thistle has been widely prescribed in traditional folk medicine for treatment of liver diseases. In this study we selected eight medicinal plants whose efficiencies had been previously demonstrated in treatment of liver diseases in ancient medicine were studied for their in vitro chelating activity. After preliminary evaluation of these plants in vitro condition, two most efficient plants were nominated for further evaluation in in vivo condition on iron-overloaded mice. The extracts with high iron chelation activity were injected intraperitoneally. Liver sections were stained by haematoxylin and eosin and Perls’ stain. Danshen and Milk thistle had maximum iron chelation activity at in vitro condition. The iron-overloaded mice treated with the extracts showed a significant decrease in plasma iron level and in plasma Fe³⁺ content. Perl stain improved sensitivity of the test significantly in order to determine the low amount of deposited iron in the liver of iron-overloaded mice treated by the extract. As evidenced by H&E staining, liver sections treated with the extracts displayed a significant decrease in extent of necrotic hepatocytes, fibrous tissues and pseudo lobules. Danshen and Milk thistle extracts exhibited satisfactory potency regarding chelating of excessive iron in iron-overloaded mice.

INTRODUCTION: Long-term treatment with red-cell transfusion effectively prevents stroke and other complications of sickle cell anemia and can sustain patients with chronic congenital and acquired refractory anemia, including thalassemia major, Diamond–Blackfan anemia, myelodysplastic syndromes, myelofibrosis, aplastic anemia, and other disorders¹,². Long-term transfusion alone inexorably produces the clinical problem of iron overload. In patients with thalassemia who undergo transfusion from infancy, iron-induced liver disease and endocrine disorders develop during childhood and are almost inevitably followed in adolescence by death from iron-induced cardiomyopathy.

In patients with sickle cell anemia, although iron-induced complications appear to develop later, eventually, liver disease with cirrhosis as well as cardiac and pancreatic iron deposition can develop¹. Iron plays a pivotal role in oxidative reactions, resulting in formation of hydroxyl free radicals from peroxidases during Fenton or Haber-Weiss...
chemistry reaction \(^3,4\), which are involved in development of cardiovascular diseases. Iron-chelating compounds can circumvent this process \(^5\). In the Fenton reaction, the hydroxyl radical (OH\(^-\)) is produced from hydrogen peroxide (H\(_2\)O\(_2\)):

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^-
\]

The superoxide radical (O\(_2^\cdot\)) acts to reduce ferric iron, which then reacts with hydrogen peroxide by Fenton chemistry to yield the hydroxyl radical:

\[
\text{Fe}^{3+} + \text{O}_2^\cdot \rightarrow \text{Fe}^{2+} + \text{O}_2
\]

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^- \text{Fe catalyst}
\]

Desferal (deferoxaminemesylate (DFO)) and Deferiporine are two drugs extensively used to chelate iron in patients with thalassemia major; however, their significant side effects has made researchers to consider natural compounds, which relatively exhibit lower degrees of deleterious side effects \(^5,6\). Chelating capacity has been reported for numerous plants and mushrooms, Including *Echium vulgare* L. and *Echium rubrum* L. \(^7\), *Quercus infectoria* \(^8\), *salvia* sp\(^9,10,11,12,13\), *Glycyrrhiza glabra* L. \(^14\), *Origanum vulgare* L. \(^12,15\), *Citrus* sp\(^16,17\), *Diospyros lotus* \(^18,19,20\), *Silybum marianum* \(^21,22,23,24\) and *Cantharellus scibarius* \(^25,26,27\) and *Pleurotus porrigens* \(^26,28\). Because of their potentially lower risk for human, herbal extracts are of great interest.

Eight medicinal plants whose efficiencies had been previously demonstrated in treatment of liver diseases in ancient medicine were studied for their *in vitro* chelating activity (Table 1). After preliminary evaluation of these plants *in vitro* condition, two most efficient plants were nominated for further evaluation in *in vivo* condition on iron-overloaded mice.

**MATERIALS AND METHODS:**

**Chemicals and standards:** Methanol (MeOH), Dichloromethane, Hexane and ferrozinewas obtained from Merck Co. (Darmstadt, Germany). Distilled deionized water was prepared by Ultrapure™ water purification system.

**Plant materials:**
The species were identified by Dr. Masood Azad bakht and deposited in the School of Pharmacy herbarium of Mazandaran University of Medical Sciences. Details on the selected plants and their herbarium numbers are given in Table 1.

The samples were transported to the laboratory and were dried in the oven at 45-6 °C for 48 hour.

**TABLE 1: THE NAMES AND CHARACTERISTICS OF A NUMBER OF MEDICAL PLANTS WITH IRON-CHELATING ACTIVITY**

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Coll. dates &amp;Localities</th>
<th>Herb. numbers</th>
<th>Tissue</th>
<th>Effective compound</th>
<th>Extraction method</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Quercus casta</em> neifolia</td>
<td>October 2012, Mazandaran province, forest of Sari</td>
<td>H 1032</td>
<td>Fruit</td>
<td>Quercetin</td>
<td>Hot distilled water (3 h)</td>
<td>(^8,26)</td>
</tr>
<tr>
<td><em>Echium amoenum</em></td>
<td>July 2013, Mazandaran province, Local market of Sari</td>
<td>K 1043</td>
<td>Flower</td>
<td>Antosianin</td>
<td>Ethanol 96% (maceration) **</td>
<td>(^7)</td>
</tr>
<tr>
<td><em>Origanum vulgare</em> L.</td>
<td>July 2013, Local market</td>
<td>H 1260</td>
<td>Shoot</td>
<td>Rosmarinic acid</td>
<td>Ethanol 50% (maceration) **</td>
<td>(^12,15,30)</td>
</tr>
<tr>
<td><em>Silibum marianum</em> L.</td>
<td>Jun 2013, Mazandaran province, forest of Sari</td>
<td>J 2012</td>
<td>Seed</td>
<td>Silybin</td>
<td>Methanol 80% (Soxhlet) ***</td>
<td>(^21,22,23,24,30)</td>
</tr>
</tbody>
</table>
**Plant Extracts:**

Plant extracts were prepared as described in Table 1. Extracts were concentrated using a rotary evaporator (under reduced pressure at 40°C) to obtain crude solid extracts, which then freeze-dried (MPS-55 Freeze-drier, Operon, South Korea) to completely remove solvents.

**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.

**Animals and experimental design:**

Thirty five male mice strain 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. All the experimental procedures were conducted in accordance with the NIH guidelines of the Care and Use of Laboratory Animals. The mice were divided into 5 groups as: (A) control, (B) iron-overloaded, (C) DFO, *S. virgata* and (D) *S. marianum*. To induce iron overloading, the mice were given 100 mg/kg/day 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. Mice in the DFO group, as the positive control, were given DFO i.p. injections of iron dextran with frequency of 5 days a week for 4 subsequent weeks with a dose of 1mg/kg/day. To evaluate the iron-chelation capacity of plant extract, the extracts were applied as i.p. injection with a frequency of 5 times a week at concentrations of 200 mg/kg/day for 4 weeks. Normal saline instead was given to the control iron-overloaded group, with the same frequency and duration. 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.

**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 mice plasma samples were analysed directly after 5 times dilution ultrapure water for iron contents. Iron was expressed as µg/L.

**Determination of plasma ferric ion (Fe³⁺):**
Plasma Fe³⁺ content was determined by kit (ZiestChem, Iran). Ferric content was expressed as µmol/L.

**Histology:**
Formalin-fixed liver specimens for histology were embedded in paraffin wax. Tissue sections were cut at 5µm and stained by haematoxylin and eosin (H&E) and Perls’ stain.

**Statistical analysis:**
Statistical analysis 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.

**RESULTS:**

**Ferrous ion (Fe²⁺) Chelating Activity Assay:**
The chelating activity was measured by monitoring the colour reduction of the red Fe²⁺/ferrozine complex. The highest iron inhibition was found in *S. virgata* and then in *S. marianum*, 94.28.13±0.53 and 84.49±0.69 %, respectively (Fig. 1).

**Content of total iron in the plasma of iron overloaded and control mice:**
Fig. 2 presents the plasma Total plasma iron content (µg/l) (registered with Atomic Absorption Spectroscopy) in different treatments.
The iron overloaded group displayed the maximum iron absorbance (5420±346.3 µ/l), showing a significant difference with the control group (p<0.001) as well as with the other treatments (S. virgata extract, S. marianum and DFO). There was no significant difference between the S. Virgata extract and S. marianum groups, while both displayed significant differences with the DFO group (p<0.001).

**Plasma level of Fe³⁺ in iron-overloaded mice:**
The plasma level of Fe³⁺ have been found to increase in iron-overloaded mice, rising from 38.48±12.32 µmol/L in control group to 120.1±10.64 µmol/l in iron overloaded mice, indicating a significant increase at statistical level of P< 0.001. There was a significant difference in the plasma iron content between the DFO and the iron-overloaded mice, but not between the DFO and the control groups. In addition, plasma iron content was found to decrease upon treatment with the extracts of S. marianum and S. virgata, showing significant differences with the control group (Fig. 3).

![Graph showing plasma Fe³⁺ levels](image)

**FIG.3: Fe³⁺ OF PLASMA IRON CONCENTRATION THAT WAS DETERMINED BY IRON KIT ((A) CONTROL GROUP, (B) IRON OVERLOADED GROUP, (C) DFO GROUP, (D) S. MARIANUM EXTRACT GROUP AND (E) S. VIRGATA EXTRACT GROUP) (MEANS ± SD, P<0.05)**

**Study of morphological changes:**

1. **Morphological changes of the liver observed by H&E staining:** Observation of H&E stained liver sections under an optical microscope showed that the hepatocellular plates in the control group were spoke wheel shaped with the central veins the center and distributed radially outward (Fig. 4A). H&E- stained liver tissues of iron overloaded mice are shown if Fig. 4B. Periportal and parenchymal inflammation, hepatics sinusoids elongation, central veins hyperemia, increased number of brown pigments surrounding the port, and focal necrosis are observed in liver parenchymal tissues. The result of H&E staining indicated that periportal inflammation, focal necrosis and genesis of brown pigments occur in the mice receiving DFO as well, but in less degree than that observed in iron-overloaded mice (Fig. 4C).

2. **Morphological changes of the liver observed by Prussian blue staining:** Fig. 5 displays the results of Prussian blue staining of liver tissues in different groups. No iron accumulation is observed in the control group (Fig. 5A). Periportal inflammation, hepatics sinusoids elongation, central veins hyperemia, accumulation

![Microscopic photographs of liver tissues stained with H&E](image)

**FIG. 4: EFFECTS OF EXTRACTS ON IRON DEPOSITION IN MOUSE LIVER WITH H&E. REPRESENTATIVE MICROSCOPIC PHOTOGRAPHS OF LIVERS STAINED WITH H&E (MAGNIFICATION 100×). ((A) CONTROL GROUP, (B) IRON OVERLOADED GROUP, (C) DFO GROUP, (D) S. MARIANUM EXTRACT GROUP AND (E) S. VIRGATA EXTRACT GROUP)**
of iron pigments around the port and parenchymal tissue, and focal necrosis in liver parenchymal tissue occur in iron-overloaded mice (Fig. 5B). As seen in Fig. 5C, DFO treatment decreases the iron accumulation in liver tissue. In comparison to the iron-overloaded group, the mice treated with the extracts of S. marianum and S. virgata show striking decrease in iron content (Fig. 5D and 5E). The minimum iron accumulation, and therefore the minimum tissue injury, occurs in S. virgata treated group (Fig. 5E).

DISCUSSION: Based on the history of Danshen and Milk thistle in treatment of liver diseases in ancient medicine in European and Asia countries, and also for their approved anti-inflammation capacities, we evaluated the iron chelating activities of these plants in iron-overloaded mice. Excess iron level in plasma has been reported to cause free radical production, lipid peroxidation and oxidative damages, resulting in iron precipitation and necrosis in liver. In this study, we evaluated the hydro alcohol extract of S. marianum and dichloromethane extract of S. virgata on iron overloaded mice and found that they significantly decreased the total plasma content of Fe$^{3+}$. This result was further confirmed by pathological analysis what indicated that iron precipitation, inflammation and necrosis had been reduced in hepatocytes upon treatment with the extracts. S. marianum’s flavonolignans, well known as silimaran (Silybin (SBN), Isosylibin (ISBN), Silydianin (SDN), Silycristin (SCN) and Taxifolin (TAX)) contribute to liver health through different mechanisms including the activation of DNA polymerases, increase of cell membrane stability, inhibition of free radicals, and increment of glutathione cellular levels. Silimarins inhibit lipid peroxidation as well.

We showed that hydroalcoholic extract of S. marianum decreases the Fe$^{3+}$ content in iron-overloaded mice. Our finding in this case corresponds to the results of Gharagozloo et al (2008) and Borsari et al (2001). Salvia has been found to contain a variety of natural compounds with significant antioxidant activity, including phenolic acids (such as rasmic acid, rosmarinic acid, cafeeic acid and lithospermic acid), flavonoids and proanthocyanidins. Among these compounds, lithospermic acid has been proven to be efficient in treatment of liver diseases. As evidenced by H&E and Perl's stainings, the extract of salvia was able to decrease iron precipitation, and tissue necrosis and inflammation. These findings correspond to the results of Gao et al (2013) and Zhang et al (2013). Therefore, reduced inflammation and increased iron chelating in mice liver tissues upon treatment with the extracts of these plant may be due to the presence of silimarins and other natural compounds, like phenols. However, more studies are needed to certify these findings.

CONCLUSION: The extracts of S. marianum and S. virgata exhibit significant iron chelating activity and are able to reduce inflammation and necrosis in mouse liver tissues. More studies are needed to exactly determine which compounds are responsible for these biological activities.

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