Brief Report

DOI: 10.5582/ddt.2018.01046

Bio-guided fractionation and iron chelating activity of agricultural residues

Farid A. Badria[1,*], Sara N. Suliman[1], Marwa Elsbaey[1], Mai H. El-Naggar[2]

[1] *Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Mansoura, Egypt;*
[2] *Department of Pharmacognosy, Faculty of Pharmacy, Sohag University, Sohag, Egypt.*

Summary **Iron overload is implicated in many disorders in the body such as heart failure, liver cirrhosis and fibrosis, gallbladder disorders, diabetes, arthritis, depression, infertility, and cancer. Even though synthetic chelating agents are available, they have several limitations such as poor oral bioavailability, short plasma half-life, high cost and numerous side effects. Therefore, the aim of this study is using agricultural residues as sources for alternative efficient, benign, and economic iron chelators of natural origin. Eighteen agricultural residues were screened for iron chelating activity using 2, 2'-bipyridyl assay. The results showed that the extract of *Mangifera indica* leaves had the highest iron chelation activity (69.7%), in comparison to ethylenediaminetetraacetic acid (EDTA) (70.3%) (standard iron chelator). The *M. indica* leaves extract was further investigated for its flavonoidcontent, phenolic content and antioxidant activity. The high concentration of phenolic (405.5µg/g expressed as gallic acid equivalent) and flavonoid (336.9 µg/g expressed as quercetin equivalent) phytochemicals in the extract, as well as its significant antioxidant capacity (96.95%) compared to ascorbic acid (91.90%) (standard antioxidant agent), suggested that the *M. indica* leaves could represent a good source for new iron chelating agents in iron overload disorders.**

Keywords: Iron overload, *Mangifera indica*, antioxidant activity, flavonoids, phenolics

1. Introduction

Iron is avital trace element of the body (*1*). It is essential for oxygen and electrons transport within cells and as an integrated part of important enzyme systems in various tissues (*2*). Since iron deficiency and iron overload are both harmful and are associatedwith several disorders, it is very important to maintain iron homeostasis in the body.

Iron overload (hemochromatosis) is caused by genetic disorder involved in a protein regulating iron absorption, or due tomultiple transfusions of iron in chronic anemia or thalassemia, and liver cirrhosis patients (*3*). Accumulation of iron in the body results in initiation and propagation of reactive oxygen species, which start to attack the cell vital macromolecules such as proteins, lipids, RNA and DNA causing DNA mutation, cell damage and ultimately cell death. The high oxidative stress status is associated with many health complications such as heart failure, liver cirrhosis and fibrosis, gallbladder disorders, diabetes, arthritis, infertility, and cancer (*2*). About 71% mortality are recorded in beta thalassemia patients who suffer from cardiac diseases due to accumulation of iron in myocardium (*4*). Iron chelators can remove the accumulated iron from body before causing irreversible tissue damage by forming soluble, stable complexes that can be excreted in feces and/or urine. Although, available iron chelators reduce iron-related complications, their severe side effects, poor oral bioavailability or short plasma half-life make them suboptimal (*5*). Deferoxamine (Desferal®) is the most common drug used for this purpose for many years (*6*). Besides being an expensive drug, its use is painful due to its administration by intravenous or subcutaneous routes. Moreover, it had a negative effect on patient's life through chronic treatment such as neurotoxicity (*4*).

**Address correspondence to:*
Dr. Farid A. Badria, Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University, Mansoura 35516, Egypt.
E-mail: faridbadria@gmail.com

Other available drugs such as deferasirox (Exjade®) and deferiprone (Ferriprox®) are also expensive and their use is associated with side effects such as gastrointestinal disorder (*4*). Several researchers have been interested in studying iron overload problems in the body and finding natural iron chelators from different sources. Catechins and curcumin were represented as natural iron chelators. They were proven to have a significant ability to reduce the level of iron overload in the body (*7-9*). Agricultural residues are considered as a potential source for economic biologically active compounds (*10,11*). Therefore, this study was designed to search for natural and efficient iron chelators with high safety margin and economic cost from agricultural residues.

2. Materials and Methods

2.1. *Plant materials*

Eighteen agricultural residues (Table 1) were collected from Mansoura city and its vicinity in August 2017 and authenticated by Prof. Ibrahim Mashaly at Ecology and Botany Department, Faculty of Science, Mansoura University. *Citrus sinesis* L. peel and leaves of all other agricultural residueswere used all over this study.

2.2. *Chemicals*

Methanol, petroleum ether, methylene chloride, ethyl acetate (EtOAc), n-butanol, sodium hydroxide, Ferric chloride, sulfuric acid, hydroxyl amine hydrochloride, Potassium acetate, and sodium carbonate were purchased from EL-Nasr Company, Cairo, Egypt. Other chemicals and reagents obtained from different sources as follow; alcoholic α-naphthol (BiochemPharma Limited, Cairo, Egypt), mercuric chloride (Noreshark, Cairo, Egypt), ferrous sulfate (Nobel Company, India), trisHCl (PrevestDenpro Limited, Digiana, Jammu, India), 2,2'-bipyridyl solution (Hopkin and Williams Essex, London, England), ethylenediamine tetra acetic acid (EDTA) (BDH Laboratory, England), aluminum chloride (Laboratory Rasayan S.D Fine, Chem. Limited, Mumbai, India), folin-ciocalteu reagent (Sigma, Louis, USA), azino-bis-(3-ethyl benzthiazolin-6-sulfonic acid), (Fluka, Germany), manganese dioxide (Oxford Laboratory, Mumbai, Maharashtra, India), ascorbic acid (Cevarol tablet, Memphis pharmaceutical Co, Cairo, Egypt). Quercetin and Gallic acid were previously isolated and identified in the Department of Pharmacognosy, Faculty of Pharmacy, Mansoura University.

2.3. *Preparation of the crude plant extracts*

The plant material was collected and washed under running tap water to remove the dust. The plant samples were then air dried and crushed into powder and stored in polythene bags for further use (*12*). Powdered plant material (5 g) was exhausted by 70% methanol (3 × 50 mL). The extract was evaporated under reduced pressure, kept in dark at about 4°C (*13*).

2.4. *Large scale preparation and fractionation of the crude extracts of Mangifera indica leaves*

The plant powder (3 kg) was extracted by the same method which used before with plant samples and yielded 200 g dry extract. The extract was dissolved in the least amount of 50% methanol. Using liquid-liquid partitioning method; it was fractionated successively till exhaustion with petroleum ether, methylene chloride, ethyl acetate and n-butanol. The fractions, in each case, were evaporated to dryness under reduced pressure, and

Table 1. The studied agricultural residues with their iron chelation activity at concentration of 1 mg/mL

Plant	Common name	Family	Iron chelation activity %
Brassica oleracea Var.capitata	Cebbage (green)	Brassicaceae	28.93 ± 4.46
***Caricapapay* L.**	**Papya**	**Caricaceae**	**34.88 ± 9.44**
Ceratonia siliqua	Carob	Fabaceae	27.25 ± 0.21
***Citrus lemon* L.**	**Lemon**	**Rutaceae**	**36.11 ± 1.30**
Citrus sinesis L.	Orange	Rutaceae	24.39 ± 0.29
Cymbopogoncitratus	Lemon grass	Poaceae	12.80 ± 0.91
Ficuscarica	**Figs**	**Moraceae**	**34.50 ± 3.78**
***Mangiferaindica* L.**	**Mango**	**Anacardiaceae**	**69.71 ± 0.27**
Morus alba	Berry	Moraceae	24.32 ± 0.93
***Populus alba.* L.**	**White poplar**	**Solanaceae**	**34.11 ± 13.13**
Prunus armeniaca	Apricot	Rosaceae	28.98 ± 0.41
Prunus persica	Peach	Rosaceae	22.60 ± 6.06
Psidium guajava	**Guava**	**Myrtaceae**	**58.38 ± 3.37**
Punicagranatum	Pomegranate	Lythraceae	19.79 ± 0.20
Pyrus communis	Pear	Rosaceae	27.54 ± 4.43
Solanum melongena	Aubergine	Solanaceae	14.0 ± 6.77
Vitis vinifera	Grape	Vitaceae	23.27 ± 3.76
Ziziphus spinacristi	**Buskthorn**	**Rhamnaceae**	**40.19 ± 2.14**

Results are expressed as mean ± SD.

kept in refrigerator for further investigation.

2.5. Determination of iron chelation activity

The metal chelating activity was assessed by bipyridyl assay method (*14*). Briefly, 250 µL of 3 Mm $FeSO_4$, 1 mL of 0.2 M 2,2'-bipyridyl solution, 1 mL of 0.2 M Tris-HCl, 400 µL of 10% hydroxyl amine, 2.5 mL of methanol and 100 µL of distillated water were added to each extract sample (250 µL, 1 mg/mL). The absorbance was determined at λ_{max} 522 nm and used to evaluate Fe^{2+} chelating activity using ethylenediaminetetraacetate (EDTA) as a standard, the results were expressed as the percentage of iron chelation activity = $(A_{control} - A_{test})/(A_{control} - A_{blank}) \times 100$.

IC_{50} of EDTA, total extract of *M. indica* leaves as well as its EtOAc fraction were determined at serial dilutions ranging from 1,000-7.8 µg/mL. The iron chelation activity was recorded at each concentration and a calibration curve was established.

2.6. *Preliminary phytochemical screening*

The plant extracts were tested for the presence of saponins, alkaloids, carbohydrates, flavonoids, and tannins using Foam test, Mayer's test, Molish's test, NaOH test, and Ferric chloride test respectively. The phytochemical screening was carried out using 0.5 mg of each extract (*15,16*).

2.7. *Determination of total flavonoid content*

The assay was performed according to the method described by Ebrahimzadeh *et al*. (2008) (*5*). To 250 µL of the extract (1 mg/mL stock solution), 750 µL of methanol, 50 µL of aluminum chloride, 50 µL of potassium acetate solution, and 1,400 µL of distilled water were added and kept for 30 min. The absorbance was measured at λ_{max} 415 nm. Quercetin was used as a positive control at serial dilutions (1,000-7.8 µg/mL). The calibration curve was established. The total flavonoid content was expressed as µg/g quercetin equivalent using standard curve equation y = 0.0048 x + 0.0012, $R^2 = 0.09995$.

2.8. *Determination of total phenolic content*

To 40 µL of the extract (1 mg/mL stock solution), 1,800 µL of Folinciocalteu reagent were added then 1,200 µL of sodium carbonate were added after 5 min. and kept for one hour in dark area. The absorbance of the solution was measured at λ_{max} 750 nm (*17*). Gallic acid was used as a standard at serial dilutions (1,000-12.5 µg/mL). The calibration curve was established. The total phenolic content was expressed as µg/g gallic acid equivalent using standard curve equation y = 0.0008 x + 0.0143, $R^2 = 0.9982$.

2.9. *Screening of the antioxidant activity*

This method was carried out according to Lissi *et al*. (1999) (*18*). The reaction mixture consisted of 2 mL of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) solution, and 3 mL of manganese dioxide solution prepared in phosphate buffer (pH 7). The mixture was shaken, centrifuged and decanted. The absorbance of $ABTS^+$ radical solution was recorded at λ_{max} 734 nm. The test absorbance was measured upon the addition of 20 µL of the test sample solution in spectroscopic grade MeOH/buffer (1:1, v/v) to the ABTS solution. The decrease in absorbance was expressed as % inhibition which is calculated from the equation: %inhibition = $(A_{control} - A_{test})/A_{control} \times 100$. Ascorbic acid (20 µL) was used as a standard antioxidant at serial dilutions (1,000-1.56 µg/mL). IC_{50} was determined by preparing a serial dilution of ascorbic acid, total extract as well as the EtOAc fraction ranging from 1,000-0.78 µg/mL. The % ABTS inhibition was recorded at each concentration and a calibration curve was established.

3. Results and Discussion

3.1. *Determination of iron chelation activity*

The results of iron chelation assay (Table 1) indicated that the leaves of *M. Indica* had had the highest iron chelation activity (69.71 ± 0.27%) that was comparable to the standard iron chelator, EDTA (70.30 ± 0.08%). In addition, *P. guajava*, *Z. spinacristi*, *C. lemon*, *C. papay*, *F. carica* and *P. alba* leave extracts showed high activities (58.38 ± 3.37%, 40.19 ± 2.14, 36.11 ± 1.30, 34.88 ± 9.44, 34.50 ± 3.78 and 34.11 ± 13.13, respectively). Meanwhile, *S. melongena* and *C. citratus* leave extracts showed the lowest iron chelation activities 14.0 ± 6.77% and 2.80 ± 0.91%, respectively. These outcomesencouraged further investigations of the different fractions of *M. indica* leaves extract to determine the most active iron chelationfraction. The obtained results showed that the EtOAc fraction had the highest activity among the tested fractions (29.28 ± 2.52%). Other fractions; petroleum ether, methylene chloride, and *n*-butanol showed 5.87 ± 0.38 %, 8.13 ± 1.65%, and 24.05 ± 2.49% iron chelation activities, respectively. In addition, the IC_{50} value of *M. indica* and the EtOAc fraction were found to be 362.7 ± 37.19 µg/mL and 150 ± 2.05 µg/mL respectively in comparison to EDTA (745.2 ± 12.72 µg/mL).

3.2. *Preliminary phytochemical screening*

Preliminary phytochemical screening of the used plant extracts was carried out to investigate the nature of the compounds responsible for iron chelation activity. Results showed presence of flavonoids and tannins in

Table 2. Preliminary phytochemical screening

Plant name	Alkaloids	Tannins	Saponins	Carbohydrate	Flavonoids & Anthraquinones
Brassica oleracea Var.capitata	+	-	-	+	+
Caricapapay L.	+	-	-	++	++
Ceratonia siliqua	-	+	-	+	++
Citrus lemon L.	+	+++	-	+	++
Citrus sinesis L.	+	+++	-	+	++
Cymbopogoncitratus	+	+++	-	+	++
Ficuscarica	-	-	-	+	++
Mangiferaindica L.	+	+++	-	+	+++
Morus alba	-	+	-	+++	+
Populus alba. L.	+	+++	-	+	+
Prunus armeniaca	+	+++	-	+	+
Prunus persica	+++	+++	-	+	-
Psidium guajava	+++	+++	-	+	+
Punicagranatum	+++	+++	-	+	+
Pyrus communis	+++	+++	-	+	+
Solanum melongena	+	+++	-	+++	+++
Vitis vinifera	+	+++	-	++	++
Ziziphusspinacristi	+++	+++	-	+	++

Table 3. Total phenolic (TPC), total flavonoid contents (TFC), and antioxidant activity of the M. indica leaves total extract and EtOAc fraction

Plant	TPC (µg/g)	TFC (µg/g)	% $ABTS^+$ inhibition
Total extract	403.59 ± 1.25	336.982 ± 3.56	96.95 ± 0.98%
EtOAc fraction	631.82 ± 1.26	616.126 ± 1.97	99.90 ± 0.29%
Ascorbic acid	—	—	91.90 ± 0.29%

Results are expressed as mean ± SD.

the active plant extracts (Table 2).

3.3. *Determination of total phenolic and flavonoid content*

Since both the total extract of the leaves of *M. indica* as well as the EtOAc fraction showed the highest iron chelation activities, they were further investigated to determine their phenolic and flavonoids contents. EtOAc fraction showed higher total phenolic and total flavonoid contents than the total plant extract (Table 3).

3.4. *Determination of antioxidant activity*

The percentage ABTS inhibition against different concentrations (µg/mL) was determined for *M. indica* leavestotal extract and the EtOAc fraction. The results showed that EtOAc fraction had higher inhibitory activity than the total extract and both had higher inhibitory activities than the positive control, ascorbic acid (Table 3). Furthermore, the IC_{50} values of the *M. indica* leaves extract and the EtOAc fraction were determined and found to be 85.29 ± 16.28 µg/mL, and 24.18 ± 1.83 µg/mL, respectively, in comparison to ascorbic acid (37.18 ± 1.83 µg/mL).

Analyzing the previous results, collectively, it could be concluded that, there is a direct relation between the iron chelating activity of the plant extracts and their content of active compounds. The activity of *M. indica* leave extract is attributed to its content of phenols, and flavonoids as represented by the phytochemical screening and studying its total flavonoid and total phenolic contents, while the chelating activity of *P. guajava*, *Z. spinacristi*, *C. lemon*, and *P. alba* was largely attributed to their tannins content only. Moreover, the good chelating activity showed by each of *C. papay* and *F. carica* was relatively attributed to their flavonoid content. In contrast, the leave extracts for each of *S. melongena* and *C. citratushave* a rich content of polyphenolic compounds and showed the weakest chelating activity. In addition, there was a direct correlation between the antioxidant activity and the polyphenolic compounds content observed by *M. indica* and its EtOAc fraction, both showed a potential antioxidant activity higher than ascorbic acid (standard antioxidant). The antioxidant activity of the EtOAc fraction was stronger than *M. indica* total extract since its total phenolic and total flavonoid contents were higher than that of *M. indica*. This indicated a direct correlation between the antioxidant activity and the polyphenolic content. In contrast, the EtOAc fraction had lower iron chelating activity than the total extract indicating no correlation between the iron chelation activity and the polyphenolic content.

The iron chelating and the antioxidant activities may not be correlated to the polyphenolic content of some plant extracts. This was also presented in other published researches (*5,19*). Overall, the leaves extract of *M. indica* that contained high polyphenolic content exhibited the best iron chelating activity and high antioxidant activity. It is worth to be mentioned that the previous literature proved the potential use of *M. indica* extract as antioxidant (*20*) and as iron chelator (*21*). We need to conduct a bio-guided study to pin point the activity of the extract, even though some literature cited that Mangiferin is the compound responsible for the activity (*22,23*).

In conclusion, the vital use of agricultural residues should be recognized, especially *Mangifera indica* leaves as potential therapeutic agents in iron overload health complications. Therefore, future strategies are in progress to determine the mechanism of action through *in vivo* and bioavailability studies. This may pose a hope for utilizing the mango leaves and/or the pure compound(s) as a valuable natural, safe, and efficient alternative for the currently used iron chelation therapies.

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(Received August 30, 2018; Revised October 24, 2018; Accepted October 25, 2018)