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2
3 **In Vitro Bioaccessibility and Bioavailability of Iron from**
4 **Fenugreek, Baobab and Moringa**

5 Kholoud K. Khoja, Mohamad F. Aslam, Paul A. Sharp, and Gladys O. Latunde-Dada*

6 Department of Nutritional Sciences, School of Life Course Sciences, King's College London. Franklin-Wilkins-Building,
7 150 Stamford Street, London, SE1 9NH, UK.

8 * Corresponding Author: yemisi.latunde-dada@kcl.ac.uk.

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11

12 **Abstract**

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

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

21 The highest amount of bioaccessible iron was obtained from moringa leaves (9.88% \pm 0.45 and 8.44
22 \pm 0.01 mg/100 g), but the highest percentage bioavailability was from baobab fruit pulp (99.7% \pm 0.13
23 and 1.74 \pm 0.01 mg/100 g) respectively. All the plant products, except for baobab, significantly
24 inhibited iron uptake from FeSO₄ and FAC, with fenugreek sprout being the most inhibitory.

25
26 **Key words: plants, in vitro, iron, availability, Caco-2**

27

28

29 **1. Introduction**

30 Iron deficiency anaemia (IDA) is a nutritional disorder affecting several population groups in the
31 world (WHO) This disorder is common amongst vulnerable infants, adolescent girls, pregnant women
32 and the elderly in most countries. The increased physiological requirements of iron for growth and
33 reproduction, within these population groups, is exacerbated by low intake and poor iron
34 bioavailability from foods (Aspuru, Villa, Bermejo, Herrero, & López, 2011). This is particularly
35 evident in populations subsisting predominantly on vegetables or plants for their sources of iron.
36 Substantial evidence has revealed that IDA has deleterious consequences on cognition, mental
37 function, work performance, and pregnancy outcomes (Muñoz & Humeres, 2012). Consequently,
38 iron supplementation and fortification of staple foods in different countries (Gera, Sachdev, & Boy,
39 2012) have been practical approaches to alleviate this critical nutritional disorder.

40 However, oral iron supplements are associated with gastrointestinal irritations and inflammation,
41 while highly bioavailable iron salts used in food fortification cause adverse sensory changes in foods
42 (Saini, Manoj, Shetty, Srinivasan, & Giridhar, 2014). Food fortification also poses a significant
43 challenge in developing economies as it relies on mass processing and distribution of staple foods
44 (Gómez-Galera et al., 2010). Consequently, sustainable food-based approaches are being advocated
45 to increase the intake of food with high iron content and bioavailability (Bouis & Saltzman, 2017).
46 In general, dietary guidelines for the sustainability of the planet advocate the consumption of
47 predominantly plant foods to meet the nutritional requirements of the world's population (Lang &
48 Barling, 2012). Recently, the EAT-Lancet Commission on Food, Planet, Health submitted that 'A
49 diet rich in plant-based foods and with fewer animal source foods confers both improved health and
50 environmental benefits' and assures sustainability of natural resources' (Willett et al.,
51 2019).Consequently, there is a surge in the use of plant parts and plant products in health care and
52 medicinal purposes (Mbah, Eme, & Ogbusu, 2012).

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

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

69

70 **2. Materials and Methods**

71 **2.1. Reagents and Chemicals**

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

75 **2.2. Plant Samples**

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

79 **2.3. Moisture Analysis**

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

85 **2.4. Determination of Mineral Content in Plant Products**

86 Samples were processed using the MARS 6 Microwave digestion system. Samples (0.5 g of
87 starting material or 5 ml of digest) and 5 ml of 15.8 M nitric acid were added into reaction vessels
88 and placed into the microwave digester. Digestion of the samples was carried out for an hour. The
89 contents were then transferred into Falcon tubes containing 140 µl of 100 ppm yttrium internal
90 standard, and the volume was made to 14 ml with deionised water. Iron in the samples was read
91 using the inductively coupled plasma optical emission spectrometry - ICP-OES (Thermo ICAP
92 6000).

93 Fractionation of the bioaccessible iron into percentages, and low-molecular-weight Fe, in the
94 digested extracts, were carried out by centrifugation (Eppendorf microcentrifuge 5417) and
95 ultrafiltration as described by Powell et al. (2014). Aqueous suspensions (0.5 ml) were centrifuged
96 (110 g, 5 min), and the supernatant represents the total bioaccessible iron (TBF) released during
97 *in vitro* digestion. To separate the low-molecular-weight Fe fraction (LMW), a fraction of the
98 supernatant was ultrafiltered through AMICON ULTRA 3 kDa molecular weight cut-off columns
99 (Merck-UFC500396) (110 g rpm, 5 min). Iron concentrations of samples were determined in ICP-
100 OES (Thermo ICAP 6000). The TBF and the LMW were calculated as follows:

101 $[(\%) \text{ Total bioaccessible Fe}] = [(\text{Fe}_{\text{supernatant after digestion}}) / \text{Total Fe}] \times 100$

102 $[(\%) \text{ Fe low-molecular-weight fraction}] = [(\text{Fe}_{\text{ultrafiltrate}}) / \text{Total Fe}] \times 100$

103 **2.5. Peptic-pancreatic *in vitro* digestion**

104 Simulated gastrointestinal digestion was performed on the samples using a procedure described
105 previously (Glahn, Lee, Yeung, Goldman, & Miller, 1998). Briefly, in dark tubes, 0.5 g samples
106 were mixed with 10 ml of saline solution (140 mmol/L NaCl and 5 mmol/L KCl) and left for 5
107 min. Then, the pH was adjusted to 2.0, using 1 M HCl. Afterwards, 0.5 ml of pepsin (Sigma-
108 P7000) (16 mg/mL) was added. Samples were incubated at 37°C on a rocking platform (150 rpm)
109 for 75 min. Following this, the pH of the samples was adjusted to pH 5.5 using solid NaHCO₃.
110 Bile extract and pancreatin (8.5 mg/ml bile extract and 1.4 mg/ml pancreatin) were added, and
111 the pH adjusted again to pH 7.0. The solution was made up to 30 ml with saline solution, and the
112 samples were incubated at 37°C for 2 hours. At the end of the incubation period, samples were
113 centrifuged at 5000 rpm for 10 min, and the supernatants were decanted and used for the
114 determination of TBF and LMW fractions. Furthermore, the digested extracts were applied to
115 Caco-2 cells to estimate iron uptake.

116 **2.6. Phytic Acid Analysis**

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

123 **2.7. Cell culture**

124 Human Caco-2 cell line was obtained from American Type Culture Collection [ATCC] at passage
125 40 and used in experiments at passage 45. Cells were sub-cultured in a 75 cm² flask to 70-80%
126 confluence. The growth medium contained Dulbecco's Modified Eagle Medium [DMEM], high
127 glucose with glutamine , 10 % fetal calf serum (FBS), 1 % Penicillin-streptomycin (100X) , 1 %
128 L-glutamine (100X) and 1 % MEM non-essential amino acids in an incubator at 37°C, 5% CO₂
129 and 95% oxygen.

130 **2.8. Cell Viability Studies**

131 Caco-2 cells were seeded at a density of 1×10^4 cells/cm² in 96-well plates. After 14 days of
132 differentiation, the medium was discarded, and the cells were washed twice with sterile phosphate
133 buffer saline [PBS] and then incubated with 100 µl of the digested extracts of fenugreek sprouts,
134 seeds, baobab or moringa for 2 h. Following this, 100 µl of fresh Modified Eagle's medium
135 [DME] along with 10 µl of Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT]
136 sterile solution (5 mg/ml MTT in PBS) were added to each well. After incubating for 3 h in the
137 dark at 37 °C, 100 µl of a solubilisation buffer in dimethyl sulfoxide [DMSO] was added and
138 incubated for 15 minutes at room temperature. To determine the MTT reaction in the cells, optical
139 density was read in a microplate reader (Bio-Tek ELx800) at 490 nm. Cell viability was expressed
140 as a percentage of the controls.

141 **2.9. Iron availability**

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

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

157 2.10. Statistical Analysis

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

162

163 3. Results

164 3.1. Mineral content and moisture in plant products

165 Levels of iron (and other minerals) in the plant samples were measured by ICP-OES (Supplementary
 166 Table 1). Iron content was highest in moringa (85.44 mg/100 g) and lowest in baobab (1.67 mg/100
 167 g). Fenugreek sprouts (19.85 mg/100 g) contained more than twice the amount of iron present in
 168 fenugreek seeds (7.75 mg/100 g). Moisture content did not vary between plant food samples (range
 169 92.5 – 95.7 %).

170 3.2. The bioaccessible and fractional low-molecular-weight iron content of the 171 digested extracts of the vegetables

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

178

179

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

180

181

Sample	Bioaccessible		Low-Molecular-Weight	
	%		%	
Fenugreek sprout	17.18 \pm 0.04 (3.41 \pm 0.01 mg/100 g)	a	9.16 \pm 0.09 (1.81 \pm 0.01 mg/100 g)	a
Fenugreek Seeds	42.71 \pm 0.22 (3.31 \pm 0.02 mg/100 g)	b	25.18 \pm 0.14 (1.95 \pm 0.02 mg/100 g)	b
Baobab fruit pulp	99.7 \pm 0.13 (1.74 \pm 0.01 mg/100 g)	c	97.8 \pm 0.01 (1.70 \pm 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
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182 Data are presented as means ± SEM of n = 3 measurements. Different letters indicate significant
 183 difference between groups (P≤0.05). Mean values represent total bioaccessible iron for each sample
 184 category while the lower type phase in bracket are the absolute quantities of iron in the samples.
 185

186 **3.3. The total bioaccessible and fractional low-molecular-weight iron content of**
 187 **the digested extracts of the vegetables with added ascorbic acid**

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

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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

199 Data are presented as means ± SEM of n = 3 measurements. Different letters indicate significant
 200 difference between groups (P≤ 0.05). Mean values represent total bioaccessible iron for each sample
 201 category while the lower type phase in bracket are the absolute quantities of iron in the samples.
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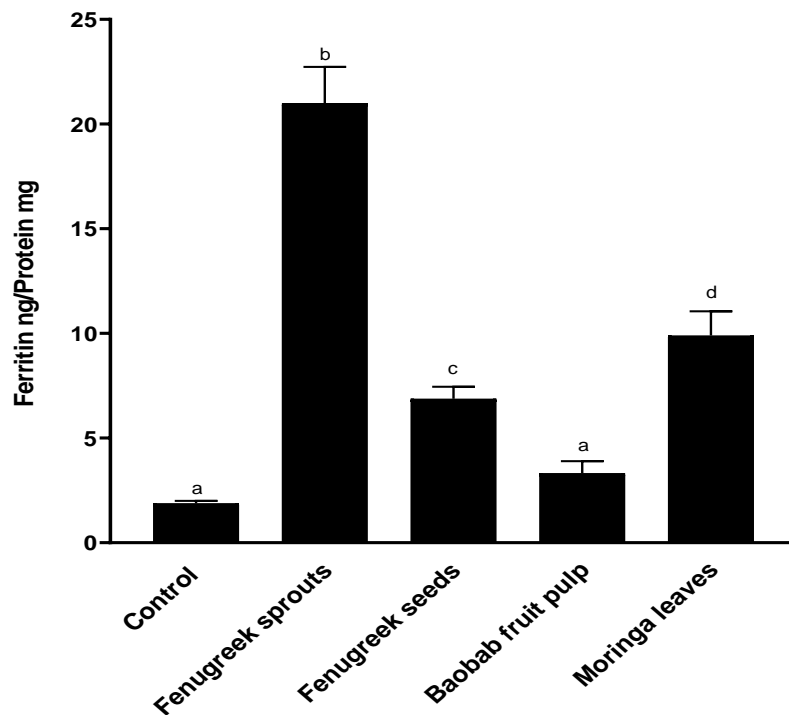
205 **3.4. Cell viability of Caco-2 cells after exposure to digested vegetable samples**

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

211

212 **3.5. *In vitro* accessibility of iron from fenugreek sprouts, seeds, baobab or**
213 **moringa in Caco-2 cells**

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



219

220 **Figure 1:** Iron uptake by Caco-2 cells from plant food digests. “Control” indicates digest system
221 components only (i.e., pepsin, pancreatin, bile extract). Values are presented as means $n = 6 \pm \text{SEM}$.
222 Data were analysed using a two-way ANOVA. Different letters indicate significant difference
223 between groups ($P \leq 0.05$).
224

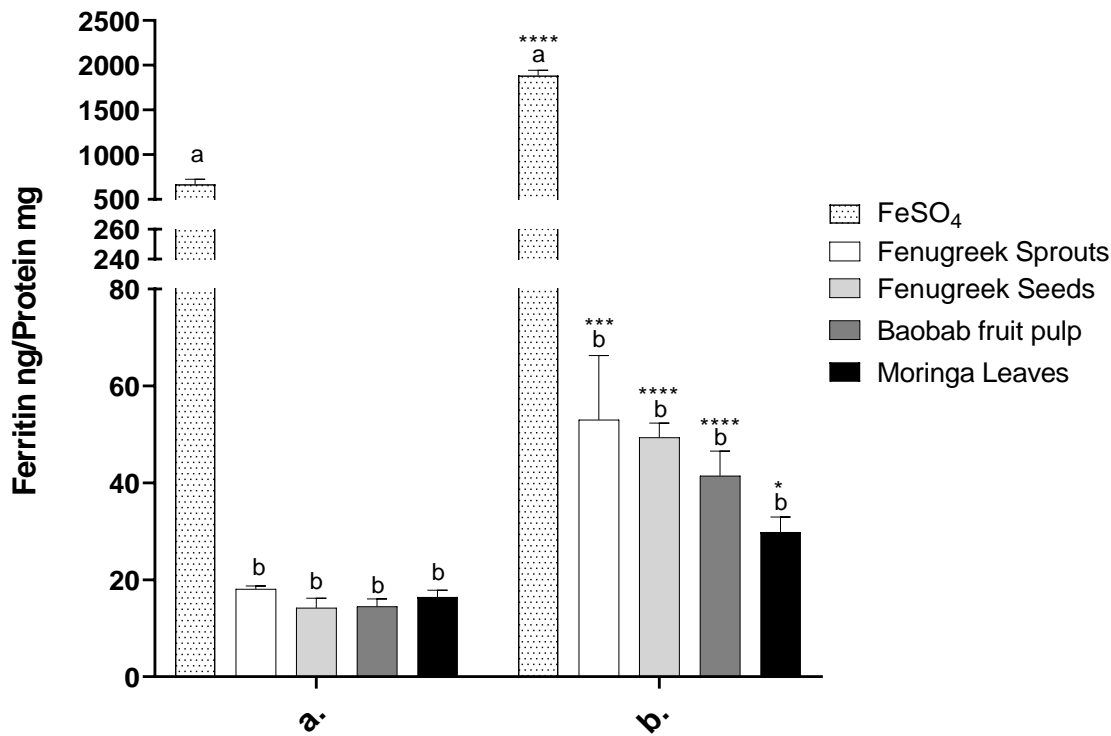
225 To enhance iron extraction during the peptic-pancreatic digestion of the plant products, ascorbic acid
226 was added to the samples during the digestion process. Iron availability from fenugreek sprouts,
227 fenugreek seeds, baobab or moringa was not significantly influenced when ascorbic acid was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

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235 **Figure 2.** Iron uptake by Caco-2 cells from vegetable samples with added ascorbic acid (AA) during
 236 digestion (a) and (b.) vegetable samples with AA added during exposure to cells. The control sample
 237 contained 50 μ M FeSO₄ and 250 μ M AA. Values are presented as means \pm SEM, n = 6. Data were
 238 analysed using a two-way ANOVA. Different letters indicate significant difference between
 239 vegetables ($P \leq 0.05$). The differences between groups (a) and (b) are denoted (* $P \leq 0.05$, *** $P \leq 0.001$
 240 and **** $P \leq 0.0001$).

241

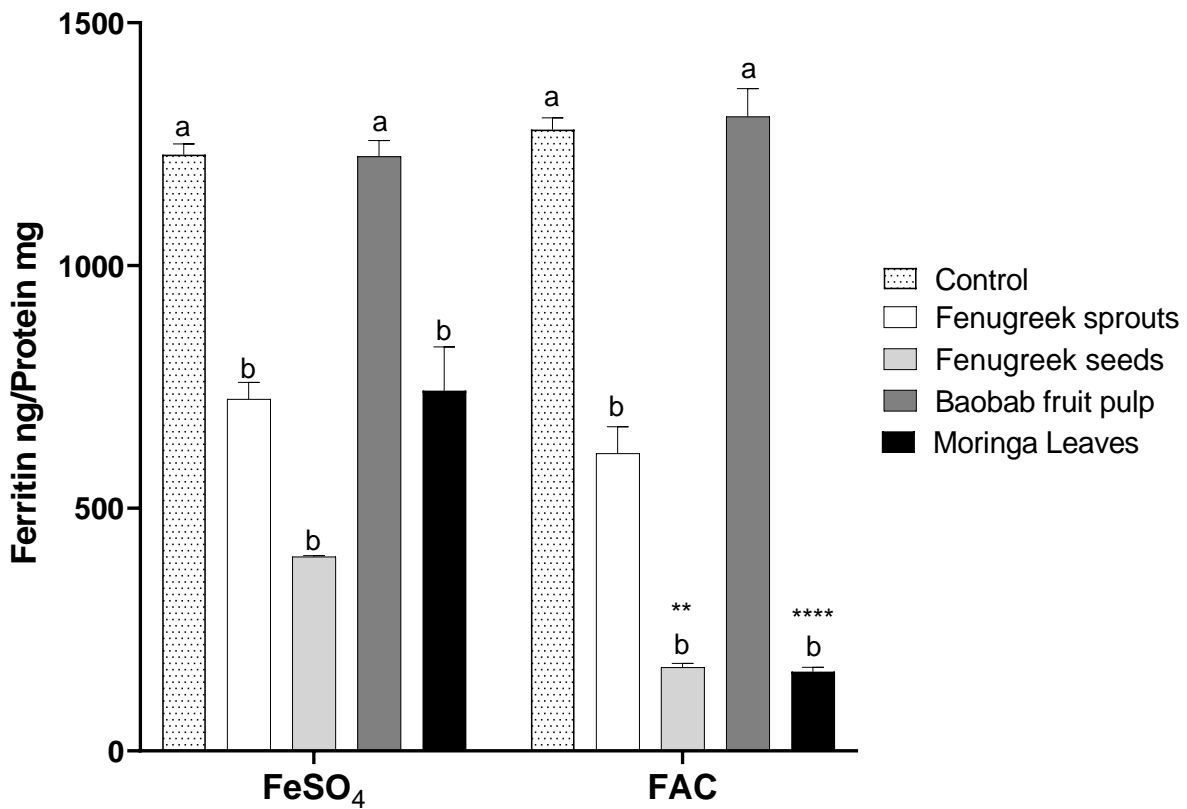
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243

244 **3.6. Modulating effects of fenugreek sprouts, fenugreek seeds, baobab or**
 245 **moringa on iron accessibility from iron salts in Caco-2 cells**

246 We next explored the interactions of the plant products on the accessibility of iron salts. FeSO₄ and
 247 FAC had comparable iron availability for uptake in Caco-2 cells (Figure 3). Except for baobab fruit
 248 pulp, the other plant products significantly ($P \leq 0.05$) reduced iron availability from both FeSO₄ and
 249 FAC in Caco-2 cells. Moreover, inhibition of iron from FAC (Fe(III)) was significantly higher than
 250 from FeSO₄ (Fe(II)) for fenugreek seeds ($P \leq 0.01$) and moringa leaves ($P \leq 0.0001$) respectively.

251



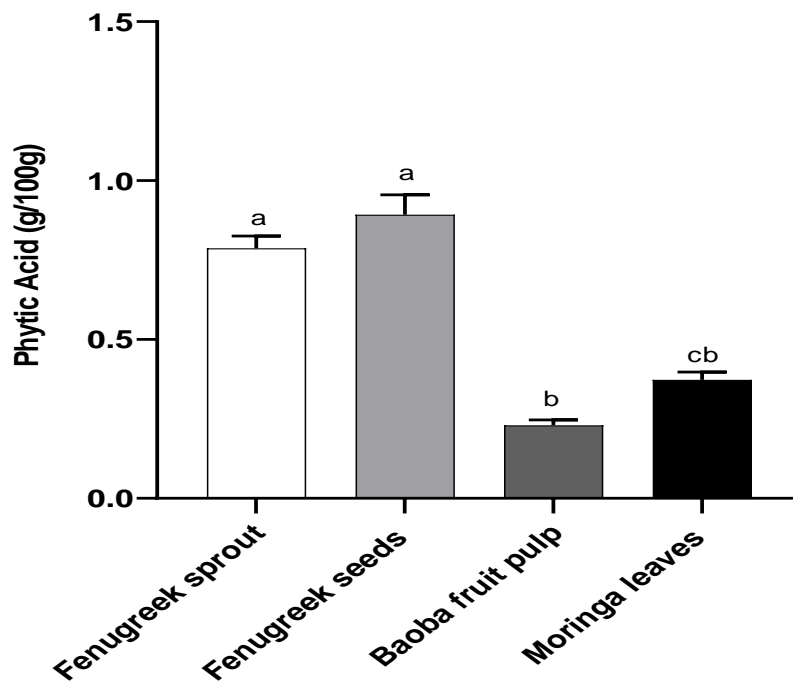
253

254 **Figure 3:** Iron uptake by Caco-2 cells from vegetable digests. Cells were exposed to the digest
 255 samples with added FeSO₄ or FAC (50 μM). Values are presented as means ± SEM, n = 6. Data were
 256 analysed using a two-way ANOVA between FeSO₄ and FAC groups and one-way ANOVA amongst
 257 the treatment groups. Different letters indicate significant difference between vegetables ($P \leq 0.05$).
 258 The differences between added FeSO₄ and added FAC groups are denoted (* $P \leq 0.05$, *** $P \leq 0.001$
 259 and **** $P \leq 0.0001$).

260

261 3.7. Phytic acids levels in fenugreek sprouts, seeds, baobab and moringa

262 Phytic acid is a major inhibitory component in plant foods. Figure 4 shows a significant level of
 263 variations in the phytate content of the plant products. Both fenugreek samples exhibited the highest
 264 levels of phytic acid and may in part explain why both plant products inhibited FeSO₄ and FAC
 265 uptake by Caco-2 cells the most (Figure 4).



266

267

268 **Figure 4:** Phytic acid content (g/100 g) of the vegetable samples. Values are presented as means \pm
 269 SEM, n=6. Data were analysed using a two-way ANOVA test. Different letters indicate significant
 270 difference between groups ($P \leq 0.05$).
 271

271

272 4. Discussion

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

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

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

287 calcium and 75% of iron requirements could be met in children aged 1-3 years from 100 g of fresh
288 moringa leaves (Price, 2007). In comparison, an equivalent weight of baobab pulp will provide 30%
289 of calcium and 23% of iron daily needs in children 4-13 years, and about 29% of the calcium
290 requirement of pregnant women (Magaia et al., 2013). Furthermore, van der Merwe et al. (2019)
291 reported that fortification of pearl millet in a cereal-based meal with moringa leaf powder, roselle
292 calyces or baobab fruit pulp contributed about 3 times more iron and zinc than from pearl millet
293 porridge fortified with provitamin A source alone. The added plant food fortificants contributed about
294 28% and 41% of the absolute iron and zinc requirements, respectively, of women of reproductive
295 age from a single meal. A recent study (Shija, Rumisha, Oriyo, Kilima, & Massaga, 2019) also
296 reported that daily supplementation of 25 g moringa flour to food intake for 6 months significantly
297 reduced anaemia prevalence in children whose average age was about one year old. Improvement in
298 haemoglobin levels for these children were significant irrespective of the degree of anaemia with
299 moringa intervention plus nutrition education compared with the control that had nutrition education
300 alone. Iron in plant foods exists predominantly as ferric non-haem iron and might be complexed with
301 other organic such as phytate and polyphenols or inorganic molecules in the matrices of fenugreek
302 sprouts, fenugreek seeds, baobab fruit pulp and moringa leaves. Iron, though relatively abundant in
303 the earth crust and most foods, is characterised by poor availability from plant sources.

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

313 Fenugreek sprouts had the highest available iron in Caco-2 cells but paradoxically, exhibited a high
314 level of phytic acid. This was an unexpected finding as the process of sprouting is known to activate
315 phytase (Ou, Cheng, Xing, Lin, Nout, & Liang, 2011) that enzymatically degrades phytate, thereby
316 enhancing iron bioavailability (Hurrell, 2004). In contrast to the current study, previous work reported
317 increased iron availability in germinated fenugreek seeds (Hooda & Jood, 2003). The ratio of phytate
318 to iron has been shown to correlate with iron dialyzability (Glahn, Wortley, South, & Miller, 2002).
319 The phytate to iron ratios were 39.66, 115.07 137.35 and 4.37, while the calcium to iron ratios were
320 9.23, 22.30, 187.25 and 27.13 respectively for fenugreek sprouts, fenugreek seeds, baobab and

321 moringa. Vitamin C in plant products in sequential order were 2 (Ahmed, 2014), 19.55 (Sharara,
322 2017), 266 and 0.11 (Sankhyan, Sharma, Seth, Chauhan, & Kulshrestha, 2013) mg/100 g. Hence,
323 baobab is abundant in both potent inhibitors and an enhancer of iron availability. In general, the
324 comparatively low solubility of iron from these plant products may be due to chemical complexation
325 with a range of inhibitory factors including phytate, polyphenols or fibre (Chadare et al., 2008).

326 The exploitation of the synergy among dietary components to enhance iron absorption promotes the
327 use of foods that are rich in ascorbic acid, a potent enhancer of iron absorption in composite meals.
328 Consequently, ascorbic acid was added to the plant products during the simulated digestion or added
329 to the digested extracts before the application to Caco-2 cells in the current study. Iron availability
330 was significantly enhanced from fenugreek sprouts, fenugreek seeds, baobab pulp and moringa leaves
331 when ascorbic acid was added to the digest before application onto Caco-2 cells but not when it was
332 added during the peptic-pancreatic digestion process. This might be due to the oxidation of the Fe (II)
333 species by ascorbic acid during heat denaturation of the digestive enzymes in the digests before the
334 application onto Caco-2 cells (Vikram, Ramesh & Prapulla, 2005). Moreover, enhanced iron uptake
335 in Caco-2 cells could be due to an augmentation of Dcytb-dependent, ascorbate-mediated
336 ferrereduction (Luo, Hill, Johnson, & Latunde-Dada, 2014). Alternatively, it could be due to the
337 complexation of iron, which is known to form insoluble complexes when the pH level is higher than
338 5.3, thus decreasing the availability and absorption of iron (Scheers, Andlid, Alming, & Sandberg,
339 2010). In support of the latter possibility, previous studies showed that there was no effect of ascorbic
340 acid on iron availability from pork meat at pH 7 during peptic and pancreatic digestion (Sørensen &
341 Bukhave, 2010). Furthermore, the study highlighted the pH dependence of iron uptake from foods
342 and revealed the significance of separating pepsin-digested and pepsin + pancreatin-digested proteins
343 during *in vitro* studies on iron availability. This exposes a technical limitation of the *in vitro* method
344 as the spatial interactions of dietary constituents in the food matrix, enzymatic digestive processes,
345 bioaccessibility and absorption of iron occur concurrently in the gastrointestinal tract of the
346 organisms.

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

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

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

366 Conclusion

367 Iron content of the dried vegetable samples (mg/100g) are 1.67 ± 0.05 , (baobab fruit pulp), $7.75 \pm$
368 0.04 , (fenugreek seeds), 19.85 ± 0.33 (fenugreek sprout) and 85.44 ± 1.22 (moringa leaves). These
369 underutilised plant foods with variable iron content which upon processing or incorporation in
370 composite cuisines containing rich sources of ascorbic acid, could potentially be employed to treat
371 improve and maintain iron homeostasis in groups at risk of iron deficiency or anaemia. Maximising
372 the potentials of these plant products requires further research in food processing, dietary formulation
373 and nutrition education of the populace.

374
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380 5. References

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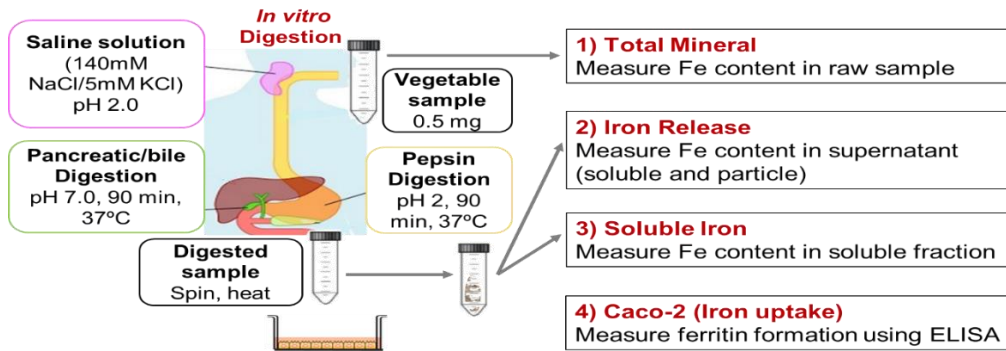
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504 **6. Supplementary**

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

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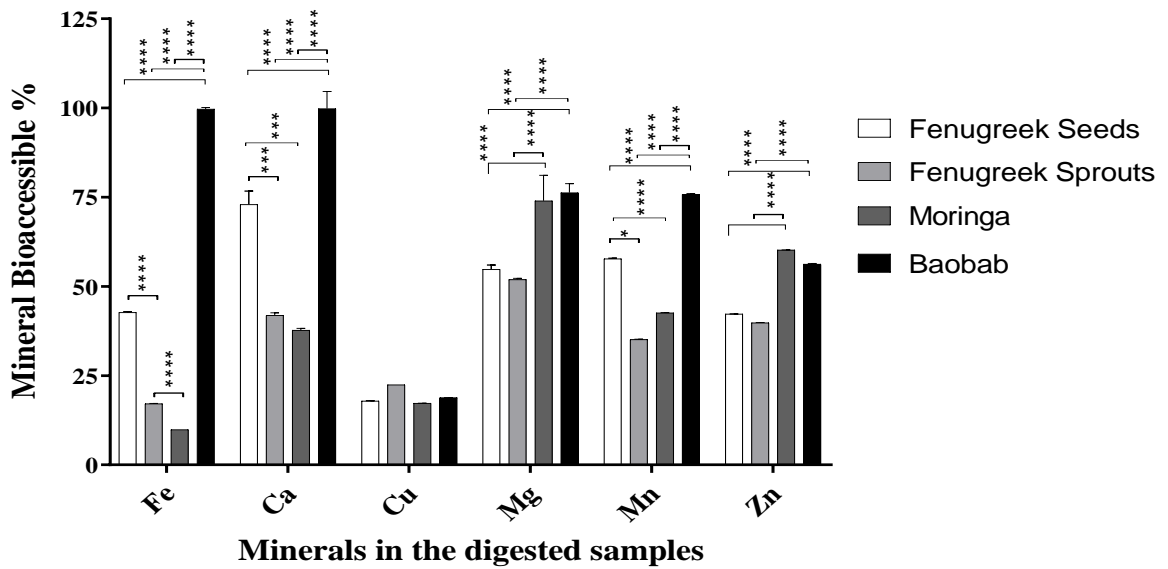
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515 **Table 1 Moisture and Mineral content of the dried vegetable samples (mg/100g)**

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

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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 ($P \leq 0.05$).

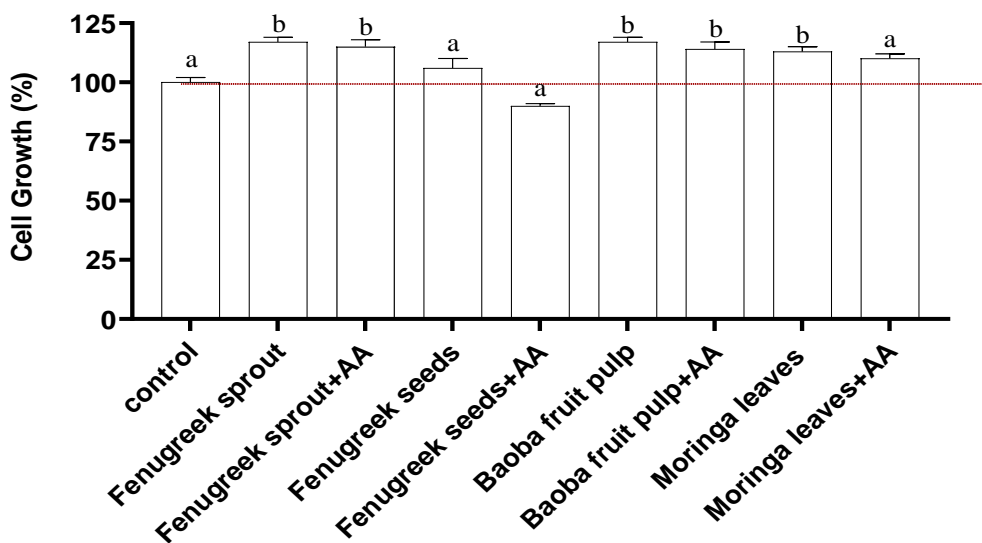


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529 **Figure 2:** Mineral bioaccessible from digested vegetable. Values are presented as means \pm SEM, n =
 530 6. Data were analysed using a two-way ANOVA. The differences between vegetables are denoted
 531 (* $P \leq 0.05$, *** $P \leq 0.001$ and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

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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 \pm SEM. Data were analysed using a one-way ANOVA test. Different letters indicate significant
 538 difference between groups ($P < 0.05$).

539