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Antioxidant and free radical scavenging activity of iron chelators

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ABSTRACT

Inside the human body, reactive derivatives of oxygen, known as reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), are constantly generated. The ROS easily cause oxidative damage to various biomolecules such as proteins, lipids and DNA leading to various disease conditions. Iron chelators function as antioxidants by scavenging ROS and also reduce the amount of available iron thereby decreasing the quantity of $^{\bullet}OH$ generated by Fenton reactions. In this study, the antioxidant activity of the iron chelators: caffeic acid (CA), 2,3-dihydroxybenzoic acid (DHBA), desferroxamine B (FOB) and benzohydroxamic acid (BHA) were determined using five different *in vitro* antioxidant assays. The antioxidant assays used were: iron binding ability, reducing ability using the potassium ferricyanide reduction method, 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity, H_2O_2 scavenging activity and $^{\bullet}OH$ scavenging activity. The standard used for the iron binding ability was Na_2EDTA whereas vitamin C was used as a standard for the remaining assays. The iron chelators showed a concentration dependent increase in their radical scavenging activities as well as their reducing ability. At the concentration of 1 mM, FOB had the highest iron binding ability of 93.7% whereas DHBA had the lowest iron binding ability of 5.0% compared to the standard Na_2EDTA which had 94.8%. The iron chelators, with the exception of BHA, showed good reducing ability than vitamin C. Caffeic acid showed significant DPPH, hydrogen peroxide and hydroxyl radical scavenging activities of 84.7%, 99.8% and 14.5%, respectively. All the iron chelators were observed to show significant activities in all five antioxidant assays.

1. Introduction

The role of free radicals in many disease conditions has been well established. Inside the human body, reactive derivatives of oxygen, known as reactive oxygen species (ROS) such as the superoxide radical ($O_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2), are constantly generated from contact with foreign chemicals in our ambient environment and/or due to a number of local metabolic processes that utilizes redox enzymes [1]. Normally, the ROS generated in humans are cleared by the presence of antioxidants in the body and there is a balance between the ROS generated and the antioxidants present. However, owing to ROS overproduction or inadequate antioxidant defense, this balance is disturbed favouring the ROS increase that end up in oxidative stress [1]. Apart from their deleterious effect, free radicals have been implicated

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in a number of biological processes which include intracellular killing of bacteria by phagocytes such as macrophages and granulocytes and these are necessary for life. Free radicals have also been associated with a number of cell signalling processes called redox signalling [2].

Although the body has effective defense mechanisms that protect it against oxidative stress, the ability of these defensive mechanisms decreases as one ages [3], creating a need to provide the body with a constant supply of antioxidants through dietary supplements. Several biomolecules such as proteins, lipoproteins, lipids and DNA are easily damaged due to the actions of ROS [4]. Epidemiological studies have found that the intake of antioxidants, such as vitamin C, reduce the risk of cardiovascular diseases, cancers [5] and neurodegenerative diseases [6].

Many radical reactions can be formed from ferrous iron (Fe^{2+}) by virtue of its ability to transfer single electrons, starting even with relatively non-reactive radicals [7]. Hence, iron chelation may be an effective therapeutic approach. However, due to absence of effective ligands, advancement in this field has been hindered. Also, there is the need for a balance between the toxic effects of chelators and their relative favourable effects against oxidative damage. Red blood cells have been found to be protected from oxidative injury by antioxidant and other supportive therapies [8,9]. Iron chelators are involved in mobilizing iron from tissues by forming soluble, stable complexes that are then passed out through faeces or urine. Iron-related complications are reduced by chelation therapy and by so doing improves quality of life and overall persistence [10]. Available chelators are deemed suboptimal due to their poor oral bioavailability, short plasma half-life and serious side effects [10]. Within this background and taking particular attention to the relative paucity of iron chelating agents, it is not storming that clinical scientists are laying much emphasis towards discovering potentially useful sources of iron chelators in order to acquire the maximum potential benefit with the least possible harm [11,12].

In this study, the antioxidant and free radical scavenging activities of the iron chelators: caffeic acid (CA), 2,3-dihydroxybenzoic acid (DHBA), desferroxamine B (FOB) and benzohydroxamic acid (BHA) (Fig. 1.1) were evaluated using *in vitro* assays including: iron binding ability; reducing ability using the potassium ferricyanide reduction method; 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity; H_2O_2 scavenging activity and $^{\bullet}OH$ scavenging activity.

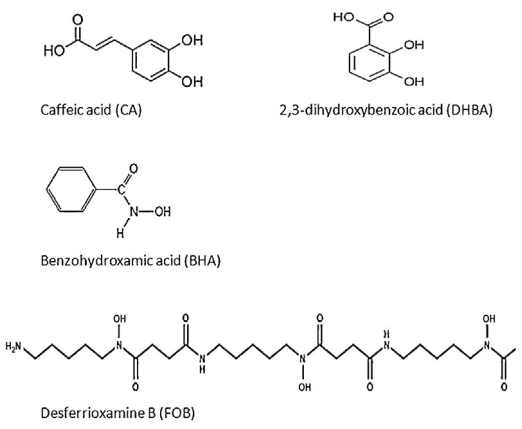

Fig. 1.1. Structures of iron chelators used in this study.

2. Materials and methods

2.1. Materials

Methanol was obtained from BDH chemicals limited, Poole-England. Caffeic acid (CA) and 2,3-dihydroxybenzoic acid (DHBA) were obtained from Sigma, Switzerland. Desferroxamine B (FOB) was obtained from Ciba-Geigy Ltd. (now Norvatis), Switzerland. Benzohydroxamic acid (BHA) was obtained from Sigma, United Kingdom. Butylated Hydroxy toluene (BHT), Ferrozine and DPPH were obtained from Sigma-Aldrich Inc., UK.

2.2. Preparation of solutions

2.2.1. Preparation of stock solutions

The stock solutions of each of the iron chelators (FOB, BHA, DHBA and CA) were prepared by dissolving a weighed amount of each chelator in a total volume of 10 ml methanol to give 10 mM concentration. Stock solutions of EDTA (10 mM), and vitamin C (10 mM) were also prepared and used as positive standards.

2.2.2. Preparation of working solutions of iron chelators

Working solutions of 1 mM and 2 mM concentrations were prepared from the 10 mM stock solutions of the iron chelators for each assay. All other reagents used were prepared by accurate dilutions from stock solutions. Reactions were carried out in duplicate. The choice of the two working concentrations was based on a data published by Gulcin in 2006 where caffeic acid was used in a concentration range of 10–20 μg/ml which translates into a molar concentration range of approximately 0.05–0.1 mM. We decided to carry out our study at 1–2 mM concentrations of the chelators to investigate their effect at a much higher concentration.

2.3. Determination of iron binding ability of chelators

Iron (II) binding ability of the chelators was determined according to the method of Dinis et al. [13] except that ferrous sulphate was substituted for ferrous chloride. Ferrozine can quantitatively form complexes with ferrous iron yielding a red colour. However, in the presence of chelating agents, there is disruption of the formation of the complex which leads to a decrease in the red colour. Measurement of colour reduction gives an estimation of the binding ability of the coexisting chelator.

The ferrous ion was monitored by measuring the formation of a red ferrous ion-ferrozine complex at 562 nm. The iron chelators (940 μl) at working concentrations of 1 mM and 2 mM were each mixed with 20 μl of ferrous sulphate (2 mM) and ferrozine added to a concentration of 0.2 mM to start the reaction. The resulting mixture was mixed thoroughly and left to stand for 10 min at room temperature. The absorbance of the solution was measured at 562 nm.

The higher the absorbance at 562 nm (which is due to the ferrous ion-ferrozine complex), the weaker the ferrous iron binding strength of the chelator. Percentage inhibition was calculated by the formula in Eq. (1) below;

$$\frac{A_o - A_s}{A_o} \times 100\% \quad (1)$$

where A_o is the absorbance of the control, and A_s is the absorbance of the iron chelator.

2.4. Determination of reducing ability of iron chelators

The reducing ability of the iron chelators was determined by the method of Oyaizu [14] with a slight modification. The reducing ability assay is based on the principle that substances, which have reduction potential, react with potassium ferricyanide ($K_3Fe^{3+}(CN)_6$) to form potassium ferrocyanide ($K_4Fe^{2+}(CN)_6$) (2), which then reacts with ferric chloride to form a ferric-ferrous complex (3) that has an absorption maximum at 700 nm.

$$K_3Fe^{3+}(CN)_{6(aq)} + Ironchelator_{[red](aq)} \rightarrow K_4Fe^{2+}(CN)_{6(aq)} + Ironchelator_{[ox](aq)} \quad (2)$$

$$K_4Fe^{2+}(CN)_{6(aq)} + FeCl_{3(aq)} \rightarrow KFe^{3+}Fe^{2+}(CN)_{6(aq)} + 3KCl_{(aq)} \quad (3)$$

An aliquot (2.5 ml) of each chelator at 1 mM and 2 mM concentration was added to test tubes containing 2.5 ml of phosphate buffer (200 mM, pH 6.6) and 2.5 ml of 1% potassium ferricyanide [$K_3Fe(CN)_6$]. The mixtures were incubated in a water bath at 50 °C for 20 min and cooled rapidly on ice. They were then mixed with 2.5 ml of 10% trichloroacetic acid (TCA) to stop the reaction. The mixtures were centrifuged at 1000 × *g* for 10 min and 5 ml of distilled water added to 5 ml of the supernatant. Ferric chloride was then added to a concentration of 0.1%. The mixture was mixed and allowed to stand for 10 min at room temperature after which the absorbance was taken at 700 nm. An absorbance at 700 nm, which is due to the formation of a Perl's Prussian blue potassium ferrocyanide-ferric complex, is a measure of the reducing ability of the chelator. Increasing absorbance at 700 nm indicates an increase in reductive ability.

2.5. Determination of free radical scavenging activity of iron chelators

2.5.1. DPPH free radical scavenging activity

The DPPH radical scavenging method is widely used to evaluate the free radical scavenging ability of natural antioxidants [15]. DPPH is a stable nitrogen-based free radical which has a violet colour that changes to yellow after reduction by either the process of hydrogen- or electron-transfer. Substances which are able to execute this reaction can be considered as antioxidants and therefore radical scavengers [16]. In the radical form, the DPPH molecule has an absorbance at 517 nm which disappears after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule [17].

The free radical scavenging activities of the chelators were estimated using DPPH according to the method of Yokozawa et al. [18]. To 1 ml methanolic solution of DPPH (0.08 mM) in Eppendorf tubes, 300 μl of the iron chelators were added. The resultant mixture was shaken thoroughly and allowed to stand at room temperature in the dark for 30 min after which the absorbance of the solution was measured at 517 nm. Vitamin C was used as the positive standard and methanol as the blank. The negative control contained DPPH and methanol but no iron chelator. The ability to scavenge DPPH radical was calculated using the formula in Eq. (4);

$$\text{Scavenging activity (\%)} = \frac{\text{Absorbance of control} - \text{Absorbance of test sample}}{\text{Absorbance of control}} \times 100 \quad (4)$$

2.5.2. H_2O_2 radical scavenging activity

The ability of the iron chelators to scavenge H_2O_2 was determined according to the method of Ruch et al. [15]. A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). The concentration of hydrogen peroxide was determined by absorption at 230 nm using a spectrophotometer. The iron chelators (500 μl each at 1 mM) were added to 1 ml of the hydrogen peroxide solution (40 mM). The absorbance of hydrogen peroxide at 230 nm was read after ten minutes against a blank solution of phosphate buffer not having hydrogen peroxide. The percentage of hydrogen peroxide scavenged by the iron chelators and standard compound were calculated using formula in Eq. (2). The procedure was repeated for the iron chelators at 2 mM concentrations but with 3% hydrogen peroxide solution.

2.5.3. $^{\bullet}OH$ radical scavenging activity

Hydroxyl radicals generated by the Fenton reaction (Fe^{3+}-ascorbate-EDTA-H_2O_2 system) were measured according to the method of Chung et al. [19] with a slight modification. Hydrogen peroxide is able to undergo a set of reaction known as the Fenton reaction to release the hydroxyl radical in the presence of iron. Hydroxyl radical scavenging activity is based on the quantification of the 2-deoxyribose degradation product, malonaldehyde, by its condensation with thiobarbituric acid (TBA) to give a yellow colour which absorbs at 532 nm.

The Fenton reaction mixture consisted of 0.2 ml $FeSO_4{\cdot}7H_2O$ (10 mM), 0.2 ml EDTA (10 mM) and 0.2 ml 2-deoxyribose (10 mM) mixed with 1.2 ml phosphate buffer (0.1 M, pH 7.4). The iron chelators (0.2 ml at 1 mM and 2 mM) were added to the Fenton reaction mixture followed by 0.2 ml H_2O_2 (10 mM) and incubated at 37 °C for 4 h. Following incubation, 1 ml TCA (2.8%) and 1 ml TBA (1%) were added to the reaction mixture and placed in a boiling water bath for 10 min for colour development. The resultant mixture was brought to room temperature and centrifuged at 1000 × *g* for 3 min and the absorbance measured at 532 nm against a blank solution. Vitamin C was used as a positive

control. Radical scavenging activity was calculated using Eq. (2).

3. Results and discussion

Reactive oxygen species cause damage to cellular biomolecules such as proteins, nucleic acids, lipids and carbohydrates [4]. Antioxidants interfere with the generation of ROS and also play a crucial role in their inactivation. Although, human cells protect themselves against oxidative damage by antioxidants, these are occasionally not sufficient to prevent ROS induced cellular damage [3].

The study investigated antioxidant and free radical scavenging potential of iron chelators based on their ability to (i) chelate metal ions, (ii) reduce ferric to ferrous ion, (iii) scavenge non biological stable free radical (DPPH) and (iv) scavenge biologically important oxidants such as H_2O_2 and $^{\bullet}OH$. Iron chelators were tested for antioxidant activity in four different *in vitro* assays namely, reducing ability (Fig. 3.2), DPPH radical scavenging activity (Fig. 3.3), H_2O_2 scavenging activity (Fig. 3.4) and $^{\bullet}OH$ radical scavenging activity (Fig. 3.5). The ferrous ion binding ability of the iron chelators was also determined (Fig. 3.1). The iron chelators did not only exhibit iron binding ability but also free radical scavenging activity. It was found that the radical scavenging activities as well as the reducing ability of all the iron chelators generally increased with an increase in chelator concentration [20].

3.1. Iron binding ability of chelators

The chelation of Fe^{2+} was estimated by the method of Dinis et al. [13].

Desferroxamine B and benzohydroxamic acid had high iron binding abilities of 93.7% and 61.5%, respectively as compared to the standard Na_2EDTA which had a chelating activity of 94.8%. Caffeic acid and DHBA had relatively low iron binding abilities of 24.4% and 5.0% respectively (Fig. 3.1).

The iron binding ability of the chelators could be related to the type of functional group used in iron chelation. The hydroxamate chelators (FOB and BHA) showed high iron binding ability whereas the catecholate chelators (CA and DHBA) showed low iron binding ability. The free radical scavenging activities of the chelators showed a different trend (Figs. 3.3–3.5). Desferroxamine B had the highest iron binding ability and this could be due to the three bidentate ligand structure within the molecule. Roosenberg et al. [21] have demonstrated that the siderophores that have three bidentate ligands inside their structure are the most effective and by virtue of that are able to form hexadentate complexes with smaller entropic variations than that caused by chelating one ferric ion with separate ligands.

3.2. Total reductive capability

A significant indicator of the antioxidant potency of a compound is its reducing ability. Benzohydroxamic acid showed the least reducing ability of 0.394 whereas CA, DHBA and FOB showed higher reducing abilities of 1.736, 1.697 and 1.442 respectively as compared to the vitamin C standard which gave a reducing ability of 1.114 (Fig. 3.2). All the chelators showed concentration dependent reducing ability, indicating that the chelators were capable of donating hydrogen atoms. All the iron chelators are capable of donating 3 hydrogen atoms per molecule with the exception of BHA which can only donate 1 hydrogen atom per molecule. The ability of BHA to donate only 1 hydrogen atom per molecule might be the reason why it showed a lower reducing ability as compared to the remaining chelators. Previous studies have correlated the reducing capacity of phytocompounds to their electron-donating ability [22]. Hence it can be stated that the effective electron (hydrogen) donating ability of the chelators contributed to the observed overall antioxidant property.

3.3. DPPH free radical scavenging activity

In the DPPH assay, the iron chelators were able to reduce the stable DPPH radical to the yellow coloured diphenylpicrylhydrazine indicating their abilities to scavenge DPPH radical. Benzohydroxamic acid had the least DPPH radical scavenging activity of 45.0% whereas DHBA had the highest activity of 84.9%. Caffeic acid had an activity of 84.7% at [1 mM] and vitamin C had an activity of 84.3% (Fig. 3.3). The iron chelators, on interacting with DPPH, might have transferred an electron to it, thus neutralizing its free radical nature as observed by Oyaizu [14].

3.4. Hydrogen peroxide scavenging activity

The highest percentage H_2O_2 radical scavenging activity of 99.8% was obtained with CA followed by BHA which had 99.2% scavenging activity. Desferroxamine B and DHBA had 50.1% and 91.1% H_2O_2 scavenging activities respectively. Vitamin C had H_2O_2 scavenging activity of 51.76%. There were no differences in the free radical scavenging activities of the iron chelators at 1 mM and 2 mM concentration (Fig. 3.4).

Hydrogen peroxide can accept protons (H^+) or electrons and by so doing be reduced to H_2O. In a H_2O_2 scavenging activity, the iron chelators act as hydrogen peroxide scavengers by donating hydrogen atoms to reduce the hydrogen peroxide to H_2O [23].

3.5. Hydroxyl radical scavenging activity

For the $^{\bullet}OH$ radical scavenging assay, BHA gave a low scavenging activity of 9.0% compared to the standard vitamin C which gave a scavenging activity of 12.2%. CA, DHBA and FOB gave higher activities. The $^{\bullet}OH$ scavenging activities of the iron chelators were in the following decreasing order: CA (14.5%) > DHBA (13.4%) > FOB (13.3%) > BHA (9.0%) (Fig. 3.5). This trend could be probably due to their hydrogen atom donating ability.

One of the natural phenolic compounds which is broadly distributed in plant materials such as fruits, vegetables and tea is caffeic acid [24]. As an antioxidant it can scavenge a number of reactive species, including DPPH [20], peroxyl and hydroxyl radicals [25]. Caffeic acid has a variety of potential pharmacological activities which could be due to

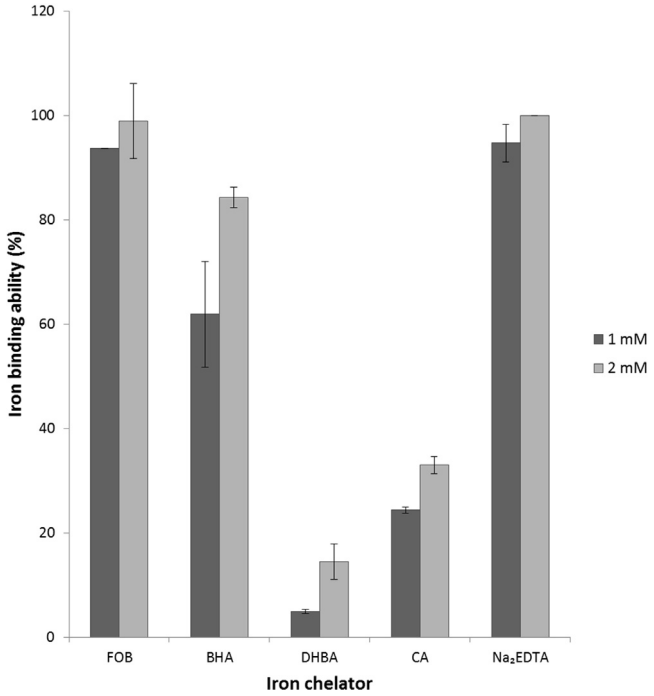

Fig. 3.1. Iron binding ability of chelators. Solutions of iron chelators and Na_2EDTA were prepared at 1 mM and 2 mM concentrations and 940 μl aliquot of each solution was used for the iron binding ability in a total volume of 1 ml reaction mixture. Each value is the average of two experiments with error bars indicating standard error of mean (SEM). Na_2EDTA was used as a positive control.

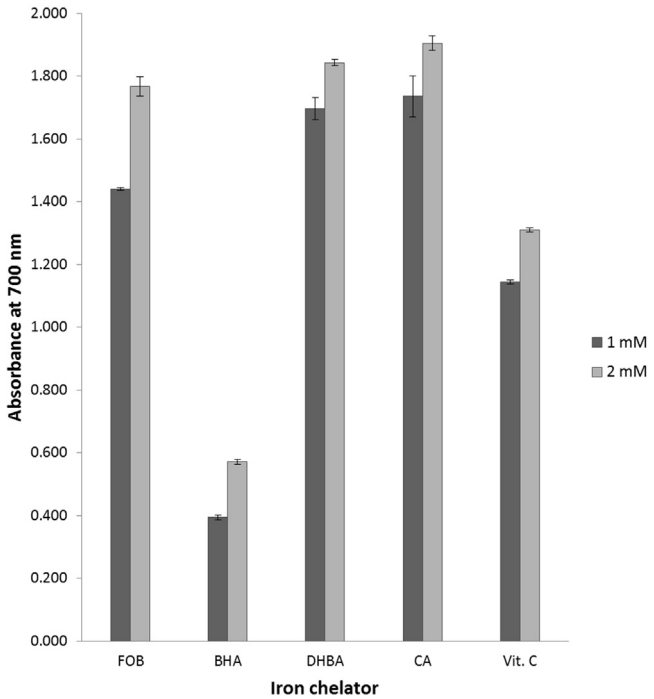

Fig. 3.2. Reducing ability of iron chelators. Solutions of iron chelators and vitamin C at 1 mM and 2 mM concentrations were prepared and 2.5 ml aliquot of each solution was used for the reducing ability assay in a total volume of 7.5 ml reaction mixture. Each value is the average of two experiments with error bars indicating SEM. Vitamin C was used as a positive control.

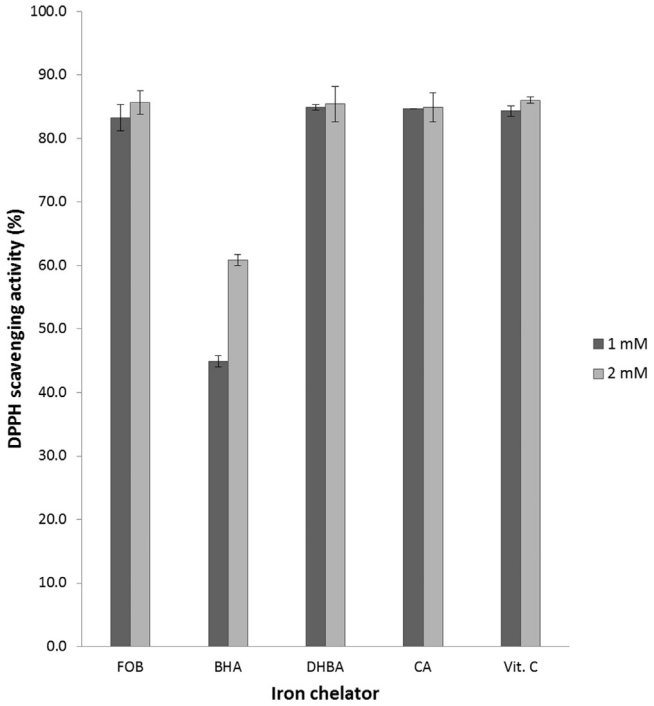

Fig. 3.3. DPPH radical scavenging activity of iron chelators. Solutions of iron chelators and vitamin C at 1 mM and 2 mM concentrations were prepared and 300 μL aliquot of each solution was used for the DPPH scavenging activity in a total volume of 1.3 ml reaction mixture. Each value is the average of two experiments with error bars indicating SEM. Vitamin C was used as a positive control.

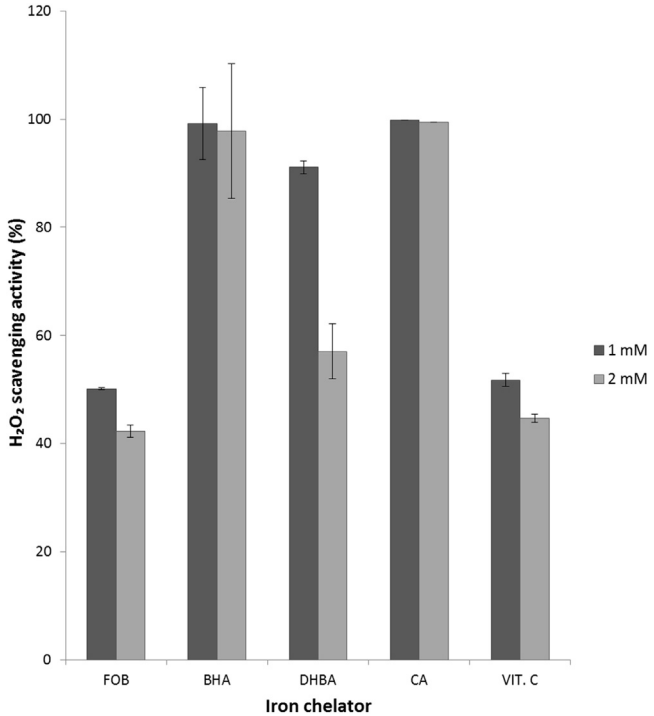

Fig. 3.4. Hydrogen peroxide scavenging activity of iron chelators. (a) and (b) represents H_2O_2 scavenging activity for the iron chelators at 1 mM and 2 mM concentrations respectively. Solutions of iron chelators and vitamin C at 1 mM and 2 mM concentrations were prepared and 500 μL aliquots of each solution was used for the H_2O_2 scavenging activity in a total volume of 1.5 ml reaction mixture. Each bar represents the mean ± SEM; all determinations were performed in duplicate. Vitamin C was used as a positive control.

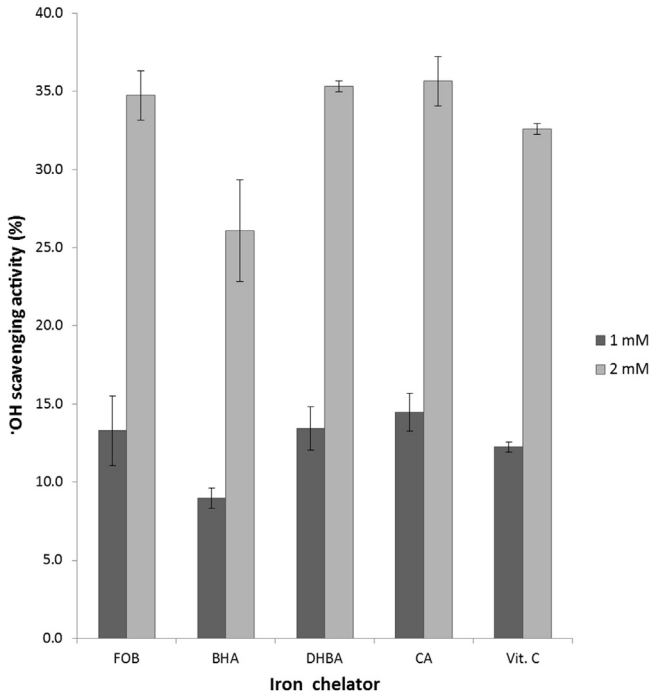

Fig. 3.5. Hydroxyl radical scavenging activity of iron chelators. Solutions of 1 mM and 2 mM concentrations of iron chelators were prepared and 200 μL aliquots of each solution was used for the •OH scavenging activity in a total volume of 2.2 ml reaction mixture. Each value is the average of two experiments with error bars indicating SEM. Vitamin C was used as a positive control.

its high free radical scavenging activity as observed in this study.

3.6. Limitations of study

While this study appears to highlight the outstanding antioxidant properties of caffeic acid, the study is limited to deductions based only on the molar concentrations of 1 mM and 2 mM for the chelators used. The observations made in the study may or may not be the same if a wider concentration range was used.

4. Conclusion

It can be concluded from the study that all the iron chelators have antioxidant and free radical scavenging activities. Caffeic acid had the highest free radical scavenging activity. This study makes valuable addition to the existing wealth of information on this indigenous natural antioxidant. Further studies would be needed to demonstrate the mechanism of the observed actions.

Conflict of interest

None declared.

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