Estimation of Vitamin C and the Fructose Levels in Some Medicinal Plants and their Effects on Iron Bioavailability in Rats

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Abstract
Iron deficiency is considered as a common problem facing the general world population. In the current research, experiments were conducted to evaluate the effects of aqueous extracts of Acacia senegal (Gum Arabic, GA), Spinacia oleracea (spinach), Zea mays (corn), and Capsicum annuum (red chili pepper) on iron and ferritin levels in rats. Vitamin C and fructose levels were first estimated in these plants by using High-Performance Liquid Chromatography (HPLC). The results showed that the GA extract contained the highest level of fructose (853 mg.L⁻¹), followed by red chili pepper (635 mg.L⁻¹), corn (521 mg.L⁻¹), and spinach (271 mg.L⁻¹) extracts. Also, the results of vitamin C estimation showed levels of 3.376, 0.645, 0.579, and 0.347 mg/ml in the extracts of spinach, red chili pepper, corn, and GA, respectively. Next, thirty male albino rats (age 8-12 weeks, weight 120-200 g) were divided randomly into six groups, each kept in a separate polypropylene rat cage. Treatments of rats with various plant extracts were conducted following a CRD complete random design, with five replicates for each treatment. The results of lipid profile test revealed that GA treatment caused the highest reduction in the serum levels of TC, TG, LDL, VLDL, and HDL in the treated rats (76.25, 55.25, 23, 15.25, and 38 mg/dl, respectively), as compared to the other plant extracts. Evidence from this study suggests that the higher serum level of fructose in the group of rats treated with all plant extracts enhances the process of ferritin formation, possibly via increasing the level of bioavailable iron.

Keywords: Vitamin C estimation, fructose estimation, medicinal plants, non-heme Iron, Anemia, Gum Arabic.
1. Introduction

Evidence showing that simple sugars, such as fructose, affect iron absorption first appeared in the 1960s. That work showed that sugar was able to chelate inorganic iron and form soluble, stable, low molecular weight complexes. It has been proposed that fructose increases iron bioavailability by increasing ferrous iron formation. These sugar-iron complexes were shown to be readily absorbed across the intestinal mucosa of rodent models. Given that intake of fructose increased worldwide in the past 40 years, especially in the western world, while at the same time iron deficiency and iron excess remains significant public health concerns, understanding the nutritional implications of iron-sugar interactions is particularly relevant [1]. Increasing iron absorption from fortified foods by adding enhancers of iron absorption may increase the efficacy of fortification strategies, particularly in diets containing inhibitors such as tannins and phytate [1]. The importance of ascorbic acid (AA) as a means of improving iron nutrition was underlined in 1970 by the U.S. Food and Nutrition Board when it suggested the promotion of “a program featuring iron, in addition to vitamin C, as a breakfast nutrient” [2]. The use of ferritin as an indicator of iron availability was pioneered by Glahn et al. [3] and it is now widely used as a surrogate marker for iron uptake (for recent examples see [4]). Ferric iron is the dominant form of iron in foods, which is far less bioavailable than ferrous iron. One of the main characteristics of ascorbic acid is its ability to reduce ferric to ferrous iron. Gum Arabic is a natural branched-chain multifunctional hydrocolloid with a highly neutral, or slightly acidic, arabino-galactan-protein complex containing magnesium, calcium, and potassium [2]. GA consists of three fractions: (a) The smallest fraction having the highest protein content is a glycoprotein which differs in its amino acid composition from that of the gum arabic glycoprotein (GAGP) complex. (b) a smaller fraction is a higher molecular weight arabinogalactan-protein complex (GAGP-GA glycoprotein) in which arabinogalactan chains are covalently linked to a protein chain through serine and hydroxyproline groups. The attached arabinogalactan in the complex contains ~13% (by mole) glucuronic acid. (c) the major fraction is a highly branched polysaccharide consisting of G-(1-3) galactose backbone with linked branches of arabinose and rhamnose, which terminate in glucuronic acid (found in nature as magnesium, potassium and calcium salts) [3]. Gum Arabic is a natural vegetable product, with hypoglycemic and prebiotic effects, having a low calorific value. It is used in drinks, meal substitutes, cereal bars, and bakery products. Also, noncarcinogenic soluble fiber dairy products and confections use acacia gum for its health benefits [5, 6, 7]. Red chili pepper is (Capsicum annuum) used as a food additive in many regions due to its pungency, aroma, and color. It has been safely consumed in large amounts in many countries. Spinach (Spinacia oleracea) is a leafy green
vegetable that contains ascorbic acid and other organic acids, which are well known promoters of iron absorption from the diet. Corn (Zea mays) is one of the world's most important cereal grains. It is a dietary staple for more than 200 million people and provides approximately 20% of the world's calories. The aim of the present study is to evaluate the effects of aqueous extracts of Acacia senegal, Spinacia oleracea, Zea mays and Capsicum annuum on iron and ferritin levels in rats.

2. Materials and methods

2.1 Collection of the plants

The plant samples that included the leaves of Spinacia oleracea, the fruits of Capsicum annuum, the kernels of Zea mays, and the powder of Acacia senegal were obtained from the local markets in Baghdad, Iraq. The first three samples were kept in an oven at 40 °C for one week, until they were hard and then grinded by a grinder to give small size pieces of 2 mm. Then, all the samples were stored in glass containers at room temperature in a dry dark place until use.

2.2 Preparation of plants aqueous extracts

An aqueous extract was prepared for each plant by applying the water extraction method without boiling [8].

2.3. Estimation of fructose in the plant samples

2.3.1 Processing

The method used for the quantitative estimation of fructose is the phenol-sulfuric acid method. In a hot acidic medium, fructose was dehydrated to 5 hydroxymethyl fufural, which forms a yellow-orange colored product with phenol and has absorption maximum at 490 nm when tested with UV spectroscopy [9].

2.3.2 Fructose level in the standardized sample

The method used to estimate fructose level in the standardized sample was applied according to a previously published study [9].

2.3.3 Fructose levels in medicinal plant samples

To have a standard comparison of samples of each type of plant, the procedure carried out to estimate fructose levels was repeated in a similar fashion with all plant extract samples. The absorbance was determined at concentrations of 0.01-0.09 µg/ml [9].

2.3.4 Vitamin C estimation

The determination of vitamin C was performed by using HPLC technique [10].

2.4 Laboratory animals

Thirty male albino Sprague Dawley rats with an age range of 8-12 weeks and a weight range of 120-200 g were purchased from Al-Nahrain University Biotechnology Research Center and housed at room temperature (25 °C). The experimental protocol was designed according to OCED, 2001 [11].

2.5 Experimental design

Rats were divided into six groups, each group contained five rats, which were subjected to oral administration of different aqueous plant extracts for 30 days [11].

Group one involved the administration of distilled water (control).

Group two involved the administration of only spinach extract (0.05 g/ml).

Group three involved the administration of spinach (0.05 g/ml) and GA e (0.1 g/ml) extracts.

Group four involved the administration of spinach (0.05 g/ml) and red chili pepper (0.1 g/ml) extracts.

Group five involved the administration of spinach (0.05 g/ml) and corn (0.1 g/ml) extracts.

Group six involved the administration of all plant extracts (0.05 g/ml of spinach and 0.1 g/ml of each of the other plants).

2.6 Collection of blood samples
Five milliliters of blood were withdrawn from the heart of the animals directly by cardiac puncture using a medical syringe. Blood samples were placed in anticoagulant-free gel tubes to obtain serum. The blood was centrifuged at 3000 rpm for 15 minutes to ensure a sufficient amount of serum that is free of red blood cells. The serum was then collected in special plastic tubes and stored at -20 °C until use [12].

2.7 Determination of iron and ferritin levels in rats
After 30 days of treatment, blood samples were collected from the experimental rats and tested for iron levels by applying the iron ferrozine colorimetric method [13,14].

2.8 Measurement of lipid profile
All lipid parameters were measured by using kits obtained from BioSystems (Spain), except VLDL concentration which was estimated by using the following equation reported by Freidwold et al. [15]:

\[
\text{VLDL} = \frac{\text{TG}}{5}
\]

Statistical Analysis
The program that was used to detect the effects of difference factors on the studied parameters was the Statistical Analysis System- SAS (2012), by using CRD complete random design with five replicates for each treatment. Least significant difference (LSD) test (Analysis of Variance-ANOVA) was used to compare between means and find statistically significant differences [16].

Results and discussion
3.1 Iron
3.1.1 The effects of plant extracts on iron and ferritin bioavailability
The results in Table 1 show that the highest level of vitamin C was 3.376 mg/ml, which was found in the extract of *Spinacia oleracea*, while the lowest level was 0.347 mg/ml in *Acacia senegal*.

**Table 1** - The concentration of vitamin C in the aqueous extracts of the studied plants, as measured by the use of HPLC analysis.

| Plant             | Vitamin C con. (mg/ml) |
|-------------------|------------------------|
| Spinacia oleracea | 3.376                  |
| Capsicum annuum   | 0.645                  |
| Zea mays          | 0.579                  |
| Acacia senegal    | 0.347                  |

The results in Table 2 demonstrate that the highest concentration of fructose (853.54 mg/ml) was found in the extract of *Acacia senegal*, while the lowest (271.72 mg/ml) was in *Spinacia oleracea*.

**Table 2** - The concentration of fructose in the studied plants

| Plant             | Fructose con. (mg/ml) |
|-------------------|-----------------------|
| Spinacia oleracea | 271.7273              |
| Capsicum annuum   | 635.3636              |
| Zea mays          | 521.7273              |
| Acacia senegal    | 853.5455              |

Moreover, the results of chemical analysis revealed that iron concentration was the highest (80.10 mg/ml) in *Spinacia oleracea* and the lowest (16.428 mg/ml) in *Acacia senegal* (Table 3).
Figure 1-Vitamin C estimation by HPLC (A; standard, B; Gum Arabic, C; red chili pepper D; spinach, E; corn.

Table 3-The concentration of iron in the studied plants

| Plant                | Iron con. (mg/ml) |
|----------------------|-------------------|
| Spinacia oleracea    | 80.109            |
| Capsicum annuum      | 23.394            |
| Zea mays             | 21.654            |
| Acacia senegal       | 16.428            |

According to Table 4, the last group of animals (G6), which was treated orally with 1 ml of each of the studied plant extracts, had significantly higher iron and ferritin levels (by 50% and 36%, respectively) compared with the control (G1) group.

Table 4-The levels of iron and ferritin in the studied groups

| Groups | Iron (mg/ml)          | Ferritin (mg/ml)        |
|--------|-----------------------|-------------------------|
|        | Means ± SE            |                         |
| G1     | 149.00 ±21.98 ab      | 153.68 ±38.78 ab        |
| G2     | 140.00 ±17.68 c       | 31.50 ±22.87 c          |
| G3     | 80.80 ±17.87 bc       | 15.93 ±6.07 c           |
| G4     | 105.33 ±4.97 bc       | 98.33 ±23.18 bc         |
| G5     | 110.60 ±16.42 bc      | 79.22 ±30.49 bc         |
| G6     | 224.00 ±57.23 a       | 209.00 ±25.27 a         |
| LSD value | 92.857 *              | 84.728 *               |

Means having with the different letters in same column differed significantly * (P≤0.05).
G1: Control (D.W), G2: Spinach, G3: Spinach + Gum Arabic, G4: Spinach + Red chili pepper, G5: Spinach + Corn, G6: Spinach + Corn + Gum Arabic + Red chili pepper.

Non-heme iron bioavailability is influenced by many dietary factors. This study suggests that fructose increases iron bioavailability in the rats of group two (treated with only spinach), which showed slightly lower serum levels of iron and ferritin compared with the control. This may be due to the type of non-heme iron in spinach, which cannot be absorbed as easily as heme iron without certain factors, such as vitamin C or fructose [17]. It has been reported that vitamin A and β-carotene can enhance iron absorption and thereby contribute to an increase in hemoglobin levels [18]. As for group three, which involved rats treated with S. oleracea and A. senegal, the levels of iron and ferritin were significantly low, which may be due to fiber content in GA [19]. Some studies showed that fibers decrease the absorption of non-heme iron[19]. This effect of fibers is reflected on dynamics of nutrients uptake and gut microflora, as well as nutrient bioavailability, microbial makeup, and gastrointestinal function. Besides, in group four (treated with S. oleracea and C. annuum), the levels of iron and ferritin were decreased. The binding of iron to phenolic compounds that are found in chili pepper prevents the absorption of non-heme iron in humans. Apparently, the known enhancing effects of chili and capsaicin on gastric acid secretion are not relevant to iron consumption. Chili was shown to have no significant effect on the absorption of iron from acid-soluble ferric pyrophosphate compared to ferrous sulfate. Furthermore, with the use of fresh chili instead of dried chili powder, as used in our research, the inhibitory effect of chili is be predicted to be lower. As a known enhancer of iron absorption, chili lyophilization is required to reduce the ascorbic acid content [20]. The daily consumption of all kinds of pepper was reported to be approximately 24 g and that for chili pepper was 9 g. The most abundant forms of capsaicinoids found in hot red peppers are capsaicin, dihydrocapsaicin, and nordihydrocapsaicin. About 70% of the burning sensation experienced from consumption of chili red peppers is attributed to capsaicin. Capsaicin is used extensively as a treatment for pain. Evidence showed that capsaicin has effects on regulating homeostasis and digestive tract, in addition to other deleterious effects. Capsicum and capsaicinoids improve hormone function and metabolism, stabilize blood glucose, reduce insulin and leptin resistance, regulate endothelial function, inhibit LDL-cholesterol oxidation, and prevent cancer due to antioxidant activity. Spicy food consumption showed highly consistent inverse associations with total mortality among both men and women after adjustment with potential risk factors [21].

In group five, which involved rats treated with S. oleracea and Z. mays, the levels of iron and ferritin were slightly higher in comparison with the control. This might be because Z. mays contains phytate and polyphenols. Phytate is known to be a possible inhibitor which chelates micronutrients (such as iron and calcium) and prevents their absorption in monogastric animals, including humans, since their digestive tract lacks the enzyme phytase [22]. Maize products, including essential fatty acids, especially linoleic acid in maize oil, play important roles in the diet by maintaining blood pressure, regulating blood cholesterol level, and preventing cardiovascular disorders. Vitamin E in maize oil is known as a key chain breaking antioxidant. Maize has comparable energy density to other cereal crops and is a relatively good source of vitamin A, but it is also rich in phytate, a compound that potently inhibits iron availability for absorption [23]. According to the results of the current study, the administration of S. oleracea, C. annuum, A. senegal, and Z. mays together into rats revealed a high increase in iron and ferritin levels at a dose of 1 ml of the extract. These results agree with those reported by other research groups [24-27]. This may be due to the high amount of vitamin C in S. oleracea and C. annuum [28]. Besides, there are many different mechanisms that suggested and explained the interaction between vitamin C and iron. One of these mechanisms is the reduction of Fe$^{3+}$ to Fe$^{2+}$ in the gastrointestinal tract, which will promote
the non-heme iron absorption. The improved iron solubility of Fe$^{2+}$ and Fe$^{3+}$ is owed to complex formation and followed by enterocyte endorsement [29]. However, the effect of A. senegal and Z. mays in increasing iron level may be due to the high amount of fructose in these plants, which is considered as an effective agent in rising iron in vivo through ferritin formation, which affects the oxidative state of iron and alters it to the bioavailable ferrous form [30]. In addition, Z. mays contains folic acid (hematopoietic factor) [31] and tannin [32], which are considered as phytochelatores and metal binding ligands that can impact iron absorption by the assessment of ascorbic acid. Such components that were mentioned above, and others, can encourage redox activities that influence iron absorption by the help of ascorbic acid [33].

3.2 Lipid profile

The data listed in Table 5 demonstrate the outcomes of the lipid profile assay. The results of the oral administration of A. senegal in group three indicate that the levels of CHO, TG, LDL, and VLDL were decreased (76.25, 55.25, 15.25 mg/dl, respectively), while HDL was increased (38mg/dl). However, group five showed raised levels of CHO, TG, LDL, and VLDL (149, 153.60, 93.00, 29.80 mg/dl, respectively) , whereas the level of HDL (26.20mg/dl) was low.

Table 5- The levels of lipid profile parameters in the studied groups.

| Groups | Serum Cholesterol (mg/dl) | Serum Triglyceride (mg/dl) | Serum HDL (mg/dl) | Serum LDL (mg/dl) | Serum VLDL (mg/dl) |
|--------|---------------------------|---------------------------|-------------------|-------------------|-------------------|
| G1     | 84.00 ±17.44 c            | 90.20 ±17.08 bc           | 38.60 ±3.66 a     | 32.16 ±15.93 bc   | 16.80 ±3.48 c     |
| G2     | 88.80 ±9.81 bc            | 39.40 ±7.10 d             | 36.00 ±1.64 ab    | 34.96 ±9.40 bc    | 17.76 ±1.96 bc    |
| G3     | 76.25 ±2.89 c             | 55.25 ±15.65 cd           | 38.00 ±1.08 a     | 23.00 ±3.32 c     | 15.25 ±0.58 c     |
| G4     | 139.67 ±13.64 ab          | 118.33 ±24.25 ab          | 30.33 ±1.45 ab    | 81.40 ±12.34 ab   | 27.93 ±2.72 ab    |
| G5     | 149.00 ±18.79 a           | 153.60 ±16.81 a           | 26.20 ±3.42 b     | 93.00 ±18.24 a    | 29.80 ±3.75 a     |
| G6     | 116.80 ±24.01 abc         | 64.20 ±16.55 cd           | 31.20 ±4.87 ab    | 62.04 ±23.89 abc  | 23.36 ±4.80 abc   |
| LSD Value | 51.40 *                 | 48.18 *                  | 10.03 *           | 49.66 *           | 10.282*           |

Means having different letters in the same column differed significantly *(p ≤ 0.05).*

G1: Control (D.W), G2: Spinach, G3: Spinach + Gum Arabic, G4: Spinach + Red chili pepper, G5: Spinach + Corn, G6: Spinach + Corn + Gum Arabic + Red chili pepper.

Lipids are defined as biological matters which are normally hydrophobic in nature and in many circumstances soluble in organic solvents [34]. The main biological purposes of lipids include energy storage, signaling, and performing as structural components of cell membranes [35]. The administration of S. oleracea in group two did not have a significant effect on lipid profile. Ko et al. [36] revealed that the administration of plant extracts of spinach is not effective at reducing blood cholesterol. The composition of the extract used in the experiments, as well as the dosages and the experimental period, can be to the reasons of these findings.

Group three showed the best influence on lipid profile. A. senegal is a soluble dietary fiber that has probiotic features [37], assisting oligosaccharides or polysaccharides to be fermented by colonic bacteria to build short chain fatty acids [38]. Other studies suggested that, due to GA viscosity [39], it can have lowering effects on lipid profile parameters in humans and animals [40]. Ali et al. [41] suggested that GA, due to its antioxidant activity, has a lowering effect on lipid peroxidation. another study reported that GA inhibits glucose absorption in the
intestine through its interaction with sodium-glucose transporter 1 (SGLT1) which is abundant in cell membrane in mice [42]. The administration of C. annuum revealed a negative effect on lipid profile in rats. It increased TC, TG, LDL, VLDL, and decreased HDL serum levels. Many studies suggested that capsaicin in the red chili pepper has a beneficial effect on lipid parameters. Al-Jumayi et al. [43] proved that the ethanol extract of red chili has positive effects on lipid metabolism. The difference between our result and those of other studies might be due to the plant extracting method. In another 35-day trial on rabbits, intubation with 8 mg capsaicin / rabbit did not have a beneficial impact on plasma cholesterol, TG and HDL-cholesterol in regular diets. These variations were due to the effects on intestinal absorption of cholesterol by capsaicin [44].

Group five, which involved Z. mays oral administration, had undesirable effects on lipid profile. Although Oyeyemi et al. [45] showed that the consumption of corn had beneficial effects on lipid metabolism, the results obtained revealed that Zea mays bran may possess a laxative activity. The different results obtained by this study might be due to the usage of the kernel instead of bran of the corn, in addition to the different method of extraction. Dapčević-Hadnaev et al. [46] showed that the corn bran has high percentage of fibers and viscosity that have a positive effect on lipid metabolism, while the usage of kernel and the water extraction method in our study had negative effects on lipid profile parameters.

The results of the last group showed negative impacts on lipid parameters. This may be due to the synergetic effects of all the components of the plant extracts, and that the positive effect of GA (as shown in group three) was reversed by the other components, which showed negative effects on lipid metabolism in other treated groups.

In conclusion, these studies suggest that plant extracts enhance iron bioavailability, possibly through the influence of either vitamin C or fructose, as measured by ferritin formation; however, the results are inconclusive. Also, the results indicated that using these plants in the modern diet has effects on iron bioavailability, without any notable pathophysiological consequences. Thus, further studies are required to test other plant extracts, or their active components, to enhance iron bioavailability.

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