In Vitro Bioaccessibility and Bioavailability of Iron from Fenugreek, Baobab and Moringa

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Abstract

Iron deficiency anaemia (IDA) is a common nutritional disorder worldwide. Sustainable food-based approaches are being advocated to use high and bioavailable dietary iron sources to prevent iron deficiency. The study investigated the bioaccessibility and bioavailability of iron from some plant products.

Total iron levels in the samples were measured by inductively coupled plasma optical emission spectrometry (ICP-OES). Fractionation of the iron from the digested extracts was carried out by centrifugation and ultrafiltration. Iron bioavailability was determined using an in vitro simulated peptic-pancreatic digestion, followed by measurement of ferritin in Caco-2 cells.

The highest amount of bioaccessible iron was obtained from moringa leaves (9.88% ±0.45 and 8.44 ±0.01 mg/100 g), but the highest percentage bioavailability was from baobab fruit pulp (99.7% ±0.13 and 1.74±0.01 mg/100 g) respectively. All the plant products, except for baobab, significantly inhibited iron uptake from $FeSO_4$ and FAC, with fenugreek sprout being the most inhibitory.

Key words: plants, in vitro, iron, availability, Caco-2

1. Introduction

Iron deficiency anaemia (IDA) is a nutritional disorder affecting several population groups in the world (WHO) This disorder is common amongst vulnerable infants, adolescent girls, pregnant women and the elderly in most countries. The increased physiological requirements of iron for growth and reproduction, within these population groups, is exacerbated by low intake and poor iron bioavailability from foods (Aspuru, Villa, Bermejo, Herrero, & López, 2011). This is particularly evident in populations subsisting predominantly on vegetables or plants for their sources of iron. Substantial evidence has revealed that IDA has deleterious consequences on cognition, mental function, work performance, and pregnancy outcomes (Muñoz & Humeres, 2012). Consequently, iron supplementation and fortification of staple foods in different countries (Gera, Sachdev, & Boy, 2012) have been practical approaches to alleviate this critical nutritional disorder.

However, oral iron supplements are associated with gastrointestinal irritations and inflammation, while highly bioavailable iron salts used in food fortification cause adverse sensory changes in foods (Saini, Manoj, Shetty, Srinivasan, & Giridhar, 2014). Food fortification also poses a significant challenge in developing economies as it relies on mass processing and distribution of staple foods (Gómez-Galera et al., 2010). Consequently, sustainable food-based approaches are being advocated to increase the intake of food with high iron content and bioavailability (Bouis & Saltzman, 2017). In general, dietary guidelines for the sustainability of the planet advocate the consumption of predominantly plant foods to meet the nutritional requirements of the world's population (Lang & Barling, 2012). Recently, the EAT-Lancet Commission on Food, Planet, Health submitted that 'A diet rich in plant-based foods and with fewer animal source foods confers both improved health and environmental benefits' and assures sustainability of natural resources' (Willett et al., 2019).Consequently, there is a surge in the use of plant parts and plant products in health care and medicinal purposes (Mbah, Eme, & Ogbusu, 2012).

In recent years, several tropical and sub-tropical plants have gained popularity in Western countries in health food shops as novel foods with nutritional potential as sources of minerals and vitamin C (Magaia, Uamusse, Sjöholm, & Skog, 2013). The legume plant, *Trigonella foenum-graecum* (Fenugreek) has long been used for culinary and medicinal purposes in countries such as India, China, Egypt and across the Middle East where it is widely cultivated (Petropoulos, 2003). Fenugreek seeds and leaves have been reported as good sources of iron, calcium and zinc (Goswami, 2012). Baobab, *Adansonia digitata*, a tropical African fruit, is used for food and beverages and medical purposes. However, it is also inherently high in polyphenols (Coe, Clegg, Armengol, & Ryan, 2013) and dietary fibre (Magaia et al., 2013), which are natural inhibitors of mineral absorption. *Moringa oleifera* (moringa) is reputed as a herb that cures a myriad of ailments and diseases which include anaemia,

asthma and skin infections in Africa, South East Asia and South America. Moringa leaves contain high levels of minerals, vitamins and phytochemicals (Anwar et al., 2007). The hematinic potential of moringa leaves has been reported to be comparable with ferric citrate in haemoglobin (Hb) repletion study in rats (Saini et al., 2014). Consequently, this current study has evaluated levels of iron in fenugreek, baobab and moringa samples and also determined the release of soluble iron following *in vitro* digestion and iron uptake in Caco-2 cells.

2. Materials and Methods

2.1. Reagents and Chemicals

Unless otherwise stated, all the reagents and chemicals used in this study were purchased from Sigma-Aldrich Ltd. (Dorset, U.K.). Solutions of enzymes were all prepared freshly just before use.

2.2. Plant Samples

Fenugreek sprout and fenugreek seeds were acquired from Plant Organic UK. Baobab fruit pulp powder was purchased from Aduna Ltd., London. Moringa dried leaf powder was acquired from Mother Nature's Garden Co., London.

2.3. Moisture Analysis

The moisture content of the samples was determined according to the AOAC (2002) method. Briefly, 2 g of samples were weighed and placed in an oven (Gallenkamp model IH-100) at 100 °C to dry for 24–48 h until constant weights were achieved. Afterwards, the percentage of moisture content was calculated for each sample. Samples (50 g) of each vegetable were ground in a classic Moulinex AR1043 grinder to fine powders and stored in sealed bags at −70 °C before analysis.

2.4. Determination of Mineral Content in Plant Products

Samples were processed using the MARS 6 Microwave digestion system. Samples (0.5 g of starting material or 5 ml of digest) and 5 ml of 15.8 M nitric acid were added into reaction vessels and placed into the microwave digester. Digestion of the samples was carried out for an hour. The contents were then transferred into Falcon tubes containing 140 µl of 100 ppm yttrium internal standard, and the volume was made to 14 ml with deionised water. Iron in the samples was read using the inductively coupled plasma optical emission spectrometry - ICP-OES (Thermo ICAP 6000).

Fractionation of the bioaccessible iron into percentages, and low-molecular-weight Fe, in the digested extracts, were carried out by centrifugation (Eppendorf microcentrifuge 5417) and ultrafiltration as described by Powell et al. (2014). Aqueous suspensions (0.5 ml) were centrifuged (110 g, 5 min), and the supernatant represents the total bioaccessible iron (TBF) released during *in vitro* digestion. To separate the low-molecular-weight Fe fraction (LMW), a fraction of the supernatant was ultrafiltered through AMICON ULTRA 3 kDa molecular weight cut-off columns (Merck-UFC500396) (110 g rpm, 5 min). Iron concentrations of samples were determined in ICP-OES (Thermo ICAP 6000). The TBF and the LMW were calculated as follows:

[(%) Total bioaccessible Fe] = [($Fe_{\text{supernatant after digestion}}$)/Total Fe] x 100

[(%) Fe low-molecular-weight fraction] = [($Fe_{\text{ultrafiltrate}}$)/Total Fe] x 100

2.5. Peptic-pancreatic *in vitro* digestion

Simulated gastrointestinal digestion was performed on the samples using a procedure described previously (Glahn, Lee, Yeung, Goldman, & Miller, 1998). Briefly, in dark tubes, 0.5 g samples were mixed with 10 ml of saline solution (140 mmol/L NaCl and 5 mmol/L KCl) and left for 5 min. Then, the pH was adjusted to 2.0, using 1 M HCl. Afterwards, 0.5 ml of pepsin (Sigma-P7000) (16 mg/mL) was added. Samples were incubated at 37ºC on a rocking platform (150 rpm) for 75 min. Following this, the pH of the samples was adjusted to pH 5.5 using solid $NaHCO_3$. Bile extract and pancreatin (8.5 mg/ml bile extract and 1.4 mg/ml pancreatin) were added, and the pH adjusted again to pH 7.0. The solution was made up to 30 ml with saline solution, and the samples were incubated at 37ºC for 2 hours. At the end of the incubation period, samples were centrifuged at 5000 rpm for 10 min, and the supernatants were decanted and used for the determination of TBF and LMW fractions. Furthermore, the digested extracts were applied to Caco-2 cells to estimate iron uptake.

2.6. Phytic Acid Analysis

Phytic acid content (total phosphorus) was measured by using a kit (Megazyme- K-PHYT, Bray, Ireland) using the protocol described by the manufacturer (McKie & MccleAry, 2016). Briefly, acid extracts of inositol phosphates from the samples were digested with phytase and alkaline phosphatase suspension was used to release phosphate from all the myo-inositol phosphate forms. The total phosphate released was measured using a modified colourimetric method, and was calculated as grams of phosphorus per 100 g of sample material.

2.7. Cell culture

Human Caco-2 cell line was obtained from American Type Culture Collection [ATCC] at passage 40 and used in experiments at passage 45. Cells were sub-cultured in a 75 cm^2 flask to 70-80% confluence. The growth medium contained Dulbecco's Modified Eagle Medium [DMEM], high glucose with glutamine , 10 % fetal calf serum (FBS), 1 % Penicillin-streptomycin (100X) , 1 % L-glutamine (100X) and 1 % MEM non-essential amino acids in an incubator at 37ºC, 5% CO_2 and 95% oxygen.

2.8. Cell Viability Studies

Caco-2 cells were seeded at a density of 1×10^4 cells/cm^2 in 96-well plates. After 14 days of differentiation, the medium was discarded, and the cells were washed twice with sterile phosphate buffer saline [PBS] and then incubated with 100 µl of the digested extracts of fenugreek sprouts, seeds, baobab or moringa for 2 h. Following this, 100 µl of fresh Modified Eagle's medium [DME] along with 10 µl of Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] sterile solution (5 mg/ml MTT in PBS) were added to each well. After incubating for 3 h in the dark at 37 °C, 100 µl of a solubilisation buffer in dimethyl sulfoxide [DMSO] was added and incubated for 15 minutes at room temperature. To determine the MTT reaction in the cells, optical density was read in a microplate reader (Bio-Tek ELx800) at 490 nm. Cell viability was expressed as a percentage of the controls.

2.9. Iron availability

Human Caco-2 cell line was used to evaluate the bioavailability of iron. These cells are derived from colon adenocarcinoma and are used as a surrogate for enterocytes in the small intestine. This model compares well with human studies and is commonly used to analyse iron bioavailability from various food types. (Glahn et al., 1998). Caco-2 cells were trypsinised and cultured in 6-well plates for 14 days to allow them to differentiate, and the medium was changed every 2 days. Before experiments, cells in 6-well plates were treated with 2 ml serum-free medium [SFM] MEM for 24 hours. Sample digests were centrifuged and heated at 100ºC for 5 min to inactivate the digestive enzymes. Serum-free medium (1 ml) was added to cells, followed by the addition of 1 ml of sample digest, and these were incubated in a rotating shaker for 2 h. Following this, 1 ml of MEM was added, and samples were incubated at 37ºC for a further 22 hours for ferritin synthesis. After the incubation, the culture medium was discarded, and cells were washed with versene (PBS + EDTA). Afterwards, 100 µl of mammalian protein extraction reagent (MPER, Thermo Scientific, UK) was added to wells and left on a shaker for 15 min for cell lysis.

Ferritin ELISA kit, Spectro Ferritin MT (Ramco Laboratories Inc., USA) was used to determine ferritin content in the cells according to the manufacturer's protocol.

2.10.Statistical Analysis

Experiments were performed in 3-6 replicates and data are shown as mean ± standard error of the means . Comparisons of iron content, solubility and ferritin concentrations in Caco-2 cells were analysed using one-way or two-way ANOVA followed by Tukey's post hoc test where appropriate, using GraphPad Prism software. The significance level was at $P \leq 0.05$.

3. Results

3.1. Mineral content and moisture in plant products

Levels of iron (and other minerals) in the plant samples were measured by ICP-OES (Supplementary Table 1). Iron content was highest in moringa (85.44 mg/100 g) and lowest in baobab (1.67 mg/100 g). Fenugreek sprouts (19.85 mg/100 g) contained more than twice the amount of iron present in fenugreek seeds (7.75 mg/100 g). Moisture content did not vary between plant food samples (range 92.5 – 95.7 %).

3.2. The bioaccessible and fractional low-molecular-weight iron content of the digested extracts of the vegetables

Percentage of TBF and LMW fraction are shown in Table 1. Although baobab fruit pulp had the highest TBF (99.7%), the absolute amount of Fe from baobab was the lowest (1.74 mg/ 100 g). In contrasts, the percentage of TBF in moringa was the lowest, but the absolute quantity LMW was the highest (Table 1). Percentage of fenugreek seeds LMW Fe was significantly ($P \leq 0.05$) higher than the sprouts; nevertheless, the quantity of Fe in both products was comparable. Interestingly, the trend of the LMW fractions from the plant products was similar to the TBF.

Table 1: Total bioaccessible and low-molecular-weight iron in digest samples.

Sample	Bioaccessible		Low-Molecular-Weight	
	%		%	
Fenugreek sprout	17.18±0.04 (3.41±0.01 mg/100 g)	a	9.16±0.09 (1.81±0.01 mg/100 g)	a
Fenugreek Seeds	42.71±0.22 (3.31±0.02 mg/100 g)	b	25.18±0.14 (1.95±0.02 mg/100 g)	b
Baobab fruit pulp	99.7±0.13 (1.74±0.01 mg/100 g)	c	97.8±0.01 (1.70±0.01 mg/100 g)	c

Moringa leaves	9.88±0.45 (8.44±0.01 mg/100 g)	d	1.96±0.04 (1.68±0.03 mg/100 g)	d

Data are presented as means ± SEM of n = 3 measurements. Different letters indicate significant difference between groups (P≤0.05). Mean values represent total bioaccessible iron for each sample category while the lower type phase in bracket are the absolute quantities of iron in the samples.

3.3. The total bioaccessible and fractional low-molecular-weight iron content of the digested extracts of the vegetables with added ascorbic acid

Adding ascorbic acid (AA) to the plant products during digestion decreased TBF and the LMW (Table.2) significantly (P≤0.05) compared with the digested sample without AA. Percentage TBF was the highest from fenugreek seeds. Moringa was the lowest of the plant products in TBF percentage although moringa yielded the highest absolute Fe. The LMW fractions from the plant digests with added AA varied significantly, and the relative proportions differ from the TBF. However, enhancing the effect of ascorbic acid became evident only when it was added along with the digests to Caco-2 cells during the iron uptake study.

Table 2: Total bioaccessible and low-molecular-weight iron when ascorbic acid was added during the digestion of the samples.

Sample	Bioaccessible		Low-Molecular-Weight	
	%		%	
Fenugreek sprout	15.4±0.13 (3.06±0.026 mg/100 g)	a	1.6±0.03 (0.32±0.007 mg/100 g)	a
Fenugreek Seeds	24.3±0.11 (1.88±0.009 mg/100 g)	b	2.7±0.09 (0.21±0.008 mg/100 g)	b
Baobab fruit pulp	19.2±0.9 (0.32±0.015 mg/100 g)	c	15.1±1.15 (0.25±0.019 mg/100 g)	c
Moringa leaves	8.1±0.02 (6.88±0.016 mg/100 g)	d	0.2±0.01 (0.16±0.009 mg/100 g)	d

Data are presented as means ± SEM of n = 3 measurements. Different letters indicate significant difference between groups (P≤ 0.05). Mean values represent total bioaccessible iron for each sample category while the lower type phase in bracket are the absolute quantities of iron in the samples.

3.4. Cell viability of Caco-2 cells after exposure to digested vegetable samples

To ascertain whether the digested extracts from the plants were cytotoxic to Caco-2 cells; the MTT viability assay was performed. The application of the boiled samples of the digested extracts of fenugreek sprouts and seeds, baobab or moringa, did not adversely affect the viability of Caco-2 cells. Indeed, in some cases, there was a significant effect on cell growth as judged by an increase in viability (Supplementary Figure 3).

3.5. *In vitro* accessibility of iron from fenugreek sprouts, seeds, baobab or moringa in Caco-2 cells

To estimate the accessibility of iron from the samples, an *in vitro* simulated peptic–pancreatic digestion was carried out followed by ferritin analysis (a surrogate marker for iron absorption) in Caco-2 cells. In contrast to the bioaccessible iron profile of the samples, fenugreek sprouts exhibited comparatively higher iron available ($P \leq 0.05$) than the seeds (Figure 1). Iron availability from the baobab fruit pulp was the lowest. The absorption of $FeSO_4$ and FAC served as positive controls.

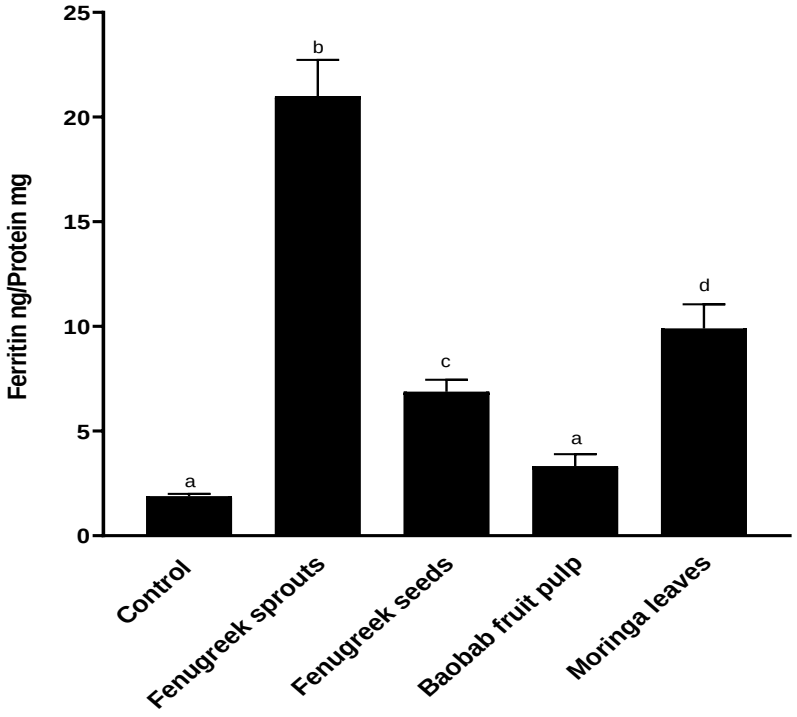

Figure 1: Iron uptake by Caco-2 cells from plant food digests. ''Control'' indicates digest system components only (i.e., pepsin, pancreatin, bile extract). Values are presented as means n = 6 ± SEM. Data were analysed using a two-way ANOVA. Different letters indicate significant difference between groups ($P \leq 0.05$).

To enhance iron extraction during the peptic-pancreatic digestion of the plant products, ascorbic acid was added to the samples during the digestion process. Iron availability from fenugreek sprouts, fenugreek seeds, baobab or moringa was not significantly influenced when ascorbic acid was included in the digestion medium (Figure 2). Ascorbic acid, however, enhanced iron availability from the plant products when added to the digest during exposure to Caco-2 cells during the iron uptake stage.

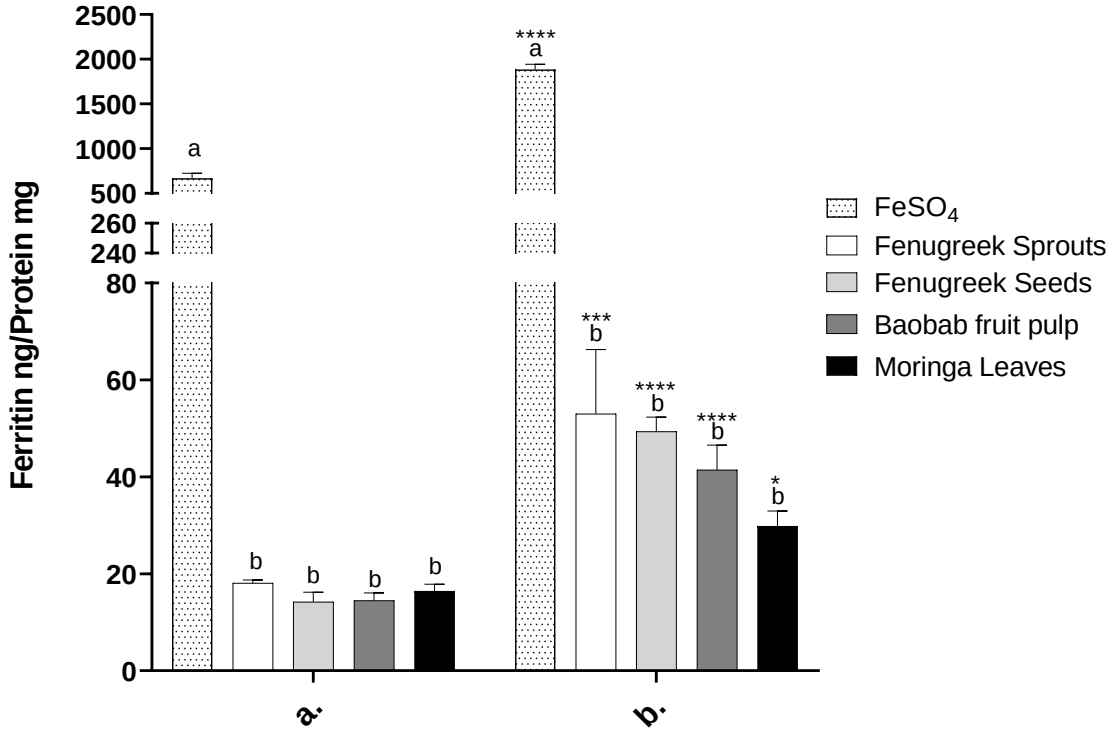

Figure 2. Iron uptake by Caco-2 cells from vegetable samples with added ascorbic acid (AA) during digestion (a) and (b.) vegetable samples with AA added during exposure to cells. The control sample contained 50 µM $FeSO_4$ and 250 µM AA. Values are presented as means ± SEM, n = 6. Data were analysed using a two-way ANOVA. Different letters indicate significant difference between vegetables ($P \leq 0.05$). The differences between groups (a)and (b) are denoted (*$P \leq 0.05$, ***$P \leq 0.001$ and ****$P \leq 0.0001$).

3.6. Modulating effects of fenugreek sprouts, fenugreek seeds, baobab or moringa on iron accessibility from iron salts in Caco-2 cells

We next explored the interactions of the plant products on the accessibility of iron salts. $FeSO_4$ and FAC had comparable iron availability for uptake in Caco-2 cells (Figure 3). Except for baobab fruit pulp, the other plant products significantly ($P \leq 0.05$) reduced iron availability from both $FeSO_4$ and FAC in Caco-2 cells. Moreover, inhibition of iron from FAC (Fe(III)) was significantly higher than from $FeSO_4$ (Fe(II)) for fenugreek seeds ($P \leq 0.01$) and moringa leaves ($P \leq 0.0001$) respectively.

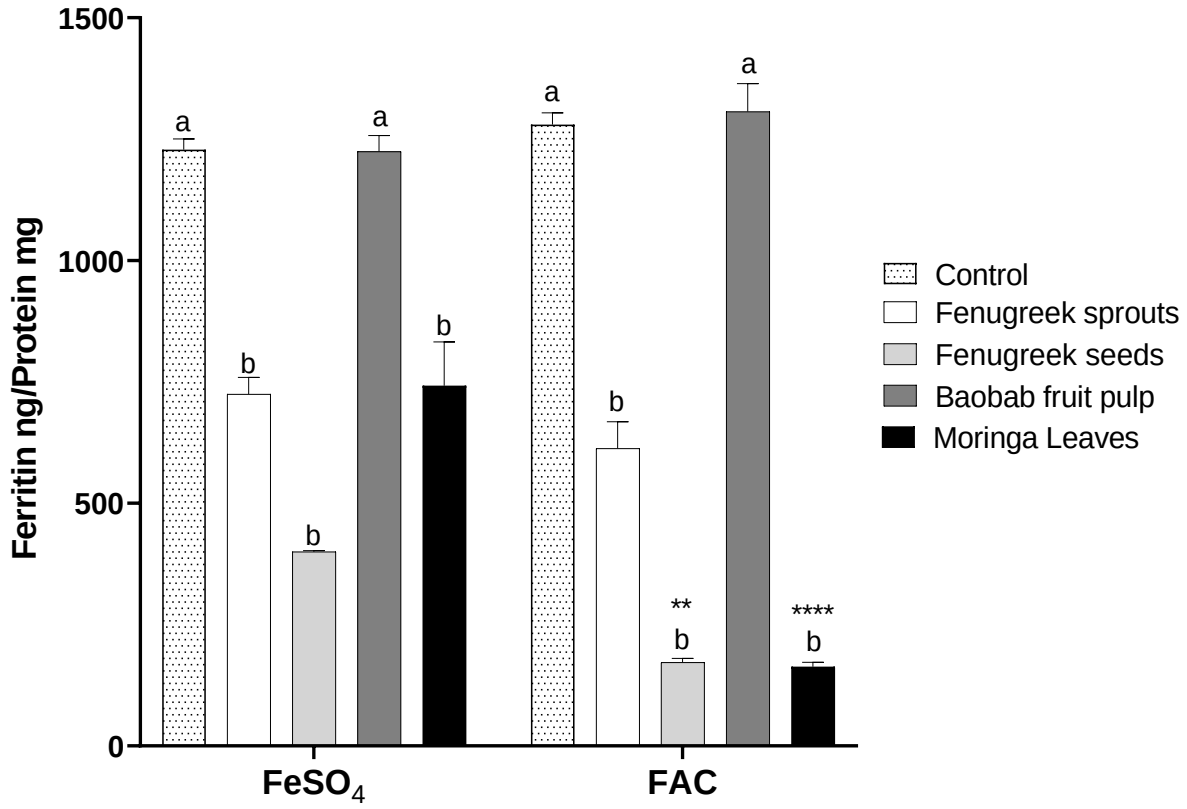

Figure 3: Iron uptake by Caco-2 cells from vegetable digests. Cells were exposed to the digest samples with added $FeSO_4$ or FAC (50 µM). Values are presented as means ± SEM, n = 6. Data were analysed using a two-way ANOVA between $FeSO_4$ and FAC groups and one-way ANOVA amongst the treatment groups. Different letters indicate significant difference between vegetables *(P≤ 0.05)*. The differences between added $FeSO_4$ and added FAC groups are denoted (*P≤ 0.05, ***P≤ 0.001 and ****P≤ 0.0001).

3.7. Phytic acids levels in fenugreek sprouts, seeds, baobab and moringa

Phytic acid is a major inhibitory component in plant foods. Figure 4 shows a significant level of variations in the phytate content of the plant products. Both fenugreek samples exhibited the highest levels of phytic acid and may in part explain why both plant products inhibited $FeSO_4$ and FAC uptake by Caco-2 cells the most (Figure 4).

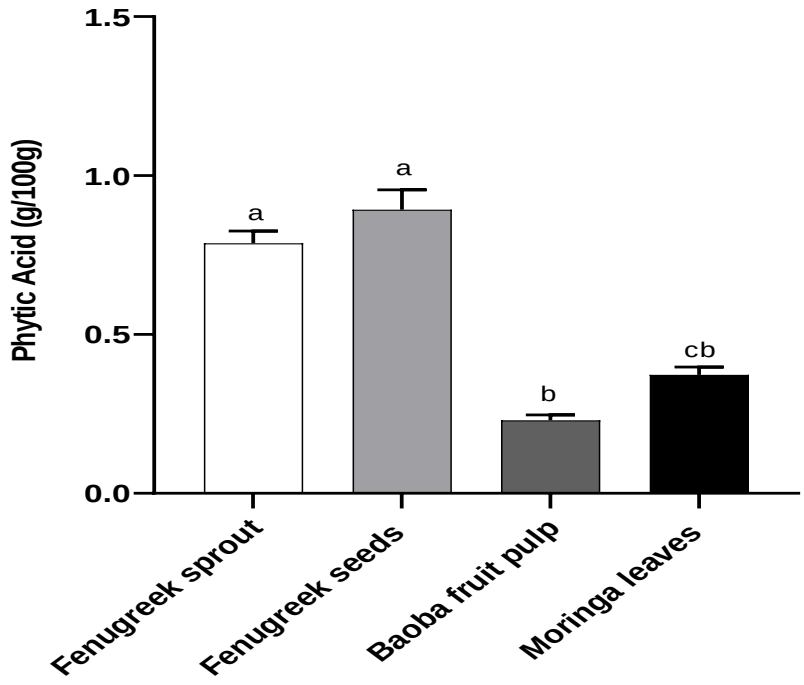

Figure 4: Phytic acid content (g/100 g) of the vegetable samples. Values are presented as means ± SEM, n=6. Data were analysed using a two-way ANOVA test. Different letters indicate significant difference between groups *(P≤ 0.05)*.

4. Discussion

. A sustainable food-based approach to tackle iron deficiency implies the consumption of plant foods that have high iron levels and that have been processed to reduce inhibitors and facilitate the natural release of enhancers of iron bioavailability in meals (Gibson, Perlas, & Hotz, 2006). The current study, therefore, investigated the potentials of fenugreek sprouts, fenugreek seeds, baobab fruit pulp, and moringa leaves as sources of minerals and of accessible iron using *in vitro* methods of analysis.

The levels of iron varied between these plant products and it was clear that they could contribute to daily intake significantly depending on dietary formulations. The differences in the mineral levels from plant products, when compared to published data could be accounted for by factors such as the seed variety, geographical location, processing types, storage conditions and analytical methods (Aslam, Anwar, Nadeem, Rashid, Kazi, & Nadeem, 2005; Chadare, Linnemann, Hounhouigan, Nout, & Van Boekel, 2008).

The iron content of moringa in the current study is higher than the published data (Price, 2007). The mineral profile of moringa leaves has been reported to be superior nutritionally to those of amaranth, mushrooms, taro leaves, cassava leaves and pumpkin seeds (Owusu, Ellis, & Oduro, 2008). All daily

calcium and 75% of iron requirements could be met in children aged 1-3 years from 100 g of fresh moringa leaves (Price, 2007). In comparison, an equivalent weight of baobab pulp will provide 30% of calcium and 23% of iron daily needs in children 4-13 years, and about 29% of the calcium requirement of pregnant women (Magaia et al., 2013). Furthermore, van der Merwe et al. (2019) reported that fortification of pearl millet in a cereal-based meal with moringa leaf powder, roselle calyces or baobab fruit pulp contributed about 3 times more iron and zinc than from pearl millet porridge fortified with provitamin A source alone. The added plant food fortificants contributed about 28% and 41% of the absolute iron and zinc requirements, respectively, of women of reproductive age from a single meal. A recent study (Shija, Rumisha, Oriyo, Kilima, & Massaga, 2019) also reported that daily supplementation of 25 g moringa flour to food intake for 6 months significantly reduced anaemia prevalence in children whose average age was about one year old. Improvement in haemoglobin levels for these children were significant irrespective of the degree of anaemia with moringa intervention plus nutrition education compared with the control that had nutrition education alone. Iron in plant foods exists predominantly as ferric non-haem iron and might be complexed with other organic such as phytate and polyphenols or inorganic molecules in the matrices of fenugreek sprouts, fenugreek seeds, baobab fruit pulp and moringa leaves. Iron, though relatively abundant in the earth crust and most foods, is characterised by poor availability from plant sources.

The digested extract solution in the current study was subsequently fractionated with a 3 kDa column to exclude the agglomerated particulate component, and the soluble fractions in the samples ranged between 9.88 and 99.7 % (Tables 1&2). Consequently, the peptic-pancreatin digestion extracts from the plant products after centrifugation consisted of micronised insoluble particulates and non-particulate soluble matter comprising aggregates and agglomerates of iron chelates of diverse sizes and bioaccessibility (Anderson & Frazer, 2017). Digested extracts from baobab fruit pulp had the highest percentage of bioaccessible iron, whichhowever, did not translate into enhanced iron f uptake by Caco-2 cells, thus indicating that some components of iron chelates or ligands though soluble might not be bioavailable (Miller & Berner, 1989).

Fenugreek sprouts had the highest available iron in Caco-2 cells but paradoxically, exhibited a high level of phytic acid. This was an unexpected finding as the process of sprouting is known to activate phytase (Ou, Cheng, Xing, Lin, Nout, & Liang, 2011) that enzymatically degrades phytate, thereby enhancing iron bioavailability (Hurrell, 2004). In contrast to the current study, previous work reported increased iron availability in germinated fenugreek seeds (Hooda & Jood, 2003). The ratio of phytate to iron has been shown to correlate with iron dialyzability (Glahn, Wortley, South, & Miller, 2002). The phytate to iron ratios were 39.66, 115.07 137.35 and 4.37, while the calcium to iron ratios were 9.23, 22.30, 187.25 and 27.13 respectively for fenugreek sprouts, fenugreek seeds, baobab and

moringa. Vitamin C in plant products in sequential order were 2 (Ahmed, 2014), 19.55 (Sharara, 2017), 266 and 0.11 (Sankhyan, Sharma, Seth, Chauhan, & Kulshrestha, 2013) mg/100 g. Hence, baobab is abundant in both potent inhibitors and an enhancer of iron availability. In general, the comparatively low solubility of iron from these plant products may be due to chemical complexation with a range of inhibitory factors including phytate, polyphenols or fibre (Chadare et al., 2008).

The exploitation of the synergy among dietary components to enhance iron absorption promotes the use of foods that are rich in ascorbic acid, a potent enhancer of iron absorption in composite meals. Consequently, ascorbic acid was added to the plant products during the simulated digestion or added to the digested extracts before the application to Caco-2 cells in the current study. Iron availability was significantly enhanced from fenugreek sprouts, fenugreek seeds, baobab pulp and moringa leaves when ascorbic acid was added to the digest before application onto Caco-2 cells but not when it was added during the peptic-pancreatic digestion process. This might be due to the oxidation of the Fe (II) species by ascorbic acid during heat denaturation of the digestive enzymes in the digests before the application onto Caco-2 cells (Vikram, Ramesh & Prapulla, 2005). Moreover, enhanced iron uptake in Caco-2 cells could be due to an augmentation of Dcytb-dependent, ascorbate-mediated ferrireduction (Luo, Hill, Johnson, & Latunde-Dada, 2014). Alternatively, it could be due to the complexation of iron, which is known to form insoluble complexes when the pH level is higher than 5.3, thus decreasing the availability and absorption of iron (Scheers, Andlid, Alminger, & Sandberg, 2010). In support of the latter possibility, previous studies showed that there was no effect of ascorbic acid on iron availability from pork meat at pH 7 during peptic and pancreatic digestion (Sørensen & Bukhave, 2010). Furthermore, the study highlighted the pH dependence of iron uptake from foods and revealed the significance of separating pepsin-digested and pepsin + pancreatin-digested proteins during in vitro studies on iron availability. This exposes a technical limitation of the *in vitro* method as the spatial interactions of dietary constituents in the food matrix, enzymatic digestive processes, bioaccessibility and absorption of iron occur concurrently in the gastrointestinal tract of the organisms.

Three indices, namely total bioaccessible iron fraction (TBF), low molecular-weight (LMW) iron fraction and iron uptake in Caco-2 cells were employed in the current study to estimate iron availability from fenugreek sprouts, fenugreek seeds, baobab or moringa. Caco-2 cells have been proven for assessing iron uptake because the cells express intestinal microvilli, enzymes and differentiation markers typical of human small intestine enterocytes (Glahn et al., 1998; Sharp, 2005). There was a positive correlation between TBF and LMW for all samples. However, no correlation was evident between TBF, or LMW and iron uptake in Caco-2 cells. Although iron must be soluble to be absorbable, variables such as the chemical nature of the soluble ligand, molecular weights and

the composition of the digest matrix could confound, in some cases, extrapolation to *in vivo* iron absorption studies (Miller & Berner, 1989). Furthermore, clathrin-mediated endocytosis and micropinocytosis have been shown to play a role in the uptake of nanoparticulate iron complexes in intestinal cells (Latunde-Dada et al., 2014).

In vitro assay of ferritin formation in Caco-2 cells after exposure to an iron source was used as a surrogate marker of iron uptake (Yun, Habicht, Miller, & Glahn, 2004). Variations of the original protocol (Glahn et al., 1998) abound in the literature, and it might now be necessary for a review to enable a form of standardisation of the methodology across different laboratories. Nonetheless, *in vitro* systems of estimating iron solubility are useful for predicting the trends of absorption or relative bioavailability which can, therefore, be used to screen or compare large quantities of food types and different varieties of plant products (Sharp, 2005).

Conclusion

Iron content of the dried vegetable samples (mg/100g) are 1.67 ± 0.05, (baobab fruit pulp), 7.75 ± 0.04, (fenugreek seeds), 19.85 ± 0.33 (fenugreek sprout) and 85.44 ± 1.22 (moringa leaves). These underutilised plant foods with variable iron content which upon processing or incorporation in composite cuisines containing rich sources of ascorbic acid, could potentially be employed to treat improve and maintain iron homeostasis in groups at risk of iron deficiency or anaemia. Maximising the potentials of these plant products requires further research in food processing, dietary formulation and nutrition education of the populace.

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6. Supplementary

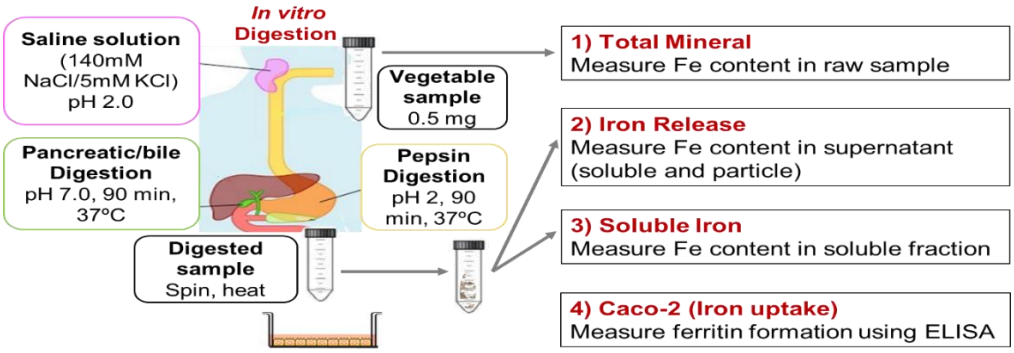
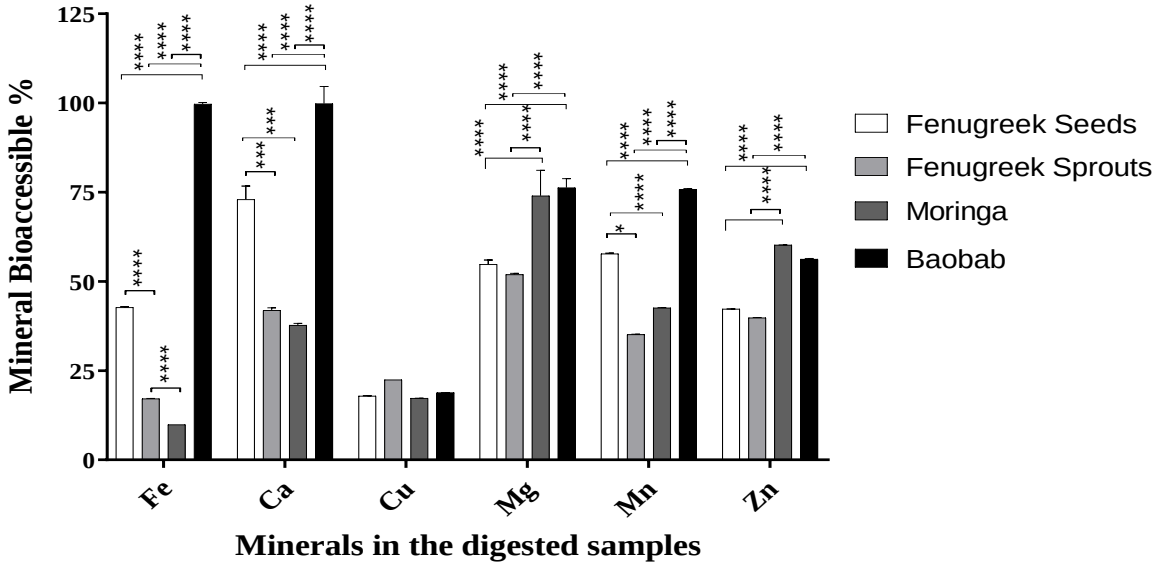


Figure 1 Schematic diagram of the in-vitro digestion procedure and iron availability determinations

Table 1 Moisture and Mineral content of the dried vegetable samples (mg/100g)

Sample	Moisture content (%)	Fe	Ca	Cu	Mg	Mn	Zn
Fenugreek sprouts	95.77 ± 0.034	19.85 ± 0.33 a	183.26 ± 0.61 a	2.67 ± 0.01 a	258.0 ± 0.30 a	2.42 ± 0.01 a	2.42 ± 0.01 a
Fenugreek seeds	92.55 ± 0.052	7.75 ± 0.04 b	172.91 ± 3.22 b	2.33 ± 0.03 a	152.89 ± 1.04 b	1.61 ± 0.06 a	1.59 ± 0.05 a
Baobab fruit pulp	94.1± 0.098	1.67 ± 0.05 c	313.05 ± 4.13 c	1.77 ± 0.03 a	172.33 ± 2.21 c	1.07 ± 0.14 a	1.079 ± 0.14 a
Moringa leaves	93.2± 0.014	85.44 ± 1.22 d	2318.27 ± 23.20 d	1.54 ± 0.03 a	639.50 ± 6.20 d	6.13 ± 0.07 b	6.13 ± 0.06 b

Mineral contents, calcium (Ca), copper (Cu), iron (Fe), magnesium (Mg) manganese (Mn) and zinc (Zn) in 100 g of sample. Values are presented as means ± SEM, n=4. Data were analysed using a two-way ANOVA test. Different letters indicate significant difference between groups ($P \leq 0.05$).



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529 **Figure 2:** Mineral bioaccessible from digested vegetable. Values are presented as means ± SEM, n =
530 6. Data were analysed using a two-way ANOVA. The differences between vegetables are denoted
531 *(*P≤ 0.05, ***P≤ 0.001 and ****P≤ 0.0001).*

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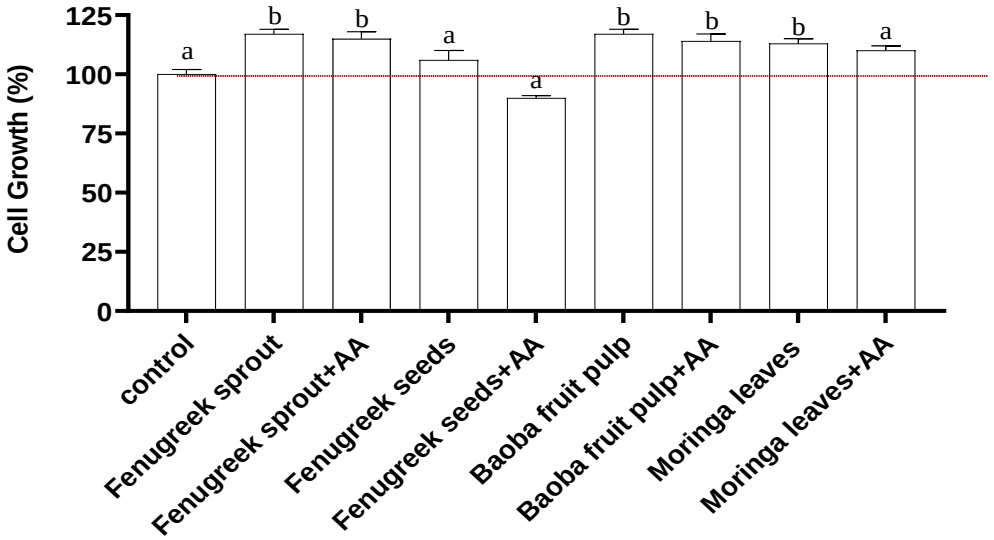

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536 **Figure 3** Cell viability of Caco-2 after exposure to digest for 2 h. Values are presented as means n =
537 4 ± SEM. Data were analysed using a one-way ANOVA test. Different letters indicate significant
538 difference between groups *(P<0.05).*
539